Ostfalia
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Applied Sciences

BIOREMEDIATION OF HEAVY METALS BY USING THE MICROALGA *DESMODESMUS SUBSPICATUS*

Bachelor´s Thesis

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Supervisor: Dr. Sander Hedda
Reviewer: Prof. Carmen Genning
Author: Hajdu-Rahkama Réka
Martikel-Nr: 1011544
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ABSTRACT

Ostfalia University of Applied Sciences
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RÉKA HAJDU-RAHKAMA
Bioremediation of heavy metals by using the microalga *Desmodesmus subspicatus*

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All around the world natural water bodies are contaminated with heavy metals from previous and recent mining activities. These contaminants not just can reach our drinking water supplies but endanger aquatic ecosystems and also other organisms. The recent technologies used for the removal of heavy metals are expensive and also causing harm to the nature. To avoid the negative impacts caused through the removal of contaminants, environmentally friendly methods, bioremediation should be applied. Using microbial processes for the removal of contaminants is still not widely used and enough studied. The objective of this work was to investigate the bioremediation properties and behavior in different environmental conditions of the microalga *Desmodesmus subspicatus*.

The heavy metal (300µg/l Pb, 30µg/l As, Cd, Hg) solution chosen was similar with the values found in the River Oker, which originate from previous mining site of the Harz Mountain in Lower Saxony, Germany. The heavy metal uptake by *Desmodesmus subspicatus* biomass was not efficient at pH 5 since the final uptakes were (4.5±0.67) µg/l As, (21.1±2.89) µg/l Pb, (7.33±1.96) µg/l Cd and (6.25±1.28) µg/l Hg (15% As, 7% Pb, 24% Cd, 21% Hg). However, its initial biosorption showed good results with Pb (<79%) and Hg (<63%).

In the conditions applied the *Desmodesmus subspicatus* can be used only for initial biosorption of Pb and Hg. Making changes like adjustment of different pH levels; applying multi culture mix, adding immobilization material, increasing the population and pretreating the cells could increase the efficiency. Also the immediate uptake of Hg and Pb by biosorption is an interesting feature as it could be used with biomass filtering not with growing cultures and lead to immediate results. The bioremediation property of *Desmodesmus subspicatus* in an environment with one or several of the previously mentioned methods applied should be further investigated.

Keywords: bioremediation, bioaccumulation, biosorption, heavy metal contamination

DECLARATION BY CANDIDATE

I hereby declare that this thesis is my own work and effort and that it has not been submitted anywhere for any purpose. Where other sources of information have been used, they have been acknowledged

Signature: ……………………………………….

Date: ………………………………………

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III GLOSSARY

As	Arsenic
Cc	cells/ml
Chl a	Chlorophyll a
Cd	Cadmium
Co	Cobalt
Cu	Copper
DM	Dry matter
DS	*Desmodesmus subspicatus*
EDTA	Ethylenediaminetetraacetic acid
Hg	Mercury
HM	Heavy metal
n	number
Ni	Nickel
OD	Optical density
Pb	Lead
ppm	parts per million
rpm	revolutions per minute
Sb	Antimony
SD	Standard Derivation of the Mean
Tl	Thallium
TRIS	Tris(hydroxymethyl)aminomethane)
UV-Light	Ultraviolet Light
$\bar{x}$	mean value
Zn	Zink
λ (Lambda)	wavelength of light

1. Introduction

1.1 Water pollution from mining industry

The mining industry has been using freshwater for recovery of the ores for many decades. This activity not just exploits the drinking water resources but as an effluent it is a great environmental hazard. The process water returned back to nature generally contains toxic heavy metals, acid-generating sulfides, waste rock impoundments and water. Usually the effluent is deposited to large free-draining piles where from it can reach natural drinking water resources, rivers and ponds and by this makes water undrinkable and kills microorganisms, fishes and plants (Moeller, 2011). Furthermore, it can cause problems in reproductive functions and health.

Different type of mines requires different amount of water. The outmost water is needed during processing concentrate metals such as copper and gold while non-metal mines like salt and gravel requires less, but the amount needed overall remains considerable. As an example 4% of Canada´s, 2-3 % of Australia´s and 1% of USA´s water usage was in the mining sector in the year 2005 (Fraser Institute, 2014).

The amount of contamination produced differs also by the type of ore being mined. The most chemically reactive ones are the sulphide ores because they are the most soluble. During the extraction of metals and preparation of minerals, different chemicals such as sulphuric acid, cyanide and organic chemicals are used. The highest chance of contamination from the process chemicals can occur when the mine is already closed but in case of natural disasters like flooding catastrophes can happen during its operational time too. Although the recent techniques are highly improved, in artisanal (ASM) or informal mining liquid mercury is still being used during such processes as recovery of gold. By the ASM process around 650-1000 tonnes mercury are released back to the environment with the effluent every year (Fraser Institute, 2014).

1.2 Present techniques to remove heavy metals from water sources

For the removal of heavy metal ions from water resources, different methods are used nowadays. The most common techniques are the addition of chemicals, Reverse osmosis,

electro dialysis, oxidation-reduction, activated carbon adsorption, evaporation recovery and solvent extraction. However, these artificial ways of removal are often expensive and moreover generate toxic chemical sludge. Thus there is need for development of natural ways like application of bioremediation processes (Juwarkar, Singh and Mudhoo, 2010). The TABLE 1 shows the different artificial techniques used to remove heavy metals from water bodies.

TABLE 1: Different ways used to remove heavy metals from contaminated water

Treatment method	Advantages	Disadvantages	Reference
Addition of chemicals	pollutants can be separated easily by centrifugation	ineffective when concentration of heavy metals is below 100 mg/l; its waste sludge is harmful to environment	Dhankhr and Hooda (2010)
Reverse osmosis	removes 90-99% of the contaminants	extra storage tank is needed; expensive	APEC d (2014); Bakalár, Búgel and Gajdošová, (2009)
Electro dialyze	effective removal of dissolved inorganics; re-generable; relatively small investment needed	pre-treatment needed to eliminate particles > 10 µm; high operational costs over long term	APEC b (2014); Lenntech b (2014)
Activated carbon adsorption	removes dissolved organics and chlorine; can be effective against microorganisms	5-10% loss of Carbon while recycling the filter; only able to remove 30-40 mg/g of Zn, Cr and Cd	Lenntech a (2014); APEC a(2014)
Evaporation recovery	no chemical needed	odors; land need; depends on meteorological circumstances; costly	Pankratz (2000)
Solvent extraction	easy removal of unwanted substance	high solvent-, investment and operational costs;	wiseGeek a (2014); Gamse (2014)

Bioremediation	environmental friendly; cost effective; no need for chemicals; highest acceptance by the public	efficiency can vary; toxic side effect may occur if the microorganism not studied well	Price et al. (2004); Beck and Jones (1995); Sharma (2012)

1.2.1 Addition of chemicals

For the removal of metals form wastewater one widely used technique is chemical precipitation. This precipitation is causing settling of the contaminants as a solid precipitate, which can be separated easily by centrifugation, filtration or other separation methods afterwards. During the precipitation a chemical agent, called coagulant, is used to create bigger agglomerates which tend to settle faster. The most commonly used coagulants are polymers (EPA, 2000).

Initially, insoluble metal hydroxides can be created from soluble heavy metals by increasing pH values via the addition of hydroxide compounds or insoluble metal sulfides by adding lime and ferrous sulfide. These insoluble metals tend to settle. Different pH values should be adjusted for precipitation of different metals. After this first step and pH adjustment, polymer coagulant can be added to accelerate the settling of the metal particles (EPA, 2000).

This technology is ineffective when the concentration of heavy metals is below 100 mg/l. Furthermore, it is harmful to the environment because it leaves behind large amounts of waste sludge, which are difficult to treat afterwards (Dhankhr and Hooda, 2010).

1.2.2 Reverse osmosis

The four filtration techniques for the removal of solids from a liquid are Reverse osmosis (RO), nanofiltration, ultrafiltration and microfiltration (United States Environmental, 2014). The mainly used filtration for heavy metal treatment is the Reverse osmosis (RO). The RO is the process when a solution is forced though a semi-permeable membrane by using pressure. This semi-permeable membrane allows the pure solvent to pass through the membrane but the solute will remain in the entering site. The separation occurs in the membrane because it has a dense barrier layer in the polymer matrix. The RO technology

is cost intensive and thus not that commonly used to treat wastewater with heavy metal content (Bakalár, Búgel and Gajdošová, 2009).

1.2.3 Electro dialysis

Electro dialysis (ED) technology uses electrical potential to influence the transportation of the ions of a solution through a semi-permeable membrane. The membrane can be, anion- or cation-selective which means that only positive or negative ions can pass through. The idea of the cation-selective membrane is that it only allows positively charged ions to flow through and the negatively charged ones are rejected. This can happen because the membrane is built up of negatively charged matter, polyelectrolytes responsible for the rejection. Several membranes can be placed in a row to allow negative or positive ions to pass through thus enabling comparatively easy separation of ions from wastewater. If a particle has no charge, it will also pass through the membrane.

The constructing material of anion-selective membranes is polystyrene with quaternary Ammonium and for cation-selective membranes con consist of sulphonated polystyrene.

If a solution has too much and big particles in it, they can plug the pores of the membrane so it is advised to remove these ones with the size over 10 µm by sedimentation, carbon filtration, flocculation or other filtration method. This technique is also rather cost intensive) like the RO described previously.

(Lenntech b, 2014

1.2.4 Oxidation-reduction

The oxidation-reduction is partially catalytic and an electrochemical mechanism. This technology is widely used for the removal of cadmium, arsenic, lead, nickel, copper, mercury, chromium, antimony and cobalt ions from water bodies. The efficiency rate of this method can be as high as 98%. The idea of it is to reduce soluble metal ions to insoluble atoms which are then electroplated onto the pores or surface of a granular (redox) media (theWater site.com, 2014).

1.2.5 Activated carbon adsorption

Active carbon is used as a solid to remove soluble substances from a solution during activated carbon adsorption process. Active carbon does have a big internal surface of 500-1500 m^2/g necessary to achieve adsorption. This activated carbon can be Granular Activated Carbon (GAC) which is mainly used during water treatment Powder Activated Carbon (PAC) (Lenntech a, 2014).

During activated carbon adsorption process the water is pumped though a column containing active carbon and leaves the column across the draining system. The nature of the substances in the water and the temperature are the factors affecting the activity of the active carbon column. While the water runs through the column, the substances are accumulating in the filter so it is important to change it periodically. This used filter can be than regenerated e.g. in case of granular carbon, the organic matter can be oxidized. Although the filter can be used again but its efficiency will be 5-10% decreased because loss of some active carbon during the cleaning. This loss must be replaced before using the filter again (Lenntech a, 2014).

This artificial technology is non-regenerable and only able to remove 30-40 mg/g of Zn, Cr and Cd which makes it expensive (Dhankhar and Hooda, 2010).

1.2.6 Evaporation recovery

Evaporation means leaving the solution with heavy metal content in evaporation ponds or using evaporation equipment to vaporize the water content and leave behind the concentrated saline liqueur with all the dissolved solids and metals. Evaporation ponds cannot be applied everywhere because of odour issues, land need, cost and meteorological circumstances of the place (Pankratz, 2000).

2.1.7 Solvent extraction

A substance can be separated from the others by using a solvent and this is called solvent extraction. This method based on the different solubility of different compounds. A solvent

is added to the mixture which dissolves all the compounds except the unwanted one. The undissolved compound can be then easily removed from the mixture (wiseGeek a, 2014).

1.3 Environmental friendly way to remove heavy metals

There is natural way for the removal of heavy metals from contaminated waters and it is called bioremediation. This method is not widely used yet but offers promising results.

1.3.1 Bioremediation

Bioremediation is the use of microbial processes for detoxification of environmental contaminants. There are different microorganisms involved in these processes such as aerobic ones which are able to degrade hydrocarbons and pesticides. The methylotrophs, aerobic bacteria, are also able to do bioremediation and they gain energy from reduced carbon compounds and multi-carbon compounds for their growth. (Juwarkar, Singh and Mudhoo 2010). The other types of microorganisms are ligninolytic fungus species which degrade extremely persistent environmental pollutants.

The bioremediation not just removes the contaminants but often makes recovery of pollutants (e.g. heavy metals) possible and has beneficial effects on the fertility of the soil and its structure. Besides the environmentally friendly properties of the bioremediation it is also cost effective compared to other techniques, which use expensive chemicals, consume high amount of energy or require expensive technology. Before implementation of bioremediation, the microbiological processes need to be well understood to avoid side effects such as degradation of chemicals to some toxic or harmful mobile substances (Price et al. 2004; Beck and Jones 1995).

1.3.1.1 Factors influencing bioremediation

Decontamination of freshwater, groundwater, marine systems, surface and subsurface soils are sometimes done by bioremediation in these days. However, for high efficiency of these processes certain factors need to be optimal. The pH, nutrient content and temperature of the media, phase of the life-cycle of the microorganism are some of the most important factors but also the composition of metals and toxic compounds;

bioavailability of pollutants; external electron availability; gene expression; bio augmentation, biodegradability of contaminants and the geological characters are also influencing the efficiency rate.

Normally, pH levels below 3 and above 9 can inhibit microbial growth by changing the gas- and metal solubility and the bioavailability of nutrients in the water. In general natural environment has pH levels between 5 and 9 so this is the optimum condition to enhance biodegradation of waste contamination for most of the microorganisms. The physical-chemical state of the contaminants, the microbial growth rates and the metabolism of the microorganisms are affected by the temperature of the environment, the media. Most of the in situ bioremediation runs under mesophilic conditions (20-40° Celsius). In this condition the doubling time of the microorganism is relatively short and the solubility of most of the metals is good. In case of conditions either too cold or too hot the microorganism might be inhibited and the metals are not available because they are not dissolved within the liquid media.

In a microbial population there are four different phases of life cycle. The first adaptation phase is the lag-phase; the external growth phase is the log-phase which is followed by the stationary- and death phases. The microorganism is most active in its log-phase because during this stage enzyme production is started, so it is adapted to the conditions of its environment and fastest increasing its own mass. Thus, during this metabolically most active phase the bioremediation works best.

Presence of some metals can inhibit some cellular processes while several inorganic nutrients such as Nitrogen, Phosphorus and some trace elements (Calcium, Sulfur, Iron, Magnesium and Manganese) are essential. But it is important to keep in mind that even though some chemicals are essential they may be toxic to the microorganism in high concentrations and slow~~s~~ down or even prevent its metabolism.

(Juwarkar, Singh and Mudhoo 2010)

1.3.1.2 Treatment technologies of bioremediation

There are two different types of bioremediation technologies, the *ex-situ* and the *in-situ* ones. The *ex-situ* technology means physical removal of the contamination for further treatment afterwards. Among the *ex-situ* processes belong anaerobic digestion, which means the biological degradation of organic matter and production of biogas (Naskeo

Environment, 2009). Also the composting processes belong here, which are defined as the decomposition of once living materials such as plants (VegWeb., 2014). Also bioreactors are employed, utilizing microorganisms in a closed system to encourage their naturally occurring biochemical processes (wise Geek b,2014). In addition, some forms of solid-phase treatment are employed like land farming, which is used for decontamination of soil.

Using *in-situ* technologies on the other hand, the treatment of the contaminated material takes place without prior removal of the contaminated material. For the treatment of contaminated water biostimulation of indigenous aquifer microorganisms and for soil decontamination bioventing, Oxygen drawing to soil in order to stimulate the growth and activity of the microorganisms are used *in-situ* techniques (Juwarkar, Singh and Mudhoo, 2010; Dhankhar and Hooda, 2010). For the removal of heavy metals from contaminated sites, phytoremediation, the application of plants are used.

There are also technologies which can be applied *in-* and also *ex-situ.* Among these belong the biosorption and bioaccumulation. In biosorption processes, certain biomass has the ability to bind and thus concentrate heavy metals on the cellular surface (Velásquez L., and Dussan). Another approach is bioaccumulation, which is the same process as done by biosorption, but in this case the HMs are carried within and onto the cellular surface. Alga, bacteria, fungi and also yeast has proven potential of removing heavy metals (Katarzyna, 2010; Ahalya, Ramachandra and Kanamadi, 2003).

1.3.1.3 Biosorption of heavy metals

While conventional treatment methods are expensive, hard to implement, and have mostly low efficiency, biosorption is an easy to employ and inexpensive technology, which minimizes the chemical or biological sludge remnant, regenerates the biosorbents and also makes the recovery of heavy metals possible.

The availability and toxicity of metals on the microorganisms used depend on the pH and temperature of the medium. There are several mechanisms for the removal of metals from a solution by activated biomass. They are intra- and extra-cellular complex reactions, ion exchange- and complexation reactions through the cell wall. At the same time, inactivated biomass is primarily removing the heavy metals by adsorbing them to the ionic groups, which might be on the cell surface (Powell et al., 1999; Ahluwalia and Goyal, 2007).

It has been reported that the microalgal biosorption of heavy metals often occurs in two stages. The initial rapid and passive uptake is the first stage which generally takes a short time of approximately 30 minutes. At this stage mainly surface adsorption is taking place by the components like carboxyl, amine, sulfate groups, Phosphate- and hydroxyl-groups of the cell wall. The second stage is the subsequent active and slow uptake stage when the metals are transported through the membrane to the cytoplasm of the cell wall. This latter stage often takes even one month (Shanab, Essa and Shalaby, 2012).

1.3.2 Biotic methods of bioremediation

For the natural removal of heavy metals from water bodies plant products or microorganisms are used. The plant products are generally originated from agricultural by-products, residues. Some examples are sunflower stalk, maize cob and husk, peat moss, wheat bran, sugar cane, exhausted coffee, wool, rice, cotton seed hulls, soybean hulls, banana pith, butter seed husk, cassava waste, chitosan and sago waste (Dhankhar and Hooda, 2010).

From the microorganisms bacteria, fungi and algae have been tested and utilized. All of these three are using functional groups on their cell walls to bind heavy metals. In general the fungal group has the biggest surface area and thus the biggest amount of cell wall material available with the functional groups to bind higher amount of metals. Using fungi has several advantages (see TABLE 2) like easy cultivation, more resistance to temperature changes (temperature change between 20-35°C has minor effects only), fast growth, large biomass production. The biosorption capacity and metal removal efficiency increases by longer retention time. However, this last property, the contact time, needs to be optimized because of influencing factors like regeneration of the biomass and efficiency of desorption. Zn, Cd, Cu, Ni and Co reduction is most efficient at low pH values but Ag^{2+}, Hg^{2+} and $AuCl_4^-$ removal are probably pH independent. As an example the pH optimum for biosorption of Pb(II) and Ni(II) is 5.0 but for Cr(VI) the pH optimum is 1.0 for *S cerevisiae* (Dhankhar and Hooda, 2010).

TABLE 2: Advantages and disadvantages of fungal biosorption

Advantages	Disadvantages	Reference
* excellent metal-binding capacity * easy to cultivate in large scale * high yield of biomass * available as industrial waste products (e.g. *Aspergillus niger* in citric acid production´s waste) * major portion is not pathogenous	* extraction procedures might results in noxious wastes	Dhankhar and Hooda (2010); Gadd (2008)
* availability of complete genomic sequence * ease genetic manipulation		Galun et al. (1984)
* low costs * possible metal recovery		Ashraf, Mahmood and Wajid (2011)

1.3.3 Efficiency of heavy metal removal by different algal species

Bioremediation of heavy metals from water bodies by different freshwater- and marine algal species has been studied for a long time. Some species are efficient with just a couple of heavy metals, some have the ability to remove certain amount from a wide range while there are also badly performing ones. The following chapters will mainly concentrate on the removal of Lead, Cadmium, Mercury and Arsenic, the heavy metals which were used during this studies´ laboratory tests.

Previously studies have been done at Ostfalia University of Applied Sciences about the heavy metal (As, Cd, Pb, Hg) removal by *Scenedesmus quadricauda* and *Chlorella vulgaris* (Valdivia, M., 2013 and Rolfes, 2014). The concentrations of different heavy metals were 300µg/l Pb, 30 µg/l As, Cd, Hg. The experimental periods differed one day (13 and 14 days). As it can be seen from the TABLE 3 *Chlorella vulgaris* showed the highest efficiency with Cd (65%) and the lowest with As (9%). *Scenedesmus quadricauda*

was able to remove higher content of As (21%) and it was most efficient with the uptake of Hg (61%). Both of the species removed similar amounts of Pb (19 and 17%).

TABLE 3: Efficiency of HM (As, Pb, Cd, Hg) removal of *Chlorella vulgaris* and *Scenedesmus quadricauda*

Efficiency (%)	**Arsenic**	**Lead**	**Cadmium**	**Mercury**	Reference
Chlorella vulgaris (14 days)	9	19	65	37	Rolfes, 2014
Scenedesmus quadricauda (at start)	34	49	63	99	Valdivia, M., 2013
Scenedesmus quadricauda (13 days)	21	17	41	61	Valdivia, M., 2013

1.3.3.1 Removal of Lead (Pb)

Some of the algal species removal was more outstanding than the others. However the studies in general do not show the efficiency rate of metal ion removal but some can be found. The exact value of removal efficiency was not available from this study, but El-Sheekh et al. reported 100% removal efficiency of a *Cyanobacterium*, *Anabaena subcylindrica* (El-Sheekh et al., 2005). The initial Pb ion concentration was not mentioned but in the study made by Chójnacka, Chójnacki and Górecka (2004) 92% efficiency by the fresh biomass and 85% by the $CaCl_2$ pre-treated *Spirulina maxima* (*Cyanobacteria*) was reported. This study showed lower efficiency of the pre-treated biomass.

The highest Pb uptake (see from TABLE 4) was observed of *Laminaria japonica* and *Lyngbya taylorii,* 349 mg/ and 304.56 mg Pb/g (mg metal ion/ g fresh biomass) (Lee et al, 2004 and Klimmek et al., 2001). But also *Ecklonia radiata and E. maxima* had good results of 281.78 and 243 mg Pb/g biomass (Matheickal and Yu, 1996; Feng and Aldrich, 2004). *Sargassum muticum* and *Fucus spiralis* removed Pb in a range of 32.3-50.4 mg/g. The report said 75% of the heavy metal uptake happened within 10 minutes (Nessim et al., 2011). Not just the previously mentioned two algal species worked fast but also *Pseudochlorococcum typicum,* which absorbed 70% of the Pb content just in 0.5 hours. In

general it can be seen that the highest heavy metal removal by Macroalgae happens in less than 120 minutes (Shanab, Essa and Shalaby 2012). A significant phenomena was reported by Baos et al. (2002), who were testing the adaptation property of *Scenedesmus intermedius (Chlorophyta),* a freshwater microalga. During tests, acid waste rich in heavy metals water (AWHM) solution with the content of Se (0.00015%), Hg (0.0015%), Ag (0.0025%), Cd (0.0025%), Bi (0.005%), Tl (0.005%), Co (0.0062%), Sb (0.05%), Cu (0.2%), As (0.5%), Pb (0.8%), Zn (0.8%), S (35-40%), Fe (34-40%) was applied and it was observed that the AWHM was toxic to this wild type, but when these cells started to die out spontaneous mutation occurred and 43 mutants per 1 million wild type cells raised. These AWHM resistant mutants were then using this acid waste as a substrate and in the absence of it they were driven to extinction (Baos et al., 2002).

TABLE 4: Pb removal efficiency by some Microalgae, Macro algae and *Cyanobacteria*

Microalgae	**Maximum removal**	**Additional Comments**	**Reference**
Chlorella vulgaris	17.2 mg/g	best pH 5.0	Sandau, Sandau, and Pulz (1996)
Pseudochlorococum typicum (Chlorophyta)	5–10 µg/ml	5,11 mg/g in 0.5 hour -> 70% efficiency	Shanab, Essa and Shalaby (2012)
Scenedesmus quadricauda var quadrispina (Chlorophyta)	5–10 µg/ml		
Schizomeris leibleinni (Chlorophyta)	65.47 mg/g		Özer, Özer and Ekiz (1999)
Scenedesmus intermedius (Chlorophyta)	n.a.	43 AWHM (acidic wastewater heavy metals) resistant mutants / million cells	Baos et al. (2002)
Macro algae			
Laminaria japonica	349.09 mg/g		Lee et al. (2004)
Laminaria hiperborea	23.9-39.5 mg/g	75% in 10 min	Nessim et al. (2011)
Bifurcaria bifurcate	18.6-32.0 mg/g		
Sargassum muticum	32.3-50.4 mg/g		

Fucus spiralis	32.3-50.4 mg/g		
Ecklonia radiate	281.78 mg/g	optimal pH 5.0	Matheickal and Yu (1996)
Ecklonia maxima	243.0 mg/g		Feng and Aldrich (2004)
Palmaria palmata	15.12 mg/g		Prasher et al. (2004)
Cyanobacteria			
Anabaena subcylindrica	n.a.	100% efficiency	El-Sheekh et al. (2005)
Spirulina platensis	16.98 mg/g		Sandau, Sandau and Pultz (1996)
Spirulina subspicatus	0.01 mg/g		Chójnacka, Chójnacki and Górecka (2004)
	95%		
Spirulina maxima	92%	best pH 5.5	Gong et al. (2005)
	85%	$CaCl_2$ pre-treated	
Phormidium ambiguum	5–10 μg/ml		Shanab, Essa and Shalaby (2012)
Lyngbya taylorii	304.56 mg/g	optimal pH 3-7	Klimmek et al. (2001)
Synechococcus sp.	30.45 mg/g		Gardea-Torresdey et al. (1998)

* mg/L: mg metal ion / ltr inoculum

1.3.3.2 Removal of Cadmium (Cd)

It has been reported by Mallick N. that to achieve higher efficiency of Cd removal by microalgae, some immobilization techniques need to be applied. These can be for example using plyurethane foam and/ κ-carrageenan gel fluized and packed bed (Mallick, 2002).

Concerning the efficiency rates of Cd removal, *Ascophyllum nodusum, Fucus Vesiculosus* and *Laminaria digitata* hold the first place with 98% when the initial metal concentration was 500 mg/l. Also *Spirulina platensis* had high performance at same Cd concentration. It was able to remove 81% of the metal ions (Sandau et al., 1996).

It can be seen from TABLE 5 that *Laminaria japonica* had high performance of Cd removal in solution with pH 4-5, it was able to take up 125.89-146.12 mg Cd/ g biomass (Mehta and Gaur 2005; Zhou, Huang and Lin 1998). After *L. japonica* the highest metal removal was seen by *Padina pavonia* (123.64 mg/g), *Sargassum subspicatus* (120.274 mg/g) and *Spirulina platensis* (120.04 mg/g) (Ofer et al., 2003; Zhou et al., 1998 and Cruz et al., 2004). *Scenedesmus obliquus and Desmodesmus pleimorphus* were tested in binary an single metal solution of Cd and Zn. *S. obliquus* performed better in binary solution. It was able to remove 58.5 % of Cd and 30.2% of Zn while D. pleimorphus worked better in single metal solution. It removed 27.7% of the initial Cd content (Monteiro et al, 2011).

It is important to mention that *Scenedesmus intermedius* was able to adapt to the toxic conditions by several heavy metals in acid waste spill and started to mutate. The rate of it was $2*12>10^{-5}$ mutants per cell division, 43 resistant mutants per 1 million cells (Baos et al, 2002).

The study made by Kumar and Oommen (2012) showed best Cd uptake at 40 ppm by *Spirogyra hyalina*after 90 min retention time.

TABLE 5: Cd removal efficiency by some Microalgae, Macro algae and *Cyanobacteria*

Microalgae	**Maximum removal**	**Additional Comments**	**Resource**
Chlorella vulgaris (green microalgae)	n.a.	efficient when immobilized in plyurethane foam and/ κ-carrageenan gel fluized and packed bed	Mallick (2002)
	490 mg/l	98% efficiency when the initial metal concentration was 500 mg/l	Sandau et al. (1996)
	4 mg/l	40% efficiency when the concentration was 10 mg Cd/l, maximum removal happened in 7 days	Sandau et al. (1996)
Pseudochlorococcum typicum	5–10 µg/ml	5,11 mg Cd/l removed in 0.5 hour -> 70% efficiency	Shanab, Essa and Shalaby (2012)
Scenedesmus quadricauda var quadrispina	5–10 µg/ml		

Desmodesmus pleiomorphus	1.92 mg/l	inhibition above 2.5 mg/l	Monteiro et al. (2011)
	58.6 mg/g (Cd^{2+})	39% efficiency (initial concentration of 150 mg/l)better removal of Cd than Zn in binary metal solution (300 mg/g Zn+Cd)	
Scenedesmus obliquus	175.6 mg/g	Binary metal solution of Cd and Zn (300-300 mg/l); 58.5% efficiency of Cd removal	Mehta and Gaur (2005); Zhou, Huang and Lin (1998) Monteiro et al, (2011)
Scenedesmus intermedius	n.a.	43 AWHM (acidic wastewater heavy metals) resistant mutants / million cells	Baos et al. (2002)
Nannochloropsis oculata	100.4 mg/g		Zhou, Huang and Lin (1998)
Spirogyra hyalina(freshwater algae)	9.832 mg/g	solution had 40 mg/l Cd content, highest bioaccumulation in 90 min; 20 mg/l solution, bioaccumulation time of 90 min	Kumar and Oommen (2011)
Macro algae			
Ascophyllum nodusum (marine brown algae)	n.a.	99.98% efficiency from effluent containing 10 mg/l Cd	Mallick (2002)
Laminaria japonica	125.89-146.12 mg/g	high capacity at pH 4-5,	Mehta and Gaur (2005); Monteiro et al. (2011)
Sargassum subspicatus	120.27 mg/g	pH 4.5	Mehta and Gaur (2005)
Padina pavonia	123.64 mg/g		Ofer et al. (2003)
Cyanobacteria			
Anabaena nodusum	91.84 mg/g	*n.a.*	Chong and Volesky (1996)
Phormidium ambiguum	5–10 μg/ml	5,11 mg Cd/l removed in 0.5 hour -> 70% efficiency	Shanab, Essa and Shalaby (2012)

* mg/L: mg metal ion / ltr inoculum

1.3.3.3 Removal of Mercury (Hg)

There have not been done as much studies about Hg removal by microalgae as from Cd, Pb and Zn. As it can be seen from TABLE 6 the highest biosorption by *Spirogyra hyalina* was achieved in 120 minutes, when the Hg concentration of the solution was 40 mg/l. *S. hyalina* was able to remove 39.212 mg/g Hg. *Phormidium ambiguum, Pseudochlorococcum typicum* and *Scenedesmus quadricauda var quadrispina* were tested in solution where Cd^{2+}, Hg^{2+} and Pb^{2+} were present. However 5 µg/ml Hg^{2+} concentration was highly toxic to all the three previously mentioned algae, in the first 0,5 hour contact time, they were able to remove 70% of the initial metal content. The initial Hg^{2+} concentration inhibited Chlorophyll A formation(?) and 20 µg/ml caused destruction of algal cell (Kumar, 2012; Metha and Gaur, 2005). *Spirulina subspicatus* was also tested but it had low efficiency as well, it could remove only 1.4 mg/g which equals 60% of the initial metal ion concentration (Chojnacka, Chojnacki and Go´recka, 2004).

TABLE 6: Hg removal efficiency by some Microalgae and *Cyanobacteria*

Species	Maximum removal	Additional Comments	Reference
Spirogyra hyaline (Micro alga)	39.212 mg/g	solution had 40 mg/l Hg content, highest bioaccumulation in 120 min	Kumar (2012)
Phormidium ambiguum (Cyanobacteria)	5–10 µg/ml	5,11 mg/g in 0.5 hour -> 70% efficiency	Metha and Gaur (2005)
Pseudochlorococcum typicum (Chlorophyta)	5–10 µg/ml		
Scenedesmus quadricauda var quadrispina (Chlorophyta)	5–10 µg/ml		
Spirulina spirulinoides. (Cyanobacteria)	1.40 mg/g	60% efficiency	Chojnacka, Chojnacki and Go´recka (2004)

* mg/L: mg metal ion / ltr inoculum

* µg/mL : µg metal ion / ml inoculum

1.3.3.4 Removal of Arsenic (As)

The acidic waste with heavy metal (AWHM) content mentioned in the previous chapters did not only contain Cd, Pb, Sb, Tl, Co, Cu, Zn but also As. The *Scenedesmus intermedius* was adapting the toxic conditions by producing resistant mutants. The rate of mutants per cell division was $2*12>10^{-5}$ which means 43 resistant mutants per one million cells. *Spirogyra hyaline, Scenedesmus intermedius and Rhizoclonium* did not show high As removal capacity (see TABLE 7). The *Spirogyra hyalina* could remove 8.719 mg/g and *Rhizoclonium* 0.105 mg/g, which results are not good enough, so they should be applied only in dense population (Kumar, 2012; Baos et al, 2002; Mallik, 2001).

TABLE 7: As removal efficiency by some Miroalgal species

Microalgae	Maximum removal	Additional Comments	Reference
Spirogyra hyalina	8.719 mg/g	solution had 40 mg/l AS content, highest bioaccumulation in 120 min	Kumar (2012)
Scenedesmus intermedius	n.a.	43 AWHM (acidic wastewater heavy metals) resistant mutants / million cells	Baos et al. (2002)
Rhizoclonium	0.105 mg/g	too small efficiency in case of small population	Mallik (2001)

* mg/L: mg metal ion / ltr inoculum

1.3.3.5 Removal of other heavy metals

The highest Zn uptake of 999.50 mg metal ion/ g biomass by microalgae was performed by the *Cyanobacteria Microcystis subspicatus* (Pradhan et al., 1998). The macoralgae *Laminaria hiperborea, Bifurcaria bifurcata, Sargassum muticum and Fucus spiralis* were not only able to remove Cd and Pb from the solutions (see chapters 1.3.3.1-2) but also some amount of Zn (23.9-32.0 mg/g, 18.6-32.0 mg/g, 32.3-50.4 mg/g and 32.3-50.4 mg/g) too (Nessim et al., 2011). Some other studies by Monteiro, Castro and Malcata (2009)

showed similar efficiency of Zn removal by another microalgal specie, *Scenedesmus obliquus.* This freshwater microalga was able to remove 112 mg Zn/g biomass content (78%) at pH 6. During the study made by also Monteiro, Castro and Malcata in 2010, *Desmodesmus pleiomorphus* was tested. It was able to remove 83.1 mg Zn/ g biomass. It was also observed that low concentration of Zn was beneficial for the growth of *D. pleimorphus.* The much-studied *Chlorella vulgaris* is also able to remove certain amounts of Zn and had high performance with Cu. There were several studies done and observed the best removal of Zn at pH 3.5 and 6.5. With this microalga the highest adsorption happened at 35°C and the highest intracellular uptake at 25°C. The tests done by Mallick showed efficiency of 53 mg Ni ions /g protein (Mallick, 2002). Another study does show 72.9% efficiency of Ni (II) removal by *Chlorella vulgaris* when the initial metal concentration was 42.4 mg/l at pH 4.5 (Dönmez et al., 1998). Concerning the Cu ions, the highest uptake performed by *C. vulgaris* cells was 420.63 mg/g protein and 437.98 mg Zn ions /g protein. These outstanding results were achieved by the acid pretreatment (HCl) of the cells (Mehta, Tripahti and Gaur, 2002). The comparative study made by Mehta and Gaur (2005) shows these results as the highest of all microalgae studied.

Dönmez et al. (1998) also tested the removal of Cu and Cr by *Chlorella vulgaris, Scenedesmus obliquus* and *Synechocystis subspicatus.* The highest Cu ion removal efficiency was by *Chlorella vulgaris* (85% at Cu concentration of 36.7 mg/l) followed by *Synechocystis subspicatus* (41.3% at 34.4 mg/l) and *S. obliquus* (34.2% at 30.1 mg/l). The efficiency order with Cd was *S. obliquus* (21.7% at concentration of 28.1 mg/l) *Synechocystis sp.* (20.7% at concentration of 29.9 mg/l) and *C. vulgaris* (18.8% at Cd concentration of 36.1 mg/l). It was also observed that when the Cu content was 10 times increased, the efficiency was raised the most by the previously mentioned weakest *S. obliquus* (2.6 times increase) and increased least by *C. vulgaris* (1.5 times increase). Another study by Metha and Gaur (2005) noted that 90% of the Cu removal by *Synechocystis sp.* is absorbed on the surface of the cells.

1.3.4 Methods to increase the efficiency of bioremediation

Some microalgae showed good results of bioremediation but in general these outcomes are not satisfactory for big scale application. However, using wild-types- or algal cells from contaminated environment showed increase of the heavy metal uptake, e.g. as by

Scenedesmus intermedius (Baos et al. 2002). In order to achieve higher efficiencies some changes need to be done on the cells or within their environmental system. The three most commonly used methods are applying non-viable cells, immobilization and pre-treatment of the cells.

1.3.4.1 Using non-viable and/ pre-treated cells

The surface of the microalgal cells contains different reactive groups like amine, Phosphate, carboxyl, imidazole, sulfhydryl, hydroxyl and sulfate groups which are functioning as ion-exchanger of the dead cells (Wilkinson, Goulding and Robinson 1990).

Even if cells are heat killed and metabolically inactive they can have same biosorption/bioaccumulation potential as viable cells with or without additional treatment. As it can be seen from TABLE 8 there are several pretreatment methods to increase the efficiency of biosorption. E.g. Mallik (2001) reports in his study that *Cyanobacterium microcystis,* which was heat-killed and formaldehyde treated, was able to biosorp the same amount of heavy metals as living cells of the same microalga. *Chlorella vulgaris* was treated in the similar way but also air dried before applying formaldehyde. This species was accumulating 80 % of the Ni and Cu applied and this potential was better than the one of the viable cells (Mallik, 2002).

The most economical and suitable pretreatment is the application of $CaCl_2$. This method was resulted 84-92% increase of Pb sorption by *Spirulina maxima* (Mehta and Gaur, 2005). Applying $CaCl_2$ showed good results with Cd and Cu too (Feng and Aldich, 2004).

The Xanthanation and Phosphorylation showed promising result of the removal of Cd and Pb. The Xanthanation of biomass consists of two steps. First the hardening of the cell wall happens by using epichlorohyrin. The second step is the introduction of xanthanate group by creating chemical reaction of the biomass with carbon disulfide (Klimnek et al., 2001 and Kim et al. 1999).

Also the pretreatment 1M KOH is used to enhance the metal uptake of algal biomass. It showed promising results of the removal of Cd (Hao, Zhao and Ramelow, 2001).

It can also be seen from the TABLE 8 that the efficiency of *Chlorella vulgaris* can be increased by applying “heat-killed, air-dried and formaldehyde treated” biomass of just pretreating with HCl (Mallik, 2002 and Mehta, Singh and Gaur, 2002).

TABLE 8: Increasing of biosorption efficiency by application of different pretreatment methods

Pretreatment method	Best efficiency reported	Treated algal specie	Resource
heat-killed and formaldehyde treated	n.a.	*Cyanobacterium microcystis*	Mallik (2002)
heat-killed, air-dried and formaldehyde treated	80% of Ni and Cu	*Chlorella vulgaris*	Mallik (2002)
heat-killed, air-dried and formaldehyde treated	84-92% increase of Pb removal	*Spirulina maxima*	Mehta and Gaur (2005)
application of $CaCl_2$	increased removal of Cd and Cu	n.a.	Feng and Aldich, 2004
Xanthanation of biomass	3 times higher removal of Pb	*Undaria pinnatifida*	Kim et al. (1999)
Phosphorylation	+581% of Cd and +109% of Pb removal	*Lyngbya taylorii*	Klimnek et al. (2001)
1M KOH	+95% Cd removal	*Ulva lactuca*	Hao, Zhao and Ramelow (2001)

1.3.4.2 Immobilization

Studies showed that immobilization of the cells in a solution can have beneficial properties concerning the resistivity of the cells against toxic conditions and the removal of heavy metals. When the cells are agglomerated in some manner of immobilization, they survive more easily within extreme pH conditions and high heavy metal content than the freely moving cells. Although the overall heavy metal removal from the solution is higher than in case of the free cells, the amount absorbed within the cell is smaller. In general there are two ways of immobilization, entrapment and absorption (Robinson et al., 1986; Bailliez et al., 1985; Tamponnet et al., 1985; Ding and Lee, 1994).

The entrapment generally happens by using some synthetic polymers or natural materials. The natural materials can be agar, carrageenan and alginate beds (e.g. Barium and

Calcium) (Mehta and Gaur 2005). Alginate beds are the most commonly used ones because of their beneficial properties of providing stable and protective microenvironment which ensures higher cell growth. Calcium alginate is the most popular alginate because it does not require heat treatment during the entrapment and it does not have any toxic effect so it is commonly used for the immobilization of microalgae. Even though alginate has many advantages, after a certain time it has decreased stability so it cannot be used in long term experiments (Robinson et al., 1986; Bailliez et al., 1985; Tamponnet et al., 1985; Ding and Lee, 1994).

The synthetic polymers are silica gel, polyacrylamide and polyurethanes. Compared to the natural immobilization materials the synthetic polymers have more toxic effects on the cells (Mehta and Gaur 2005).

As an example of Cd removal by *Chlorella emersonii* – immobilized in alginate fixed bead - much more heavy metal content stayed on the surface of the cells (and also some was taken up by the immobilization material) than was the case in free cells. Because of this phenomenon, the immobilization material needed to be removed together with the cell mass after the treatment period (Wilkinson, Goulding and Robinson 1990). A study of Hg removal by Mallik (2002) shows 90% of the Chlorophyll content remaining in the immobilized cells even after 3 months inoculation period, while free-cells displays pheophytization just after 7 days. The reason for this difference might be because the entrapped cells have lower rate of respiration, longer lag-phase and are metabolically less active (studies made with *Chlorella vulgaris* by Robinson et al., 1985). Also the higher stability of protein- Chlorophyll complex during application of immobilization can be a reason.

Adsorption can happen physically or chemically by the help of some solid supports such as coral stone and polyurethane foam. It is important to keep in mind that the immobilization might not be suitable for the production of the species which cannot be grown in high densities and strictly photoautotrophic because if these cultures are exposed to optimum light intensities so it would make the system expensive (Largeau et al., 1980 and Richmond, 2007).

Generally the collateral incidence of immobilization is volatilization. Through volatilization the toxicity of the liquid is reduced. This is desirable form one point of view – since it gives some small protection level against toxic effects and enables better cell growth, e.g. to

Chlorella – and unwanted on the other hand – the air-liquid interface needs to be minimized to prevent the loss of Mercury. While volatized Hg may enter the liquid phase again without immobilization of biomass, by using packed-bed reactors the volatized Hg may be accumulated in the biomass along the reactor. Some other immobilization materials than alginate are mentioned in the report of Wilkinson, Goulding and Robinson (1990) like agar and agarose. From these three the lowest volatilization rates were reported of the agarose by Wilkinson, Goulding and Robinson (1990).

But also κ-carregeenan gel fluidized and polyurethane were tried before. These two immobilization materials showed high efficiency with *Chlorella vulgaris* during the treatment with solution containing Cd, Cr and Zn (Mallik 2002). Gupta et al. (2000) also mentions Polyacrylamide gel, Polyurethane, Polysulfone and Calcium alginate applied with different species.

Other techniques, which can be applied as immobilization are flocculation, crosslinking, entrapment in polymetric matrix and covalent binding to carriers (Metha and Gaur, 2002).

1.3.5 *Desmodesmus subspicatus:* A suitable freshwater green alga to remove heavy metals

Desmodesmus subspicatus (DS) is a freshwater green alga which occurs as a free-floating plankton in the water column (Shubert, 2014). Planktonic algae can be defined as a seasonal succession in temperate lakes. The trophic status of the lake has effect on the size of the colony and appearance of DS (Bellinger and Sigee, 2010). This green alga can be found worldwide and often causes algal blooms in the present of excess nutrients so it is a good bio-indicator. Although, DS has larger size than some other green algal species it can be seen only under microscope. *Desmodesmus subspicatus* is a photosynthetic organism like other freshwater green algae because it has green Chlorophyll pigments surrounded by a chloroplast (Shubert, 2014).

1.3.5.1 Taxonomy, morphology and reproduction

Desmodesmus subspicatus comes from the genus Desmodesmus which was formerly named Scenedesmus. The Desmodesmus family has two subgenii by differentiation with microscope. The non-spiny form is called *Scenedesmus subspicatus* and the spiny form is

named *Desmodesmus subspicatus* (the microscopical picture under 1000x magnification can be seen on Figure 1).

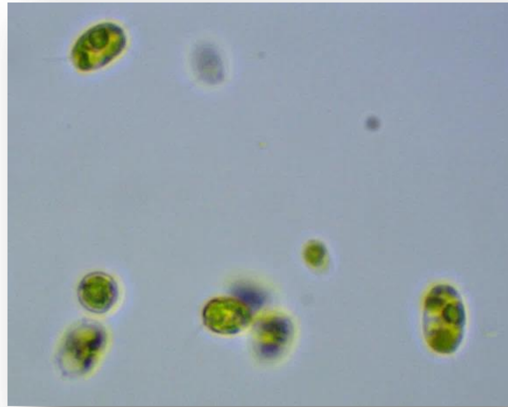


Figure 1: DS cells under 1000x magnification of Motic (Type: BA310) microscope

The subgenus DS can be characterized by its flat colony formation. These colonies are generally 2-, 4-, or 8-celled but rarely 16-, 32-celled ones also can occur. The cells are often lying parallel to each other and joined laterally. The shape varies between ellipsoid and ovoid with rounded apices and they have long spines or teeth on their surface. The cell has main spines which are longer than the rest. The cell wall of DS is toothed or spiny, granular and there is presented ribs and/or wart-like projections. The chloroplast of DS is parietal with a single pyrenoid.
Primarily during the reproduction of *Desmodesmus subspicatus* auto spores are released by the fracture of the lateral cell wall as an asexual mechanism (Shubert, 2014).

1.3.5.2 Behavior

Desmodesmus subspicatus has the ability to change its form as a response to the changing environmental conditions and by this it is exhibiting phenotypic plasticity.

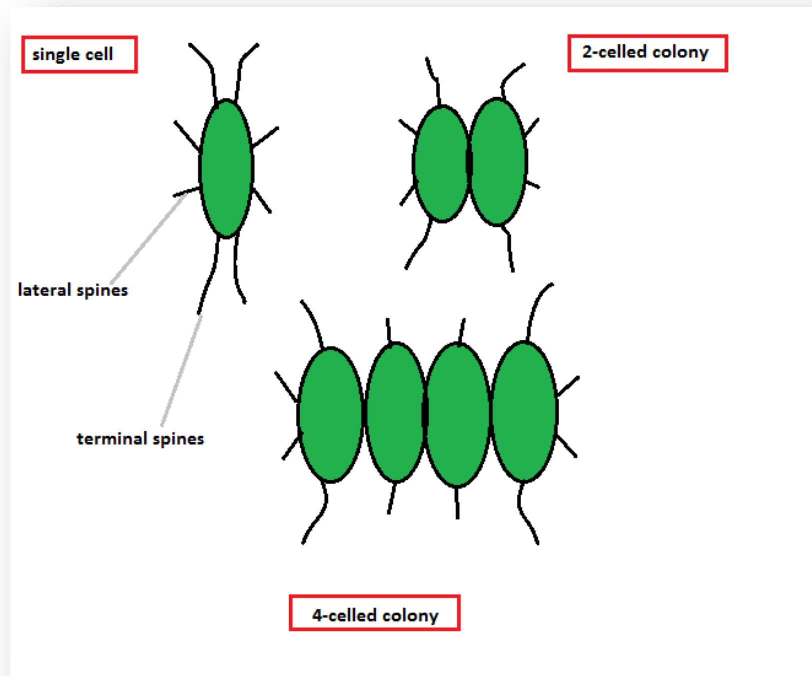


Figure 2: Different morphs of *Desmodesmus subspicatus* (Natural History Museum, 2014)

Depending on the changing environmental conditions the DS can have different morphs (see them on Figure 2). The most resistant form of DS is the colonies but the single cells, unicells are also common. This latter one means that within the colony an unicell is produced and it has more spines but has the same wall morphology as a cell of the colony. The unicell can occur also spineless. By increasing the Phosphorus or Nitrogen concentration, the morphological variation can be triggered. When the DS is isolated and grown in culture media free of other organisms with enough nutrients, clonal culture is formed by the unicells (Natural History Museum, 2014).

1.3.5.3 Beneficial properties

In general microscopic green algae have nutritious properties because they contain organic material, minerals and vitamins. But it is not only a nutrient source but also a source of energy. Green algae can be converted to biofuel because of their oil production and storage (Shubert, 2014). This type of algae is also a bioindicator, in the presence of excess nutrients it can cause algal blooms (Bellinger and Sigee, 2010). Freshwater green

algae can also play crucial role in maintaining the stability of an ecosystem because of their heavy metal absorbance property (Algae Control Program, 2014).

1.4 Chlorophylls: pigments responsible for photosynthesis

Chlorophylls are pigments which are essential for running photosynthesis. Two types of photosynthetic pigment can be distinguished. One of them is the green pigment, which is essential for plants, algae and *Cyanobacteria* to perform oxygenic photosynthesis. The other type is Bacteriochlorophylls, which exist in bacteria and responsible for anoxigenic photosynthesis. In the green pigments there is a porphyrin ring. This ring contains a ring-shaped molecule, which is surrounded by free migrating electrons around. Because of these freely moving electrons it is rather easy for the porphyrin ring to lose or gain electrons. During this interchange of electrons the light energy of the sunlight is captured (Munns, Schmidt and Beveridge, 2010).

Different kinds of Chlorophylls can be distinguished. First of all Chlorophyll a, which is the most common and important of all. This type of Chlorophyll exist in all plants, algae and *Cyanobacteria* with the ability to photosynthesize. The ring-shaped molecule of the Chlorophyll a is passing its energized electrons onto other molecules, which are then able to produce carbohydrates. The other type of these greenish pigments is Chlorophyll b (see the adsorption spectrum for Chlorophyll a and b from Figure 3). This type of Chlorophyll exists only in plants and green algae. The last type of Chlorophyll is Chlorophyll c, which occurs in *Dinoflagellates* and photosynthetic *Chromista* (University of California, 2006). There are also Chlorophyll types d and f, which only exist in *Cyanobacteria*. These Chlorophylls absorb infrared light (Munns, Schmidt and Beveridge, 2010).

The best absorbing wavelengths for plants, red- and green algae are between the range of 400-700 nm (Jabr, 2010). As it can be seen from Figure 3, Chlorophyll a and b are absorbing light energy on slightly different wavelengths. While Chlorophyll a has peaks at 450 nm and at 680 nm, Chlorophyll b has at 430, 470 and 640 nm. Chlorophyll b mainly absorbs blue, orange and red lights. Chlorophyll a is able to absorb also the same colours of lights but it works the best with the reddish one.

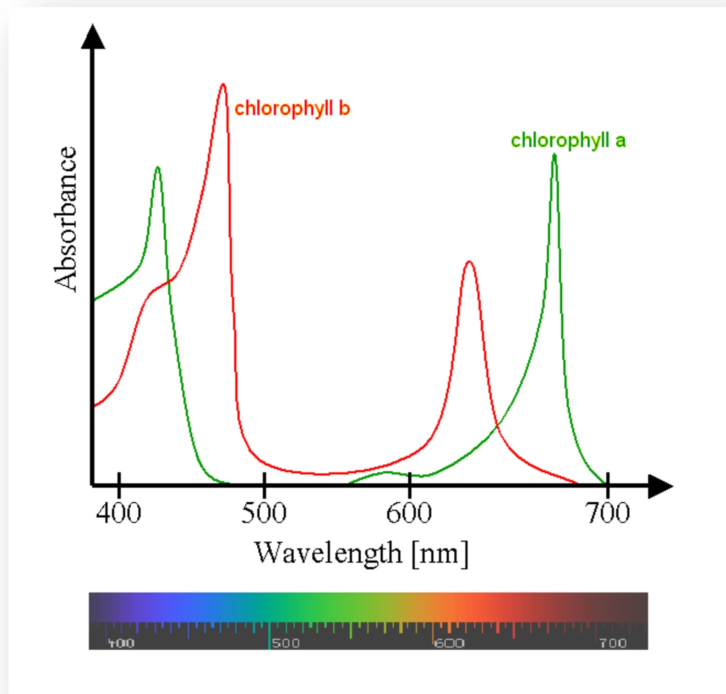


Figure 3: Adsorption of light energy at different wavelengths for Chlorophyll a and b (Pugliesi, 2008)

1.5 Research area: Harz Mountain

The Harz Mountains are located in North-Germany in the region of Lower-Saxony and Southwest from Berlin (see Figure 4). The Mountains are part of the northernmost German low mountain ranges. They are 30-40 km wide, 120 km long and the highest peak is 1142 m, the Brocken Mountain.

The folding of the Harz Mountains occurred in the late paleozoic Variscan orogeny and it rise in the Jurassic/Cretaceous age. Plutonites and metamorphic rocks (gneiss, gabbro, granite and hornfels) are the rocks present and at the western and southern parts due to the vaporization in the Permian age, gypsum, dolomite and anhydrite came up to the Mountains surface. The predominantly sulfuric ores contain different metals such as Mercury, Iron, Silver, Thallium, Arsenic, Copper, Cobalt, Gold, Lead, Cadmium, Magnesium, Zinc and some others (Gishler, 2008 and Ernst et al., 2004).

Figure 4: Harz Mountains on Germany´s map (Harz/Saxony-Anhalt, 2014)

1.5.1 Previous mining activities

Mining activities have been carried out for many centuries in the Harz Mountains. The earliest records available are from 968 (lead-zinc ore) but some slags and ores are said to be from the third and fourth centuries. The mining of sedimentary ores started in Rammelsberg Mountain in the tenth century. The mine with its 30 million tons of deposited ores became one of the world´s richest and most productive mining site. Around 1-2% of the world´s production of Zn and Pb came from this mine. Until the 19th century old techniques like stamping and separation by washing were used, which caused in 1820 25% of the lead to be lost to the rivers. Also there was no air filter used yet in the 19-20th century and the Oker- and Innerste dams were built only in 1960´s. Without the dams the process water was returned straight back to the environment. In 1978 the lead smelter Frankensharmhütte, 1988 Rammelsberg Mine and 1992 the Bad Grund Mine were closed because of their exhaustio (Gishler, 2008; Ernst et al., 2004; Gäbler and Schneider, 2000).

1.5.2 Heavy metal pollution of the environment

In this chapter the pollution caused to the environment within the Harz Mountain will be introduced by mainly focusing on a 12.5 km study area of Gäbler and Schneiders´ (2000).

The extraction of the ores happened with simple technology, the lack of air filters and reservoirs (until 1960´s) caused long term problems to the environment (see the mining sites on Figure 5). The heavy metal content from washing the stamped ores or by the dust from the ore deposition could easily reach the rivers. As, Cd, Pb, Cu, Tl and Zn were heavily loaded to the rivers Innerste, Oker, Nette, Ruhme, and Oder (see the river basins and floodplains also on Figure 5). Some of the heavy metal content settled within the river´s and the rest was transported during flooding to floodplains even as far as 100 km. After the construction of Innerste-, Grane-, Oker-, Söse- and Oder reservoirs and closing the mining sites, the leaching from mine dump heavy metal sources to the rivers was improved but some considerable amount of Cadmium, Zinc and Lead emission is still detectable. Recently Pb in the fishes of Oker and in the bones and livers of bats in Lower-Saxony is still found (Ernst et al., 2004; Gäbler and Schneider, 2000).

The negative sides of mining activity are not only recognizable on the aquatic system but also on the flora. First there was just deforestation because of the need for wood but later on, when the mines were operating, even bigger harm was caused to the nature. The dust and the water bodies from the extraction were carrying heavy metal content to the surrounding environments and the soil became too toxic for coniferous and broad leaved forests. Because of these toxic conditions forests gradually disappeared and vegetation of metal resistant herbs and grasses was established in their place (Ernst et al., 2004).

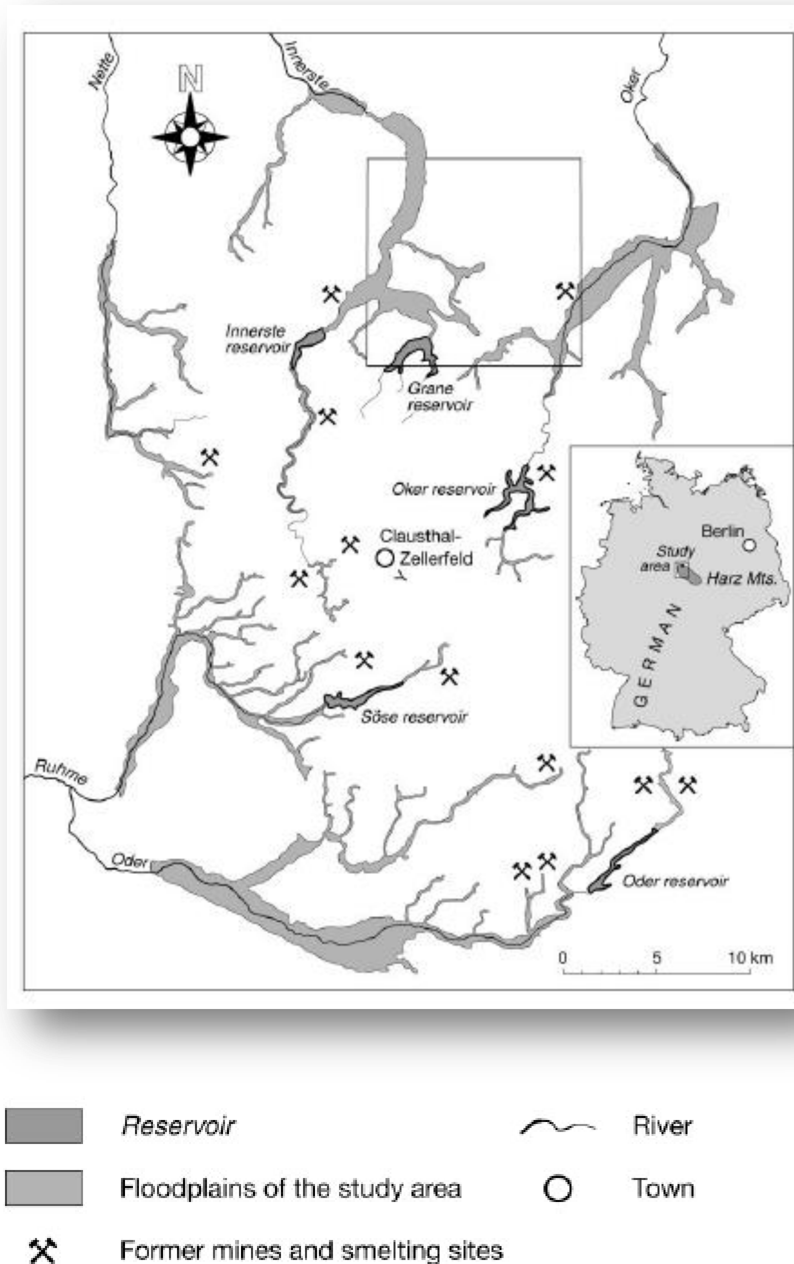


Figure 5: Waterways and mining activities in the Harz Mountains (Dreschhoff, 1974; Gäbler and Schneider, 2000)

The 12.5 km study area (see Figure 6) of Gäbler and Schneider was located around Goslar and consisted of the Innerste floodplain, Oker floodplain, Grane floodplain and some sections of the Innerste-, Oker- and Grane rivers. With respect to the heavy metal mobility, the study defined four risk categories (See Figure 6). Locations within the I-III categories are not causing any harm if no change of the present use of the area/land will take place. The least polluted sites (I) without detection of mobile heavy metal fraction

were found North at the far-most floodplain of the Innerste river. The heavy metals content of these sites are below the BW III category (see TABLE 9). The Innerste- and Oker floodplains had low risk (II) to the environment but the Grane floodplain can be hazardous (III) in case of a pH drop below 6.2, which needs a lot of attention considering it is an agricultural area. One of the samples taken from site II and also from site III had heavy metal concentration above BW III. The highest risk (IV) was shown at the catchment area of the Oker and Innerste rivers. These areas of the category IV are considered as hazard to the environment (Gäbler and Schneider, 2000).

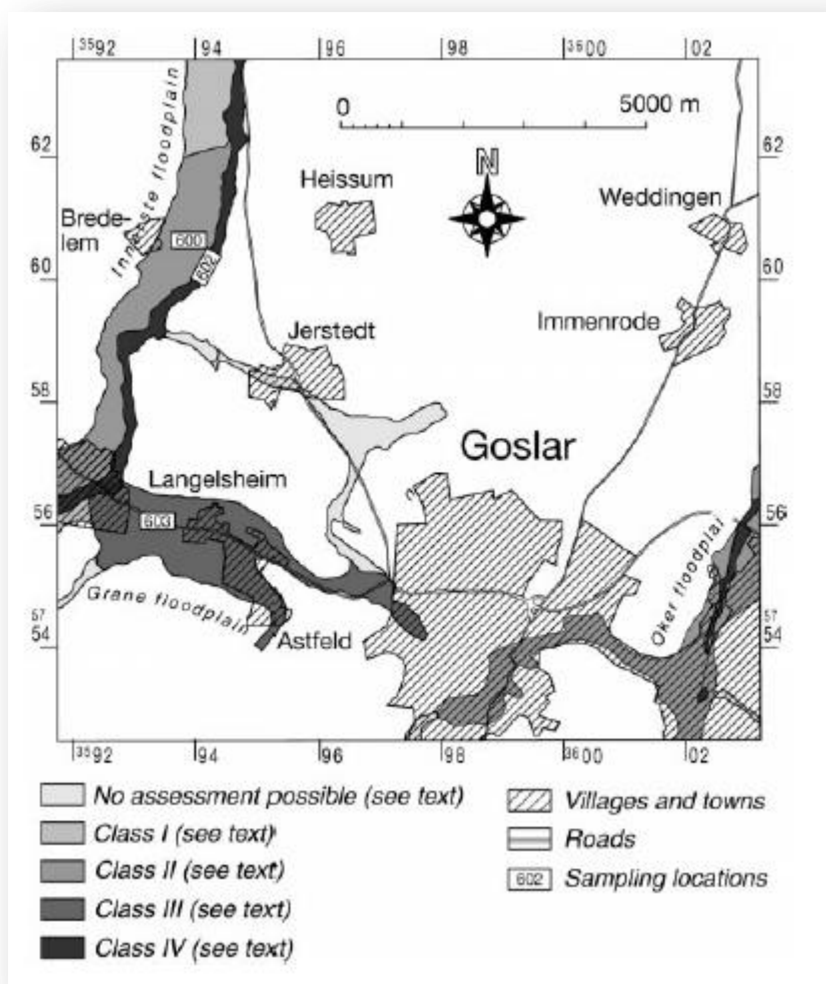


Figure 6: Locations of different risk categories (Dreschhoff, 1974; Gäbler and Schneider, 2000)

TABLE 9: Guideline values for agriculturally used soils by Eikmann and Kloke (Eikmann and Kloke, 1993)

Guideline value	Pb (mg kg^{-1})	Cd (mg kg^{-1})	Zn (mg kg^{-1})	Cu (mg kg^{-1})
BW I	100	1	150	50
BW II	500	2	300	50
BW III	1000	5	600	200

*BW: Bodenwert in German, which means Acceptable value of soil (Gäbler and Schneider, 2000)

2. Materials and methods

2.1 Laboratory testing

The laboratory tests of this thesis work had four phases (see the exact times of the different phases with the measurements done from Figure 7). The first phase was the "inoculation-phase", where a preparation of the stock cultures necessary for inoculation of the subsequently studied cultures took place. After isolation of single cells and raising the first cell cultures in an Erlenmeyer flask a photobioreactor (INFORS AG, Type: CH-4103) was used to produce the amounts of cell cultures necessary for the subsequent studies.

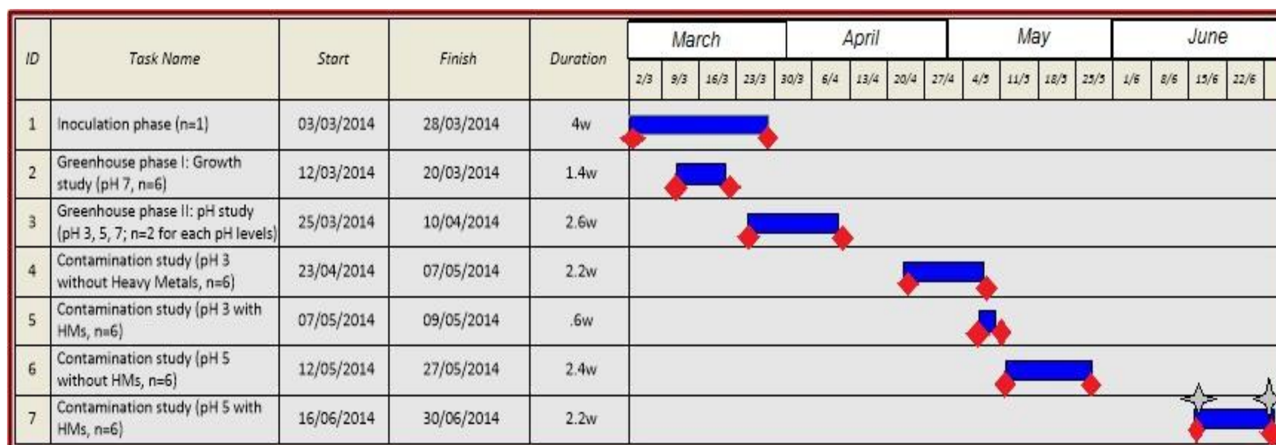
The "Greenhouse phase I: Growth study" served to observe the growth and cultural development behavior of the microalga *Desmodesmus subspicatus* (abbreviation DS) when cultivated within semi-field environmental conditions of a greenhouse without artificial light or heating, since later the use of algal cultures outside of laboratory environments should be possible for decontamination purposes. 5 liters bioreactors (n=6) with the same conditions ensured were employed for that purpose.

The third phase was the "Greenhouse phase II: pH study" in order to establish the pH sensitivity of the microalga *Desmodesmus subspicatus* by monitoring cultural growth within the greenhouse. This was necessary, since many of the heavy metal contaminants are not soluble and thus not readily accessible for the algae at pH levels at or above 7. Three pH levels (3, 5 and 7; n=2 reactors each) were tested in duplicates using 5 ltr. bioreactors.

The last phase was the "Contamination study". Here cultures were monitored in two separate experimental runs under a closely controlled laboratory environment within a light incubator (BINDER GmbH, Type: KBW 400) using six 1 ltr. bioreactors for each run. The first run consisted of control cultures to establish the growth behavior of *Desmodesmus subspicatus* under these conditions. Within the second run selected heavy metal concentrations (300µg/l Pb, 30µg/l As, 30µg/l Cd, 30µg/l Hg) resembling heavy metal contamination values found within the river Oker in 2002 (Gewässergütebericht Oker, 2002) were added to each of the bioreactors, thus creating six replicates of this run.
During all the phases the culture growth parameters as optical density, cell count by Thoma-chamber, coulter count and Chlorophyll a measurements were monitored on a daily basis.

Starting from the second phase samples of approximately 35 ml were taken to assess nutritional status (PO_4, NH_3, NO_2, NO_3) at the start and end of each phase and stored for further analysis in the freezer at -18°C.

As an addition in the third and fourth phases, extra samples were taken and frozen for determination of dry matter (DM) of the samples before and after treatment.



ID	Task Name	Start	Finish	Duration
1	Inoculation phase (n=1)	03/03/2014	28/03/2014	4w
2	Greenhouse phase I: Growth study (pH 7, n=6)	12/03/2014	20/03/2014	1.4w
3	Greenhouse phase II: pH study (pH 3, 5, 7; n=2 for each pH levels)	25/03/2014	10/04/2014	2.6w
4	Contamination study (pH 3 without Heavy Metals, n=6)	23/04/2014	07/05/2014	2.2w
5	Contamination study (pH 3 with HMs, n=6)	07/05/2014	09/05/2014	.6w
6	Contamination study (pH 5 without HMs, n=6)	12/05/2014	27/05/2014	2.4w
7	Contamination study (pH 5 with HMs, n=6)	16/06/2014	30/06/2014	2.2w

Figure 7: Timelines for the different study phases. Blue bars represent the daily sampling for assessment of OD, Cell count, pH and Chlorophyll a values, red turned square represents sampling for nutrient analysis (NO_4, NO_3, NO_2, PO_4) at the beginning and end of study, grey star indicates sampling for heavy metal analysis of media and biomass within the treatment group.

2.1.1 Inoculation phase

2.1.1.1 Isolation of *Desmodesmus subspicatus*

Microalgae are isolated from one individual cell or filament of a genetically clone propagated culture. Filaments or single cells can be picked up by using a dissecting microscope and a micropipette and then transferred to an agar plate with a sterile medium (Richmond, 2007). This isolation step was done by a laboratory assistant of Ostfalia University of Applied Sciences.

Light energy is essential for algal growth and metabolism so the source of it needs to be ensured during the isolation too (Richmond, 2007). During the isolation of *Desmodesmus*

subspicatus, INFORS AG CH-4103 photobioreactor was used (see chapter 2.1.1.2 Using photobioreactor).

2.1.1.2 Enrichment of the culture

Double concentrated ES-media solution mixed with ionized water (see the recipe for one liter of the media in the Appendix 1) was prepared for the 3 liter volume of a photobioreactor. To the media approximately 1 million cells/ liter of DS needed to be added, but before the right amount could be calculated, the cell count of the culture needed to be done by using Thoma-chamber (see the method in chapter 2.2.1). When the amount of cells in the inoculum was given, the amount to be added was calculated by the Eq. 1 seen below. (1)

$$c_1 \cdot V_1 = c_0 \cdot V_0 + c_m \cdot V_m$$

C_1: required cell concentration
V_1: required volume
C_0: cell concentration of the inoculum
V_0: volume of the inoculum
C_m: cell concentration of the media
V_m: volume of the media

2.1.1.3 Using photobioreactor

A photobioreactor is a system, which ensures optimal light and Oxygen conditions for a culture. The greatest proportion of light (more than 90%) passes through the transparent reactor´s wall and reaches the cells but does not impinge directly on their surface. Because of this property and its closed system the contamination inside the reactor is minimized (Richmond, 2007).

For preparation of stock cultures of *Desmodesmus subspicatus* the photobioreactor (INFORS AG, Type: CH-4103) seen on Figure 8 was used. The DS culture was inoculated (1 million cells/ml medium) and cultivated in this reactor for 28 days, when the culture achieved its stationary phase.

Around the reactor there are 8 strip lightings established and each of them were switched off approximately for 6 hours. The switching was done manually so the lights were left on for the whole weekend. The temperature of the medium was kept on 28° Celsius by the help of a water ring around the inner reactor. The pH was kept at 7 and adjusted via CO_2 addition. Also stirring was established at 180 rpm and continuous aeration applied.

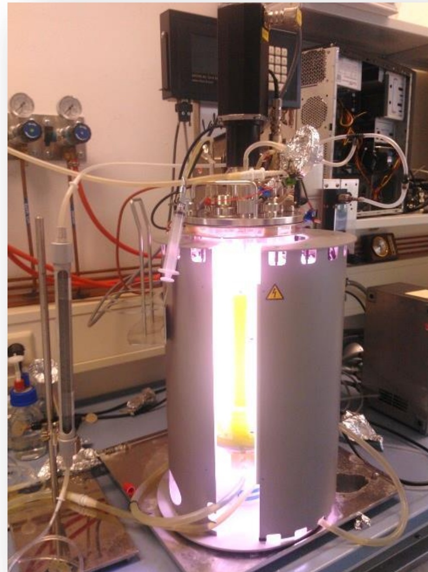


Figure 8: Photobioreactor used for the preparation of *Desmodesmus subspicatus* stock soulution for inoculation in the different experimental runs (Growth conditions: 6 hrs light/ 18 hrs dark, 28˚C, stirring 180 rpm, pH7, CO_2 adjusted, continuous aeration)

2.1.2 Greenhouse phases

This phase was run to see the development of *Desmodesmus subspicatus* culture in natural light and temperature. In the greenhouse there were 2 experimental runs. The first was done with 6 bioreactors at pH 7 through 8 days and the second with pH 7 (n=2), pH 5 (n=2) and pH 3 (n=2) through 16 days.

2.1.2.1 Greenhouse phase I: Growth study

This Growth study phase was run to see how the DS cells are reacting to semi natural conditions within glass tube reactors outside in a greenhouse. No additional light or heat was applied.

This time not the photo bioreactor was used for growing the culture but 6 tube reactors (see 2.5.2.1 Experimental setup chapter) each with a volume of approximately 5.5 liters. 3x10 liter ES media (preparation see Appendix 1) with 30 g/10 l $NaHCO_3$ as an additional C source were prepared and divided up between the six reactors. The volume of inoculum needed for 1 million cells/ml and optical density of 0.1 λ (according to Eq. (1) 2.1.1.2 and Eq. (2)) were calculated. The Eq. 1 explains how the DS stock solution to the reactors was calculated by using cell count and the Eq. 2 by using optical density of the culture. After calculating the needed volume the both ways, the mean value of them was taken. The previously calculated volume of stock culture (9 days old) from the photobioreactor was then added to the media. But before adding it, the mixture was aerated for 30 minutes and then the same volume of media was removed to maintain the 5 liters volume to be put to the reactors. Also to ensure the same starting conditions within the reactors, the pH of the solutions was adjusted to pH 7 by buffering them with HCl (60%). To each of the reactors 5 liters of solution were poured in the way that the 1st 10 liter portion went to the reactors 1-2, the 2nd to the reactors 3-4 and the 3rd to the reactors 5-6.
The duration of this pilot study phase was 9 days.

$$c_1 \cdot V_1 = c_0 \cdot V_0 + c_m \cdot V_m \qquad (2)$$

C_1: required optical density
V_1: required volume
C_0: optical density of the inoculum
V_0: volume of the inoculum
C_m: optical density of the media
V_m: volume of the media

2.1.2.2 Experimental setup

All together 6 tube reactors were set up outside in the greenhouse. The reactors were made of glass and each of them had a volume for 5,5 liters of liquid (see Figure 9). Inside the tubes two plastic pipes were installed, one for aeration and one for sample taking. To each of the aeration tubes air stones were attached to ensure the Oxygen flow throughout

the whole content of the bioreactors. The aeration was run by using two small scale aquaristic pumps (see on Figure 9). Due to technical issues with the pumps it was not possible to adjust any flow rate so it slightly differed in the reactors. Besides the pipes, a data logger (Hobo Pendant Data Logger, Type: 64K-UA-002-64) was inserted into the first reactor and another one also inside the greenhouse to provide environmental data (temperature, sunshine intensity and duration as constant measurement parameters).

Figure 9: Tube reactors used during the Greenhouse phases. On the right picture the greenhouse, reactors (middle) and aquaristic pumps (left bottom).

2.1.2.3 Greenhous phase II: pH study

Within this phase the reaction of DS cultures in terms of growth and culture development to different pH values of 3, 5 and 7 was tested. For each pH level 10 liters of media (see double concentrated ES media from Appendix 1) inoculated with 1 million cells of DS/ml and 30 g/10 l $NaHCO_3$ as an additional C source were prepared. The different pH levels were adjusted same way as mentioned in chapter 2.5.2. The reactors 1 and 2 were started with pH 7, the 3 and 4 with pH 5 and the reactors 5 and 6 with pH 3. The setup was the same as in the “Pilot study phase” and the duration of this third phase was 14 days.

2.1.3 Laboratory phase: Contamination study

The laboratory study phase consisted of two experimental runs (Control and treatment) for either pH 3 or 5 adjusted media. First the control cultures were established in the chosen pH and in the second round heavy metal solution with the content of 30 µg/L As, Cd and Hg and 300µg/L Pb was added to the freshly prepared cultures. Both of the rounds were run with pH 3 and 5.

2.1.3.1 Control samples with chosen pH levels

In this test phase 6 times 1 liter glass reactors were used (see on Figure 10). The inoculum was prepared same way as mentioned in the “2.1.2.1 Greenhouse phase I: Growth study” chapter but this case calculated for 6 liters. At this time the cultures were taken from the best performing reactor of the ”Greenhouse phase II: pH study” with different pH levels.

Figure 10: Glass reactors inside the light incubator

The glass reactors were cleaned with ionized water before adding the inoculum. Aeration was established by using air filters with pipes attached to the glass walls (see Figure 11). The aeration of the pump was adjusted to 10 l/h to each of the reactors to ensure proper Oxygen supply for development of the cells. Also a magnetic stirrer was added to each reactor for stirring and a top to have a controlled system. The ready reactors with the inoculum in them were placed onto magnetic plates providing 150 rpm stirring inside a light

incubator (BINDER GmbH, Type: KBW 400). Inside the light incubator the temperature was set up to 28 ˚C, light conditions to 14 hours and dark for 10 hours as an imitation of light conditions in the natural environment (see the set up on Figure 10).

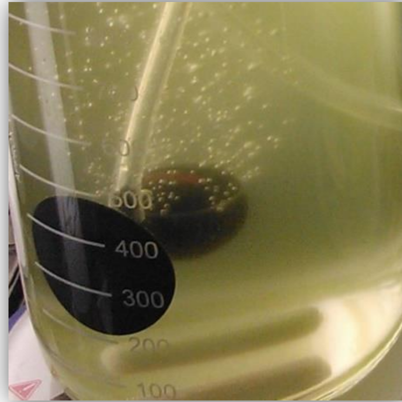


Figure 11: Aerotation by using air filter (flowrate adjusted to 10 L/hour)

Closer to the strip lighting the light can penetrate better through the glass walls so to avoid the different light condition to the reactors, the place of them were switched daily. The general scheme was to rotate the reactors by one and every second day changing the direction. On the Figure 12 the blue and red arrows are showing the directions of daily switches.

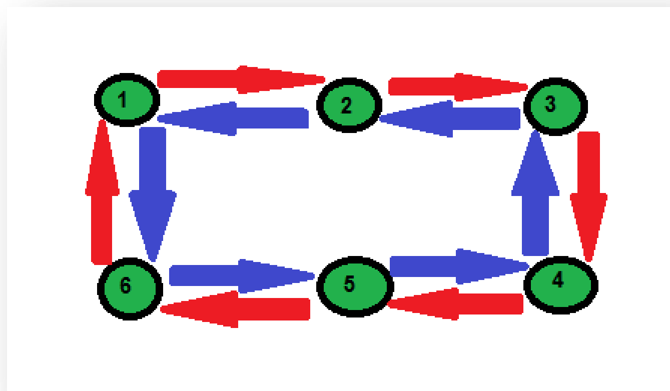


Figure 12: Daily rotation of the reactors inside the light incubator

This test phase was first run with pH 3 and later on with pH 5.

2.1.3.2 Heavy metal content phase

The set up and conditions inside the light incubator were the same as in the previous chapter 2.1.3.1. The only difference was the addition of heavy metals. A heavy metal solution containing 10 ml (100mg/l) Pb and 1 ml (10 mg/l) As, 1 ml (10 mg/l) Cd and 1 ml (10 mg/l) Hg was prepared. From 6 liters of the prepared inoculum (see preparation from 2.1.2.1 Pilot phase) 18 ml of sample was removed and replaced with 18 ml (3ml solution / ltr inoculum) of heavy metal solution, resulting in a heavy metal contamination with a concentration of 30 µg/L As, Cd and Hg and 300µg/L Pb.

At the beginning of the "Heavy metal content phase" 2 x 40 ml of sample was taken from the starting culture with HM. To preserve the sample, couple drops of 65% HNO_3 was added and placed to the freezer (-18˚C). Samples were also taken from the end cultures (see the steps from "2.2.3 Heavy metal pollution analysis").

This test phase was first tried out with pH 3, but because of culture failure to adapt and grow, it needed to be run also with pH 5.

2.1.3.3 Monitoring of heavy metal (As, Cd, Hg, Pb) polluted culture with pH 3

Even though the *Desmodesmus subspicatus* cultures did not perform well in the HM polluted medium with pH3, the culture with the best results was saved and stored for further monitoring and future cultivation. The preparation of the Culture was the following.

The remaining of the culture from the light incubator was filled up with 2xES medium and placed on a magnetic stirrer near the laboratory window. The stirring was adjusted to speed of approximately 100 rpm. No additional aeration was added so the top of the reactor was slightly closed to ensure the Oxygen supply. The culture was stored for 5 weeks when 288 ml of the supernatants ($1*10^6$ cells/ml, 0.1λ) was removed and mixed with 2xES calculated for 500 ml (see concentration from Appendix 1). The new culture was also placed near to the original culture with HM and pH3. The air supply and stirring was same as of the original culture. After 11 days retention time, microscopic pictures were taken with 1000x magnification of Motic (Type: BA310) microscope. Picture of the color change of the new cultures was also made.

2.2 Methods

Different tests needed to be done to follow the development of the DS culture. Some of these tests were run daily (optical density, Chlorophyll a, cell counts, pH measurement), some after the phases (dry matter measurement, nutrient analyses) and some (heavy metal analyze) only after the heavy metal contamination phase.

2.2.1 Growth parameter measurements

The measurements to monitor the development of the cultures were run on a daily basis. These consisted of Optical Density (OD), Cell counts by Thoma-chamber and Coulter Counter, pH and Chlorophyll a detection. As an addition the increase of biomass was also monitored by measuring the dry matter (DM) contents of the cultures at the beginning and at the end of the test periods.

2.2.1.1 Optical density (absorbance)

For the measurement of optical density as a parameter for monitoring the population development of the microalgae investigated, a Spectrometer (Unicam UV/Vis Sectrometer) is used. First light is directed through a fiber optic cable into the spectrometer via an entrance slit, which labels the light when it enters the spectrometer. After this, the collimation of the divergent light happens by a concave mirror which directs it then onto a grating. This grating separates the different components of the light by their varying angles. These components are then focused by another concave mirror and give an image onto the detector. After all, the photons are converted within the detector into electrons, which are then read and digitized (B&W Tech, 2014).

Before the optical density measurement, the Spectrometer (Unicam UV/Vis Sectrometer) was calibrated with ionized water samples at the wavelength of 750 nm. After the calibration, the left-hand side ionized water carrier was changed with one containing the sample from the bioreactor and also measured on the wavelength of 750 nm.

2.2.1.2 Cell count by Thoma-chamber

The counting chamber contains two frames with one central squares (each of them has an area of 1 mm^2). The central squares can be seen entirely with 100x magnification of a microscope but for the cell counting the 400x magnification is used. Each of these squares contains 16 smaller squares which are also divided to 16 even smaller ones. Before using the microscope a coverslip is put over the frames and small amount of sample is injected under to both of the frame areas. The suspension of the samples under the coverslip reaches a height of 0.1 mm. Considering this, the total volume of sample over each frames is 10^{-4}ml (1 mm^2 * 0.1 mm) (University of the Basque Country, 2014).

In the laboratory the microscope used was Carl Zeiss (Type: Axiostar plus) and the principle for the determination of the cell density by the Thoma-chamber was to count the cells in 1st, 6th, 11th and 16th squares (see Figure 13) in both of the frames and then the calculation was done by using the Eq. 3 seen below. (3)

$$cell\ density = \left[\frac{cell\ count}{2*4*6} * dilution * 1000ml\right] \div 0.00025$$

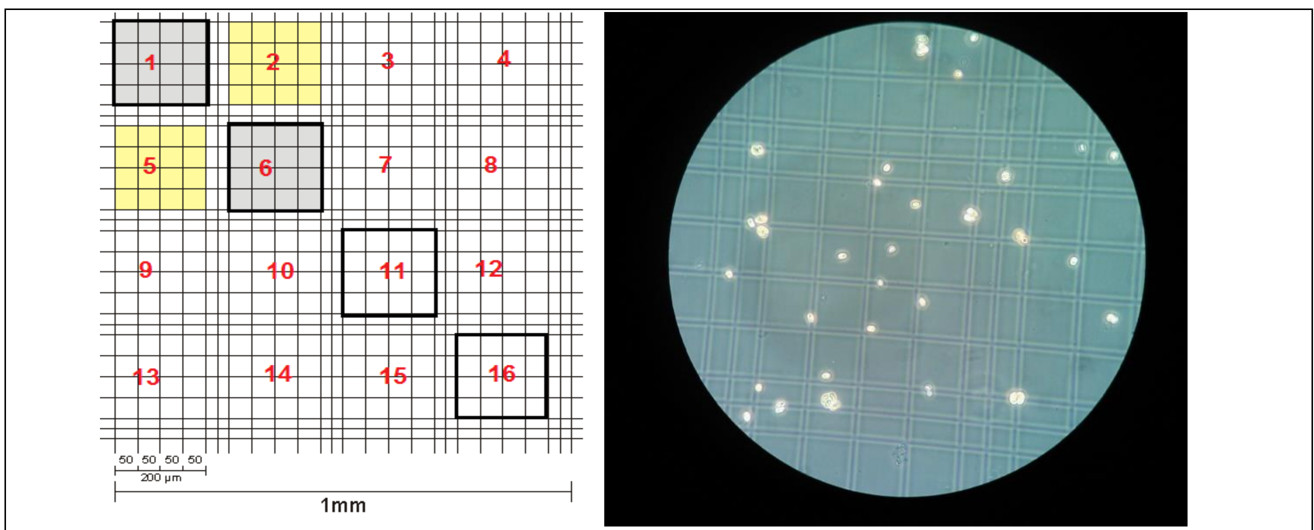


Figure 13: Frame of Thoma-chamber with counting square (Alcibiades, 2006) and the same under 400x magnification of microscope (Carl Zeiss, Type: Axiostar plus)

2.2.1.3 Coulter count

The Coulter Counter technology was originally developed to count blood cells but nowadays it can also be used to measure the density of wide range of samples such as toners, clay, minerals, coating materials, explosives, algal biomass etc. The principle of this technology is to mix some sample with some electrolyte and immerse the electrodes (inside and outside the aperture) of the Coulter counter into the mixture. A “sensing zone” is created by the aperture and when the electrolyte containing small amount of sample passes through the aperture, the particles can be counted (Beckman Coulter, 2014).

During the density measurement of *Desmodesmus subspicatus*, 19.8 ml NaCl solution was put to 200µl of sample in a beaker and the Coulter counter´s electrodes were immersed in it. Before starting the program, the particle size range was set to 3000-6000µm. The Coulter Counter used for the cell count is shown on Figure 14.

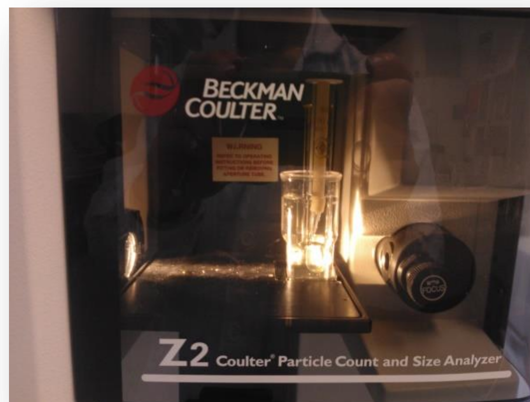


Figure 14: Beckman cell counter (Beckman Coulter, Type: Z2) in operation - used in this studies´ laboratory tests

2.2.1.4 Chlorophyll a

The development of the cell can be seen from its Chlorophyll a content. To be able to measure this concentration, the Chlorophyll a need to be extracted the following way.

For the Chlorophyll a measurement, 0.5 ml of sample and 4.5 ml Methanol were injected into a test tube under the hood and then covered with metal cap. The next step was to vortex the sample well and then leave it in 70°C water for 10 minutes. When the time was

over, the sample was put into a centrifuge (Eppendorf AG, Type: 5418) at 3000 rpm for 5 minutes. After the preparation steps of the sample, the Spectrometer (Unicam UV/Vis Sectrometer) first was calibrated with Methanol samples at the wavelength of 665 nm and then the left-hand side Methanol sample was changed with the previously prepared sample containing culture. The mixture was first measured at 665 nm and also at 750 nm. The best absorbance of Chlorophyll a happens at 665 nm (see "1.4 Chlorophylls" chapter). To reduce the error of measured value the turbidity needed to be measured at 750 nm. When the values were given by the spectrometer, the calculation of Chlorophyll a content of the culture was done as it is showed in Eq. 4.

$$\textbf{Chlorophyll a} = \frac{[extinction\ (650nm) - extinction\ (750nm)] * 13{,}9(calibration\ factor) * 0{,}5ml}{4{,}5ml} \quad (4)$$

To minimize the error of measurements, Methanol-sample mixtures were prepared in duplicates from each reactor.

2.2.1.5 Dry matter (DM) content

To see how much the dry matter content of the solution was changing by the end of the treatment period, samples from each of the reactors at the beginning and at the end of the experiments were taken. For this measurement first the weight of the crucible was checked and noted with its serial number. Than the sample from the bioreactor was added, weighted and inserted to the oven for 48 hours at 105°C. When the sample was cooled down, its weight was checked and noted. The DM content was then calculated by using the Eq. 5. (5)

$$\boldsymbol{DM\ (g)} = weight\ of\ sample\ from\ oven - weight\ of\ crucible$$

$$\mathbf{DM}\ (\%) = (\text{weight of sample from oven} \div \text{weight of wet sample}) * 100$$

2.2.1.6 pH measurement

From the "Greenhouse phase II: pH study" the pH of the samples from each reactors were measured on daily basis. This was the first measurement done on the samples daily to

avoid change of pH because of different environment (temperature, contact with air). For this WTW GmbH (Typ: pH 530) was used.

2.2.2 Monitoring Nutritional Status

The availability of nutrients is one of the most important factors besides light and temperature to ensure optimal conditions for the cells. The Phosphate, Ammonium, Nitrate, and Nitrite content of the samples were tested at the beginning and end of each test phases to ensure these conditions had been met throughout culturing period. From the results of test it can be seen how much of the available nutrients were consumed by the cells during the test phases. Also connection between cell growth and nutrient availability can be visualized. These tests were done concerning the recommendations of DIN-ISO 15923.

2.2.2.1 Phosphate content (PO_4-P)

The procedure for the detection of PO_4-P content of the samples from the reactors was the taken from DIN-ISO 15923. One test tube was prepared to each of the samples, one for a blank sample and also one to each of the different concentrations (0.1, 0.5 and 1.0 mg/l) of PO_4 standard curve. First of all 5 ml of the samples were injected to all of the tubes for the samples, 5 ml of distilled water to the tube for the blank sample and 5 ml of the different PO_4 concentrations were added to their own test tubes. The second step was adding 1 ml of the PO_4-mix (see recipe from Appendix 2) to each of the test tubes. After injecting the PO_4-mix, all the test tubes were vortexed and incubated for 10 minutes at 30-40 ˚C. The next step was the measurement of PO_4 by using Spectrometer at 880 nm wavelength. When the values were inserted into an Excel sheet, a linear trendline was drawn from the value of blank sample and the three different PO_4 concentrations to establish a standard curve. The value of the blank sample was subtracted from the measured values of the samples and using the equation of the slope, the amount of PO_4-P contents were calculated in mg/l.

2.2.2.2 Ammonium content (NH_4-N)

The procedure for the detection of NH_4 content of the samples from the reactors was taken from DIN-ISO 15923. One test tube was prepared to each of the samples, one for a blank sample and also one to each of the different concentrations (0.1, 0.5 and 1.0 mg/l) of NH_4. First of all 10 ml of the samples were injected to all of the tubes for the samples, 10 ml of distilled water to the tube for the blank sample and 10 ml of the different NH_4 concentrations were added to their own test tubes. The second step was adding 1 ml of the NH_4-1 reagent (see recipe form Appendix 2) to each of the test tubes. After injecting the NH_4-1 reagent, all the test tubes were vortexed and incubated for 10 minutes at 30-40 ˚C. The next step was the addition of 1 ml of NH_4-2 reagent (see recipe form Appendix 2) to each of the test tubes and then vortexing them. The mixtures were incubated again for ten minutes and after that, their NH_4 contents were measured by using Spectrometer at 660 nm wavelength. When the values were inserted into an Excel sheet, a linear trendline was drawn from the value of blank sample and the three different NH_4 concentrations to establish a standard curve. The measured values of the samples (blank value subtracted from each) were inserted to the equation of the slope, which gave then the amount of NH_4 content of the samples in mg/l.

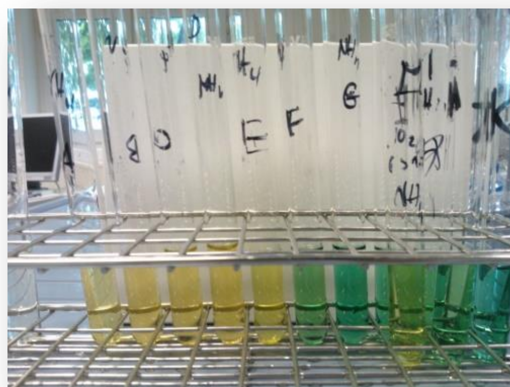


Figure 15: Different colors of samples as indicators of NH_4 content. Yellowish color indicates small NH_4 concentration and darker color of greenish blue responds to higher concentrations.

2.2.2.3 Nitrate content (NO_3-N)

The procedure for the detection of NO_3 content of the samples from the reactors was taken from DIN-ISO 15923. One test tube was prepared to each of the samples (used dilution 1:25 and 1:50), one for a blank sample and also one to each of the different concentrations (0.4, 0.8 and 1.2 mg/l) of NO_3 standard curve. First of all 1 ml of TON1 (see recipe from Appendix 2) was injected to all of the test tubes. The second step was adding 20µl of samples + 980 µl distilled water to the test tubes of the samples with dilution factor of 1:50 and 40 µl sample + 960 µl distilled water to the test tubes with 1:25, 1 ml distilled water to the tube for the blank sample and 1 ml of the different NO_3 concentrations of the standard curve were added to their own test tubes. The test tubes were then vortexed well and incubated for 3 min at 30-40 ˚C. After incubation, 1 ml of TON2 reagent (see recipe form Appendix 2) was added to each of the test tubes. After injecting the TON2 reagent, all the test tubes were vortexed and incubated for 8 minutes at 30-40 ˚C. The next step was adding 1 ml of coloring agent, Farbreagent (see recipe form Appendix 2) to each of the test tubes and then vortexing them. The mixtures were incubated again for 5 minutes and after it, their NO_3 contents were measured by using Spectrometer at 540 nm wavelength. When the values were inserted into an Excel sheet, a linear trendline was drawn from the value of blank sample and the three different NO_3 concentrations to establish a standard curve. The measured values of the samples (blank value subtracted from each) were inserted to the equation of the slope, which gave then the amount of NO_3 content of the samples in mg/l.

2.2.2.4 Nitrite content (NO_2-N)

The procedure for the detection of NO_2 content of the samples from the reactors was taken from DIN-ISO 15923. One test tube was prepared to each of the samples, one for a blank sample and also one to each of the different concentrations (0.1, 0.5 and 1.0 mg/l) of NO_2 standard curve. First of all 1 ml of TON1 (see recipe from Appendix 2) was injected to all of the test tubes. The second step was adding 1ml sample to their own test tubes, 1 ml distilled water to the tube for the blank sample and 1 ml of the different NO_3 concentrations of the standard curve were added to their own test tubes. The test tubes were then vortexed well and incubated for 3 min at 30-40 ˚C. After incubation, 1 ml distilled water was injected to all of the test tubes, they were vortexed and incubated for 8 minutes at 30-

40 ˚C. The next step was adding 1 ml of coloring agent, Farbreagent (see recipe form Appendix 2) to each of the test tubes and then vortexing them. The mixtures were incubated again for 5 minutes and after that, their NO_2 contents were measured by using Spectrometer at 540 nm wavelength. When the values were inserted into an Excel sheet, a linear trendline was drawn from the value of blank sample and the three different NO_2 concentrations to establish a standard curve. The measured values of the samples (blank value subtracted from each) were inserted to the equation of the slope, which gave then the amount of NO_2 content of the samples in mg/l.

2.2.3 Heavy metal pollution analysis

The preparation of the samples for heavy metal analysis was the following. The culture to be harvested from each reactor was centrifuged and the supernatant removed. Six 25 ml glass tubes of the Kjeldatherm Digestion System were cleaned with 2% HNO_3 and then with ionized water. The cleaned glass tubes were weighted and filled with the leftovers (algal pellets). The tubes containing the algal pellets were weighted again and put to an oven to dry at 105˚C for 24 hours. The dried samples were weighted and their DM content calculated (see calculation in chapter 2.2.1.5 , Eq. 5). Considering the weight of DM, HNO_3 and 37% HCl were added to the dried samples (see the ratio in Appendix 3). After the addition of acids, the glass tubes were filled up with distilled water and placed under the reflux condensers of the Kjeldatherm Digestion System.

After 2 hours at 110 ˚C, the tubes with the samples were removed and the content of condensation trap (HNO_3 with the volatile substances vaporized) were added. This mixture was then filtered into a glass flask with 0.45µm filter paper. This digestion step was done according to the DIN EN ISO 15587-1 standard.

The supernatants and the samples from the digestion step were analyzed by Gesellschaft für Bioanalytik MBH laboratory by following the DIN 38412 German standard.

The heavy metal pollution analysis was modified by using Chlorophyll extraction (see chapter 2.2.1.4 Chlorophyll a) method rather than Chlorophyll measurement by fluorescence as recommended in the DIN 38412 due to missing technological options.

Finally the heavy metal contents of the algal pellets were calculated by using the Eq. 6.

$$c1 = \frac{\left(\frac{c2}{0,001}\right)}{\frac{V}{m}} \div 40 \qquad (6)$$

m = Dry weight [g]

V = replacement volume [ml]

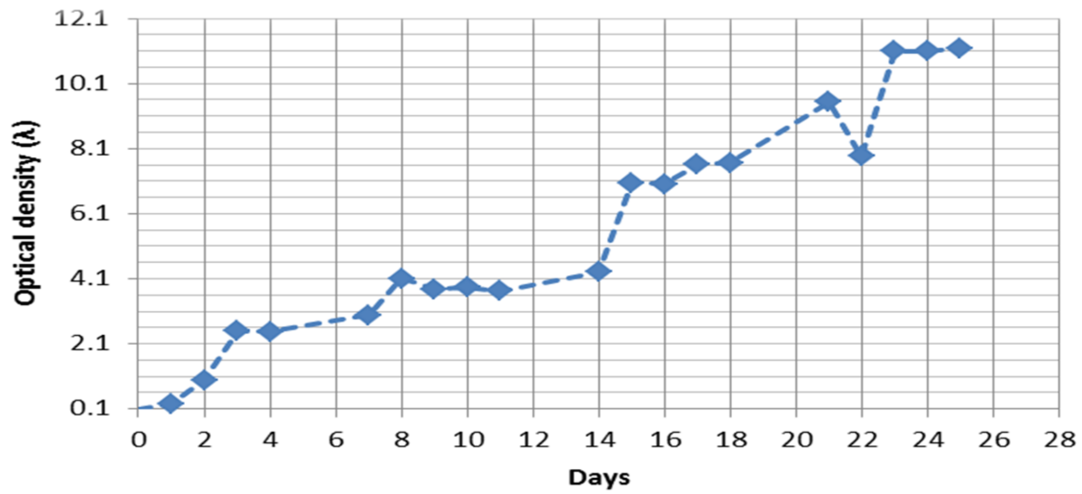
c_1 = concentration of Heavy Metal [µg/l]

c_2 = HM concentration in biomass [mg/kg TS]

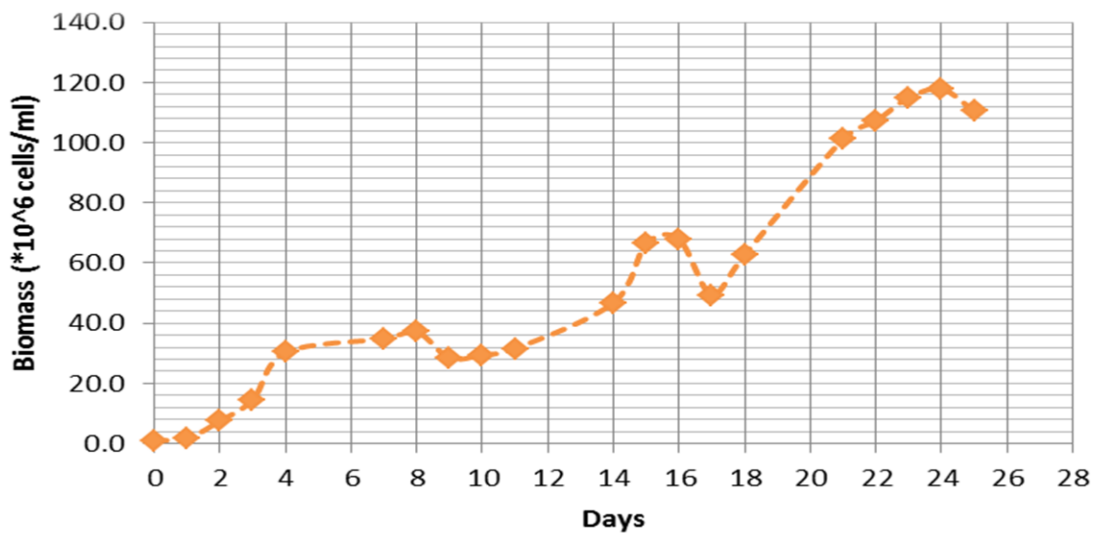
3. Results

3.1 Inoculation phase

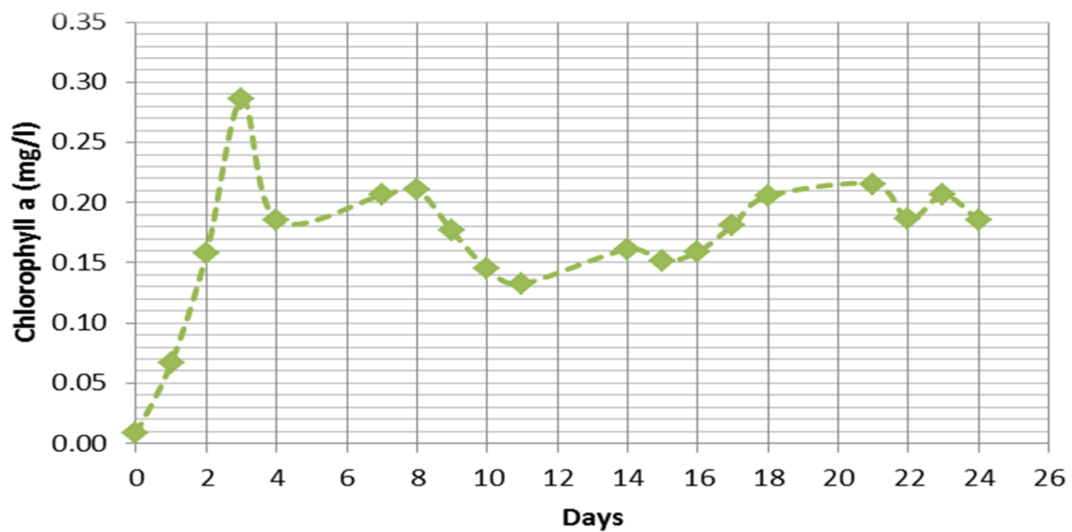
It can be seen from Figure 16, pictures A and B that the optical density and cell growth of *Desmodesmus subspicatus* showed the same tendency. On the same Figure it is shown that the adaptation to new environment took one day (between day 0 and 1), the log-phase approximately three-four days (day 1-3 or 1-4). The log-phase was followed by the stationary-phase, which ended around days 8-9 when fall of all the parameters occurred (see Figure A, B and C). The culture obviously started to die out so on day 14 Nitrogen was added as nutrient to the inoculum. After the addition of nutrient, all the parameters started to increase again. The peak of optical density was achieved on day 23 (11.19λ), of biomass on day 22 ($117.8*10^6$ cells/ml) and of Chlorophyll a on day 6 (0.29 mg/l). The Figure C shows that the Chlorophyll a production was the most intense of the fresh culture (1-5 days old). The data from the daily measurements can be seen in the Appendix 4.



A)



B)



C)

Figure 16: Optical density (A), Cell count (B) and Chlorophyll a (C) content of the culture of inoculation phase (pH=7, n=1)

3.2 Greenhouse phase I: Growth study

The temperature of the green house was measured through the culturing week. As the Figure 17 shows, each day the minimum temperature stayed above 0˚C, which was satisfactory to cultivate the *Desmodesmus subspicatus* culture inside the greenhouse. The highest temperature was 53˚C and the lowest 2˚C. The peaks of the temperature were approximately around 2 pm and the lowest ones were around 6 am, except on day 3-4 when it was at 1 am.

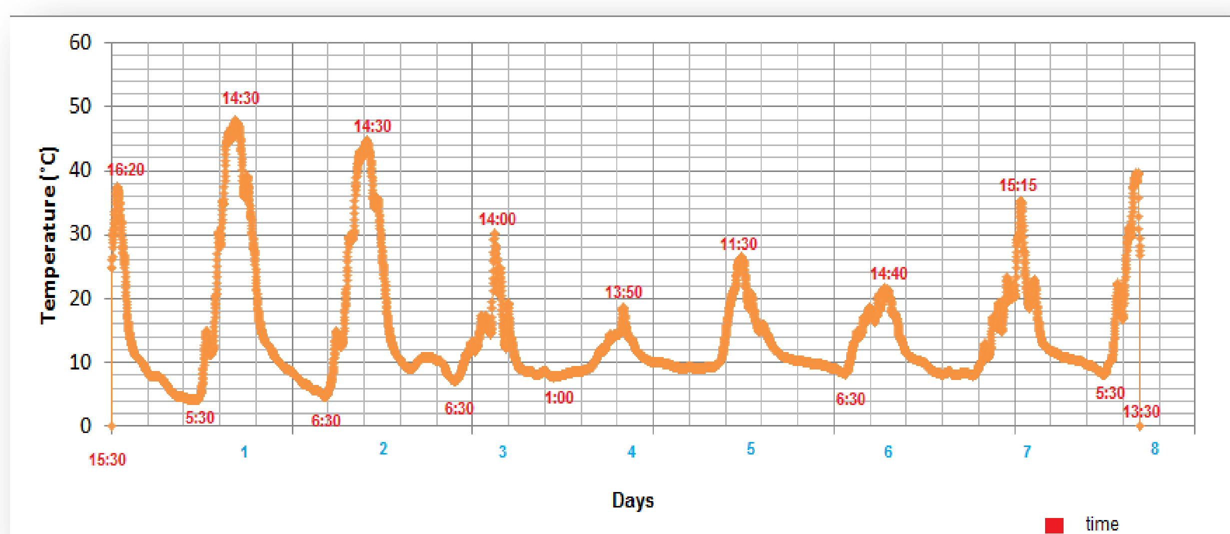


Figure 17: Temperature changes in the green house during the days, duration of the measurements was 1 week (12-20th March) and the frequency was 2 minutes. The peaks are showing the warmest time of the day, which was around 2 pm, and the scoops are the night times with their lowest temperature around 6 am.

At this phase the growth of the biomass was studied inside of a greenhouse. The TABLE 10 shows that the DS cultures fast adapted the new conditions. Because of the fluctuations of the temperature between days and nights, the cultures were growing slower than the ones of the photobioreactor (see data of 3.1 Inoculation phase). The optical density was approximately 4.5 fold higher and the biomass triple after 6 days. It can be also seen from the same Table that the Chlorophyll a showed different tendency than the culture of the photobioreactor. However the Chlorophyll a was around 4 times higher after 6 days, it had fluctuations daily (see data of measurements from Appendix 5).

The TABLE 10 and the Figure 18 also show that the optical density of the six cultures was (0.591±0.137)λ after 8 days. The standard deviations (SD) of the optical densities show (see Figure 18) that the gap between the development of cultures became bigger and bigger every day. The highest biomass was 3.4*10^6 cells/ml (8th day) and of Chlorophyll a 0.064 mg/l (day 7).

TABLE 10: Results of Greenhouse phase I (pH7) tests (OD±SD, n=6). All the reactors had same conditions and their starting pH was 7. The duration of this phase was 8 days (including 2 days at weekend when no measurement was done).

Days	**0**	**1**	**2**	**3**	**6**	**7**	**8**
OD (λ)	0.135	0.153	0.200	0.371	0.432	0.483	0.591
SD (OD)	0.002	0.014	0.019	0.059	0.085	0.106	0.137
Biomass (*10^6 cell/ml)	1.2	1.2	1.2	2.3	2.3	2.0	3.4
Chlorophyll a (mg/l)	0.011	0.004	0.015	0.007	0.041	0.064	0.043

*SD: standard deviation of optical densities

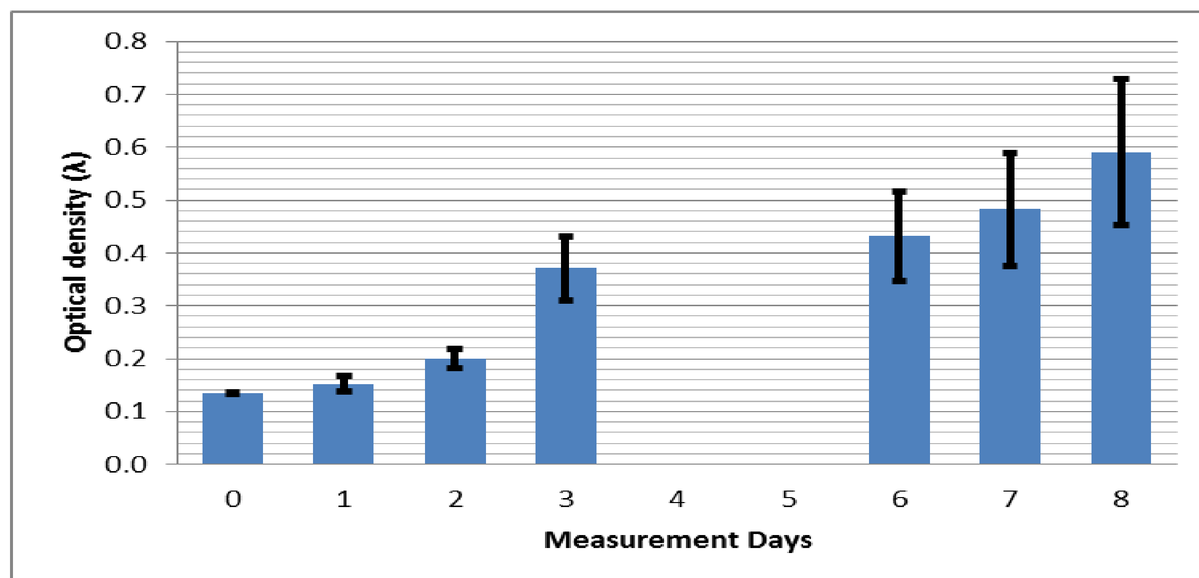


Figure 18: Optical density on day 0 and mean values (n=6) of OD (λ) from day 1 to 8. The starting pH of the cultures was pH7 and each condition applied to the reactors were the same. The error bars are the ±SD.

3.3 Greenhouse phase II: pH study

The "Greenhouse phase: pH study" was run by three different pH levels (pH 7, 5, 3). The aim of this phase was to see how the *Desmodesmus subspicatus* cultures can adopt these different pH conditions.

To see how the temperature of the cultures is changing depending on the temperature of the greenhouse, 1 data logger was established inside one reactor with pH7 and 1 to the shelf inside the greenhouse. The Figure 19 shows the temperature changes between days and nights inside the greenhouse and the Figure 20 inside the reactor.

The tendency of temperature changes did look similar to those of the greenhouse and the cultures however day/night fluctuations were much less pronounced inside the reactors. The maximum temperature achieved inside the greenhouse was 57˚C and the minimum 2˚C. In the meanwhile the max of the culture was 33˚C and the min 5˚C. Even though the high temperature inside the greenhouse, the culture´s temperature stayed at normal level, below 34˚C. Also the minimum temperature stayed above satisfactory level for the culture´s growth. The average temperature inside the greenhouse (19˚C) and the culture (17˚C) were very similar.

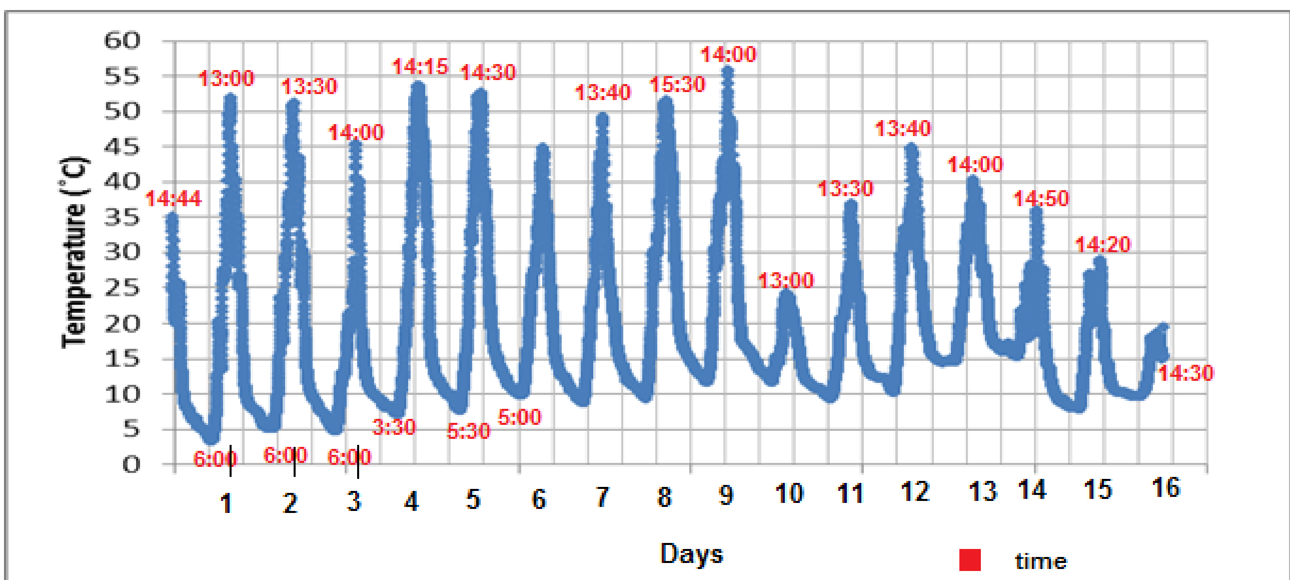


Figure 19: Temperature changes between days and nights inside the greenhouse. Duration of data logging was 16 days (25th March – 10th April 2014). The data logger was put on a shelf which was approximately 2 meter high. Measurements were taken every 2 minutes.

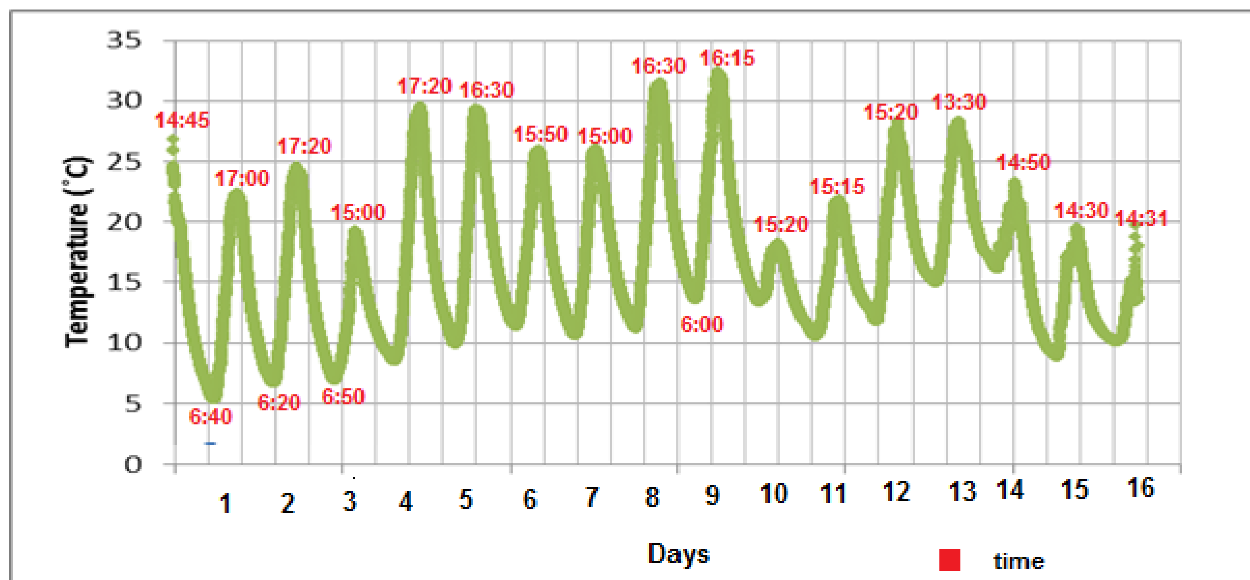


Figure 20: Temperature changes between days and nights inside the reactor 1 (pH 7). Duration of data logging was 16 days (25th March – 10th April 2014).

Figure Nr picture A, B and C show that the lag-phases for the cultures in pH 7, 5 and 3 were similar. Although the gradients of the OD curves differed, each of the cultures had exponential growth. During the whole pH study phase, the reactors with the same starting pH level behaved the same way except of the pH 3. However the lag-phase of these 2 cultures were similar, difference occurred between the 2 cultures at day 6 and after this day the gap became bigger and bigger.

From the Figure 22, picture A, B and C can be seen that the best and worst performing cultures were both from the reactors (C) with starting pH3. Taking into consideration the mean values of the cultures (belonging to the same starting pH level), the best pH was 5.

The Figure 21 visualizes how dense the cultures were by the end of the experiment compared to the starting cultures.

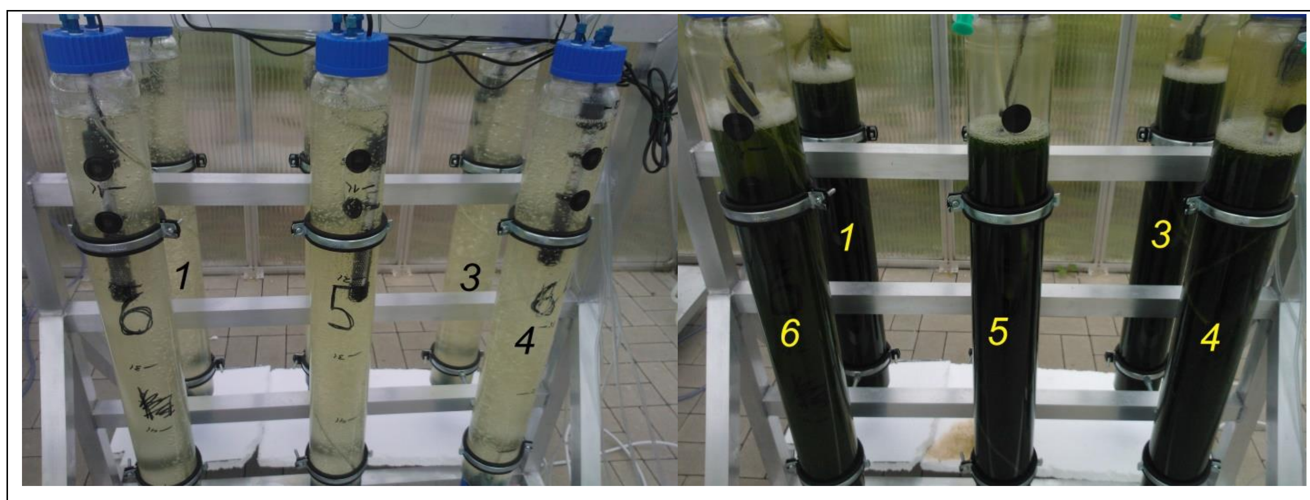


Figure 21: Fresh 1 day old cultures (on left) and same cultures after 16 days (on right). The pictures were taken from the same spot. Cultures 1 and 2 were at pH 7, Cultures 3 and 4 at pH 5 and Cultures 5 and 6 at pH 3. The Culture 2 is not shown from any of the figures because the sealing was not accurate so the reactor was leaking and empty by the day 16.

The increase of biomass of the cultures with pH 7 (Cultures 1 and 2) and with pH 3 (Cultures 5 and 6) were similar (see TABLE 11). The average increase of cultures with pH 7 was 6.5%, 3.7% of cultures with pH 5 and 6.3% of cultures with pH 3.

TABLE 11: Increase of the biomass (DM) of the cultures between day 0 and day 16 (n=6, pH 7, 5, 3)

pH adjusted	**pH 7**		**pH 5**		**pH 3**	
Culture	**Culture 1**	**Culture 2**	**Culture 3**	**Culture 4**	**Culture 5**	**Culture 6**
Change of DM (**%)**	6.25	6.81	3.88	3.49	6.58	5.97

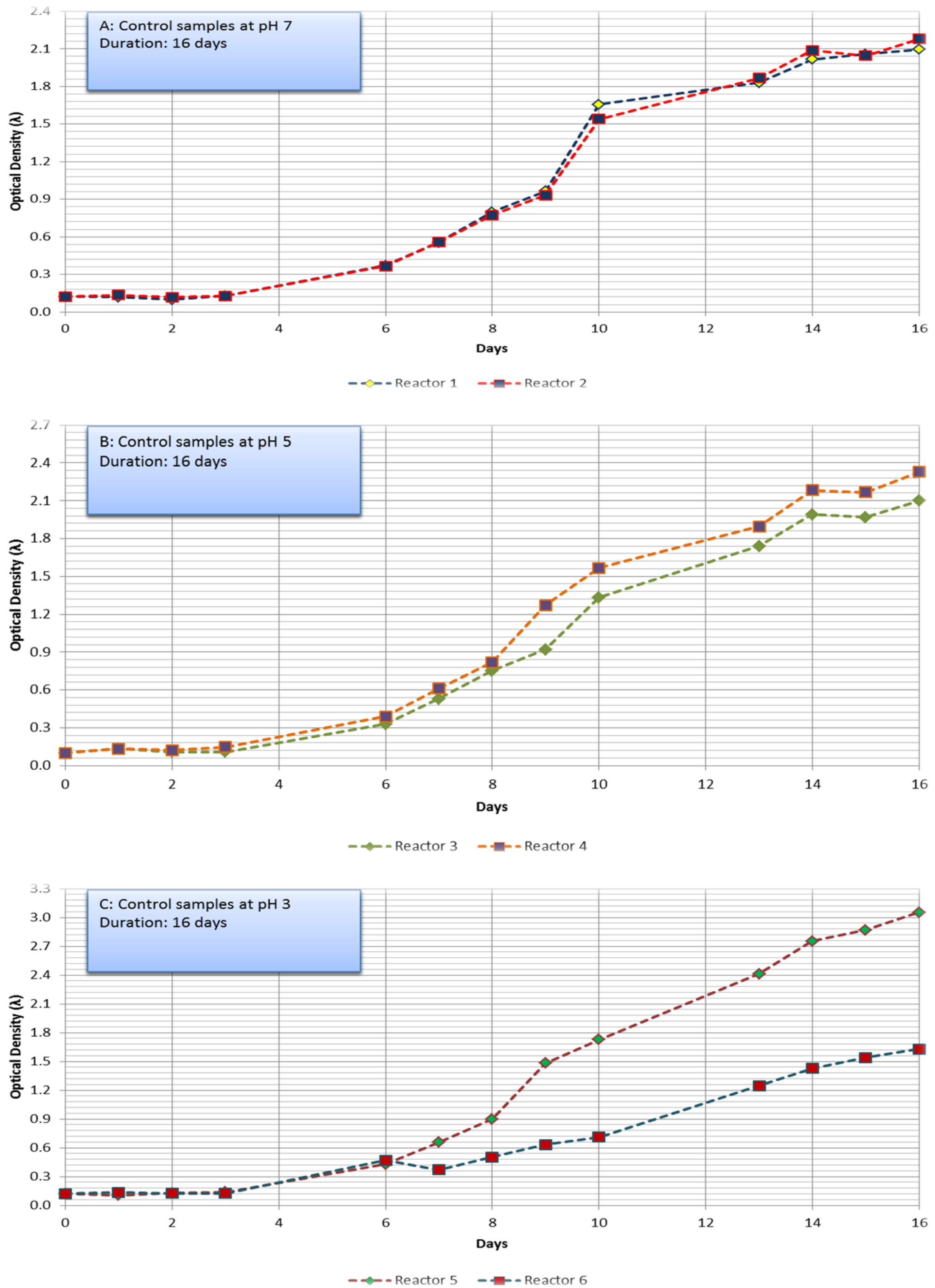


Figure 22: Change of Optical Density of the cultures in pH 7 (A), pH 5 (B) and pH 3 (C) under natural light conditions in 16 days (n=2 of for each pH).

3.3.1 Reactors with pH 7

Neutral conditions were adjusted to two reactors (1 and 2) at the beginning of "Greenhouse phase: pH study" experiment. The TABLE 12 shows the results of daily measurements applied on samples from the two reactors. The mean value of optical density of the 16 days old cultures increased 21 fold. The highest change (ΔOD = 1.7x) was between the 9 and 10 days old cultures. The highest OD achieved was (2.14±0.042)λ. The TABLE 12 Figure 22 A and Figure 23 also show (SD) that the biggest deviation (0.058λ) between the ODs of the two cultures was on day 10. The highest biomass ($15.5*10^6$ cells/ml) was of the 15 days old culture. The pH after increasing above 9 remained slightly alkaline (pH 9.2-9.6).

TABLE 12: Mean values of daily measurements of the samples inside the greenhouse (OD±SD, n=2, pH7, duration =16 days). On the weekends no measurement was done so the "Days" in the table means number of measurement day not the age of the culture.

Days	**1**	**2**	**3**	**6**	**7**	**8**
OD (λ)	0.128	0.109	0.128	0.369	0.556	0.785
SD (OD)	0.009	0.011	0.001	0.002	0.002	0.013
Biomass (*10^6 cell/ml)	1.0	0.6	0.5	2.7	3.9	4.9
Chlorophyll a (mg/l)	0.004	0.001	0.008	0.021	0.042	0.055
pH	9.19	9.29	9.28	9.35	9.44	9.53
Days	**9**	**10**	**13**	**14**	**15**	**16**
OD (λ)	0.948	1.598	1.848	2.052	2.052	2.136
SD (OD)	0.016	0.058	0.018	0.036	0.006	0.042
Biomass (*10^6 cell/ml)	6.6	12.5	13.6	13.9	15.5	13.5
Chlorophyll a (mg/l)	0.080	0.112	0.152	0.160	0.167	0.185
pH	9.57	9.50	9.40	9.48	9.39	9.41

*SD: standard derivation of the mean

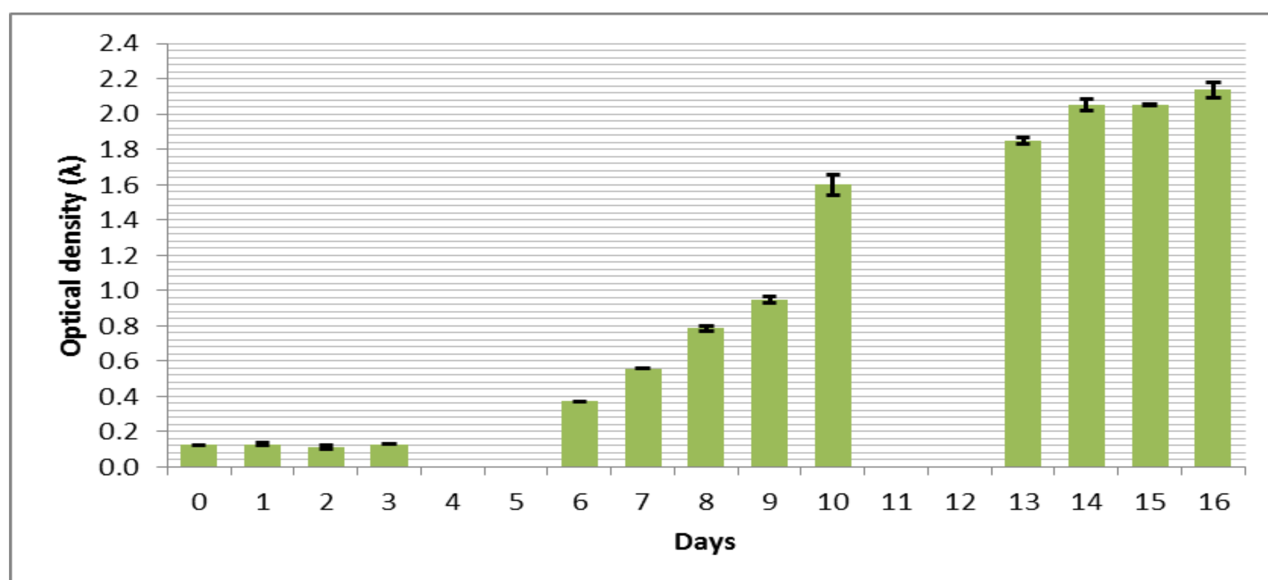


Figure 23: Optical density of the cultures with their SD (error bars) on the measurement days (n=2, starting pH = 7, duration = 16 days)

NH_4-N was the only parameter, which increased by the end of the experimental period of both cultures. The TABLE 13. below shows that the NH_4 content of the Culture 1 increased by 8.6 fold (+1.156 mg/l) and by 6.3 fold (+0.751 mg/l) of the Culture 2. The NO_2-N was growing by 33 fold (+0.361 mg/l) of the Culture 1 but slightly decreased of the Culture 2 (-0.009 mg/l). The other parameters (NO_3 and PO_4) decreased a lot by the end of the experimental period. The NO_3 content of both cultures decreased nearby the same amount (approx. 21 mg/l). The fall of PO_4 was around double of the Culture 2 (-2.610 mg/l) than of the Culture 1 (-1.212 mg/l).

TABLE 13: Nutrient (NH_4, NO_2, NO_3, PO_4) content of the cultures in the greenhouse at the beginning and end of control experiment in pH 7

	Start value	Culture 1	Change (Culture 1)	Culture 2	Change (Culture 2)
NH_4-N (mg/l)	0.12	1.16	1.04	0.87	0.75
NO_2-N (mg/l)	0.01	0.37	0.36	0.00	0.00
NO_3-N mg/l)	29.6	8.48	-21.1	8.27	-21.3
PO_4-P (mg/l)	1.14	-0.07	-1.21	-1.47	-2.61

3.3.2 Reactors with pH 5

At the beginning of “Greenhouse phase: pH study” experiment, pH 5 was adjusted to two reactors (3 and 4). The TABLE 14 shows the results of daily measurements applied on samples from the two reactors. The changes of OD can be read from TABLE 14 and seen from Figure 22 B and Figure 24. The highest OD (2.214±0.114)λ was achieved by the end of the experiment (16 days) and this result was around 22 fold higher than the starting OD of the cultures. The highest SD (0.176λ) was of the 9 days old culture. The most dense biomass (18.3*10^6 cells/ml) and highest Chlorophyll a content (0.218 mg/l) was of the oldest cultures. By the end of the experiments with the “pH Study” phase, the biomass increased 18 fold compared to the starting value. The TABLE 14 also shows how the pH was changing. Already after the first day, the pH of the cultures was around 8 and it stayed between 8 and 9.5 through 15 days.

TABLE 14: Mean values of daily measurements of the samples inside the greenhouse (OD±SD, n=2, pH5, duration =16 days)

Days	**1**	**2**	**3**	**6**	**7**	**8**
OD (λ)	0.133	0.115	0.129	0.359	0.570	0.786
SD (OD)	0.001	0.006	0.020	0.031	0.041	0.035
Biomass (*10^6 cell/ml)	0.6	0.8	0.6	2.9	3.4	5.5
Chlorophyll a (mg/l)	0.003	0.002	0.008	0.020	0.042	0.058
pH	7.84	8.23	8.24	8.53	8.75	9.28
Days	**9**	**10**	**13**	**14**	**15**	**16**
OD (λ)	1.096	1.449	1.818	2.088	2.067	2.214
SD (OD)	0.176	0.117	0.078	0.096	0.099	0.114
Biomass (*10^6 cell/ml)	8.5	7.8	14.1	14.4	14.0	18.3
Chlorophyll a (mg/l)	0.051	0.121	0.204	0.182	0.189	0.218
pH	9.45	9.08	8.77	8.82	8.75	8.69

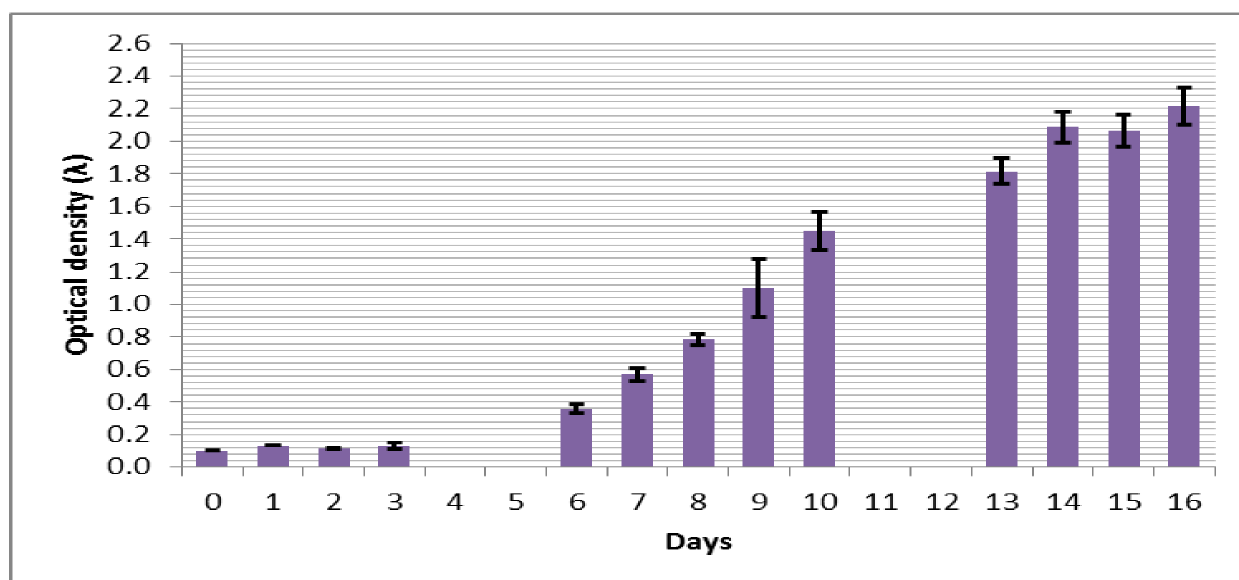


Figure 24: Optical density of the cultures with their SD (error bars) on the measurement days (n=2, starting pH = 5, duration = 16 days)

As TABLE 15 shows, the NH_4 and NO_2 content of both cultures increased by the end of the experiment. The NH_4 of the Culture 3 was 7 fold higher (+0.79 mg/l) and 5.7 fold higher (+0.63 mg/l) of the Culture 4. The NO_2 concentration increased 47 fold (+0.47 mg/l) of Cultures 3 and 48 fold (+0.48 mg/l) of Culture 4. Both of the NO_3 and PO_4 contents of cultures decreased by the day 16. The change of NO_3 was -6.01 mg/l of Culture 3 and -10.94 mg/l of Culture 4. The PO_4 content of Culture 3 fell 8.1 fold (-1.62 mg/l) and 9.3 fold (-1.87 mg/l) of Culture 4. From all the parameters only the PO_4 concentration of both 16 days old cultures was negative.

TABLE 15: Nutrient (NH_4, NO_2, NO_3, PO_4) content of the cultures in the greenhouse at the beginning and end of control experiment in pH 5

	Start value	Culture 3	Change (Culture 3)	Culture 4	Change (Culture 4)
NH_4-N (mg/l)	0.11	0.90	0.79	0.74	0.63
NO_2-N (mg/l)	0.00	0.47	0.47	0.48	0.48
NO_3-N mg/l)	19.94	13.93	-6.01	9.00	-10.94
PO_4-P (mg/l)	0.20	-1.42	-1.62	-1.67	-1.87

3.3.3 Reactors with pH 3

At the beginning of “Greenhouse phase: pH study” experiment, pH 3 was adjusted to two reactors (5 and 6).

The optical density of the cultures (see TABLE 16 and Figure 25) were increasing until the end of the experimental phase (day 16) and its highest value was (2.341±0.713)λ. The highest change (+0.4λ) occurred between days 8 and 9. Comparing the starting and end values of OD, there was a 16 fold increase. The gap between the development of the Culture 5 and 6 was growing (see also from Figure 22, picture C) from day 6, which is also visualized by the value of SD in Figure 25.

The biomass was also increasing until day 16 and had its peak at $18.6*10^6$ cells/ml, which means approximately 18 fold growth from the starting culture. The highest change ($+2.5 *10^6$ cells/ml) seen from TABLE 16 occurred between days 8 and 9.

The Chlorophyll a was in tendency behaving the same way as the other parameters. The peak of Chlorophyll a content was 0.188 mg/l and its highest change between days 6 and 7. The amounts on the different days are showing that adaptation occurred until day 3 followed by an increase of growth up to day 14. The highest change (+ 0.048 mg/l) was at day 6-7. The pH of the cultures slightly increased above 8 and after day 7, it remained between pH 8 and 9.6.

TABLE 16: Mean values of daily measurements of the samples inside the greenhouse (OD±SD, n=2, pH3, duration =16 days)

Days	1	2	3	6	7	8
OD (λ)	0.120	0.127	0.132	0.449	0.513	0.702
SD (OD)	0.015	0.001	0.007	0.020	0.143	0.197
Biomass (*10^6 cell/ml)	0.8	0.7	0.8	3.1	3.5	5.0
Chlorophyll a (mg/l)	-0.001	0.002	0.008	0.020	0.068	0.049
pH	5.65	6.49	6.82	7.86	8.51	9.58
Days	9	10	13	14	15	16
OD (λ)	1.058	1.219	1.830	2.091	2.204	2.341
SD (OD)	0.424	0.509	0.582	0.663	0.664	0.713
Biomass (*10^6 cell/ml)	7.6	9.1	17.5	16.0	17.9	18.6
Chlorophyll a (mg/l)	0.071	0.102	0.147	0.167	0.171	0.188
pH	9.38	8.37	8.83	8.94	8.73	8.47

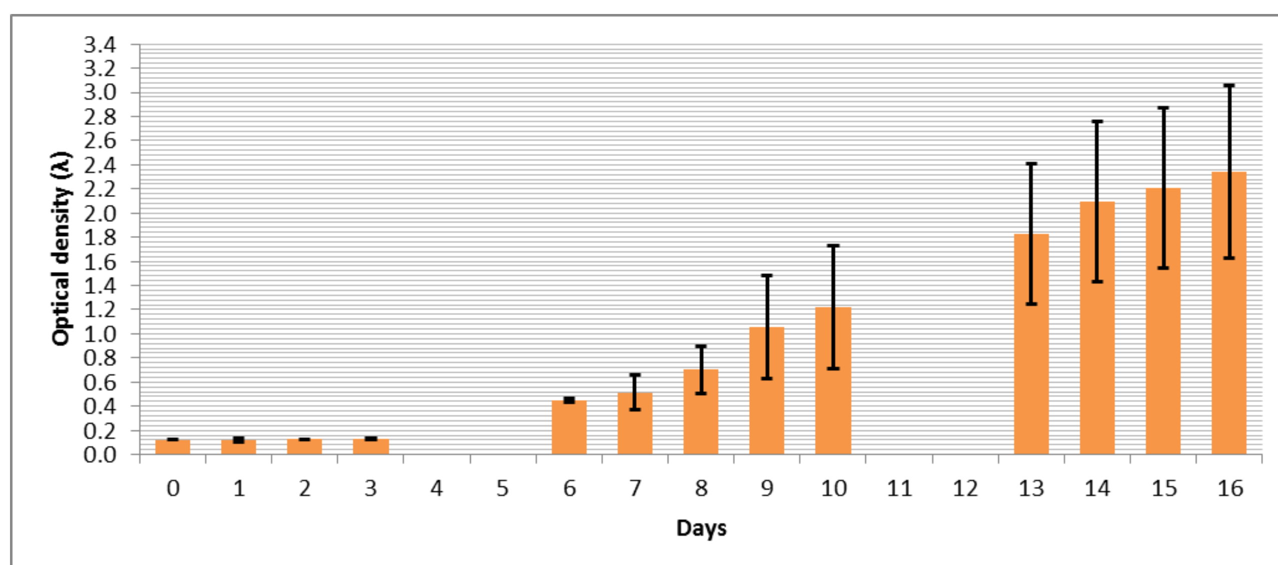


Figure 25: Optical densities of the cultures with their SD (error bars) on the measurement days (n=2, starting pH = 3, duration = 16 days)

The NH_4 end concentration in the two cultures differed (see TABLE 17). While it increased for Culture 5 (+0.17 mg/l) it was falling for Culture 6 (-0.09 mg/l). There was no change of the NO_2 concentrations of any of the cultures. The NO_3 and PO_4 contents of both cultures were falling by the end of experimental period. By the day 16, the Culture 5 lost over half

of its starting NO_3 concentration and its PO_4 was -2.09 mg/l, 4.5 fold smaller than the initial value. The Culture 6 behaved similar way and lost over half of its NO_3 content. The final concentration of NO_3 was 10.97 mg/l. The end PO_4 content of the Culture 6 was -1.77 mg/l which is 4.8 fold smaller than its starting value.

TABLE 17: Nutrient (NH_4, NO_2, NO_3, PO_4) content of the cultures inside the greenhouse at the beginning and end of control experiment in pH 3

	Start value	Culture 5	Change (Culture 5)	Culture 6	Change (Culture 6)
NH4-N (mg/l)	0.61	0.79	0.17	0.52	-0.09
NO2-N (mg/l)	-0.01	-0.01	0.00	-0.01	0.00
NO3-N mg/l)	24.10	8.15	-15.94	10.97	-13.13
PO4-P (mg/l)	0.46	-1.63	-2.09	-1.77	-2.23

3.4 Laboratory phase: Contamination study

This Contamination study phase had two experimental runs (control and treatment) for either pH 3 or 5 adjusted media. First the Control Cultures were established at pH 3 and tested for 15 days to see how the cultures develop. After the control study, new cultures were prepared at pH3 and heavy metal solution (300µg/l Pb and 30µg/l As, Cd, Hg) was added to each. The HM concentrations were similar with the values of River Oker.

The highest increase of biomass measured as dry matter (DM) was visible in the control cultures with pH 5 (see TABLE 18). The smallest amounts of DM were of HM contaminated cultures with pH 5. Some error occurred with the weighting of the Culture 5 so the weight of the crucible was the same as the DM. The average DM content of control cultures with pH 5 was 12.73%, of HM contaminated cultures with pH 5 was 4.12% (the Culture 5 was not taken into consideration) and 6.24% of control cultures with pH 3.

TABLE 18: Increase of the biomass (DM) of the cultures in pH 5 control cultures, in heavy metal (As, Cd, Hg, Pb) contaminated cultures with pH 5 and control cultures with pH 3 (n=6 for each). The durations of different experiments were 14-15 days.

%	Culture 1	Culture 2	Culture 3	Culture 4	Culture 5	Culture 6	x̄	SD
pH 5	13.72	13.21	13.3	12.2	12.13	11.8	12.73	0.71
pH 5 + HM	5.12	5.62	2.55	2.8	-8.63	4.52	4.12	1.23
pH 3	6.85	6.64	6.67	6.87	5.24	5.14	6.24	0.74

3.4.1 Control cultures with pH3

The Figure 26 visualizes how the cultures developed by the end of the experiment (14 days). It can be seen that the end cultures were healthy (right picture) and much more dense (dark green color) than the starting cultures.

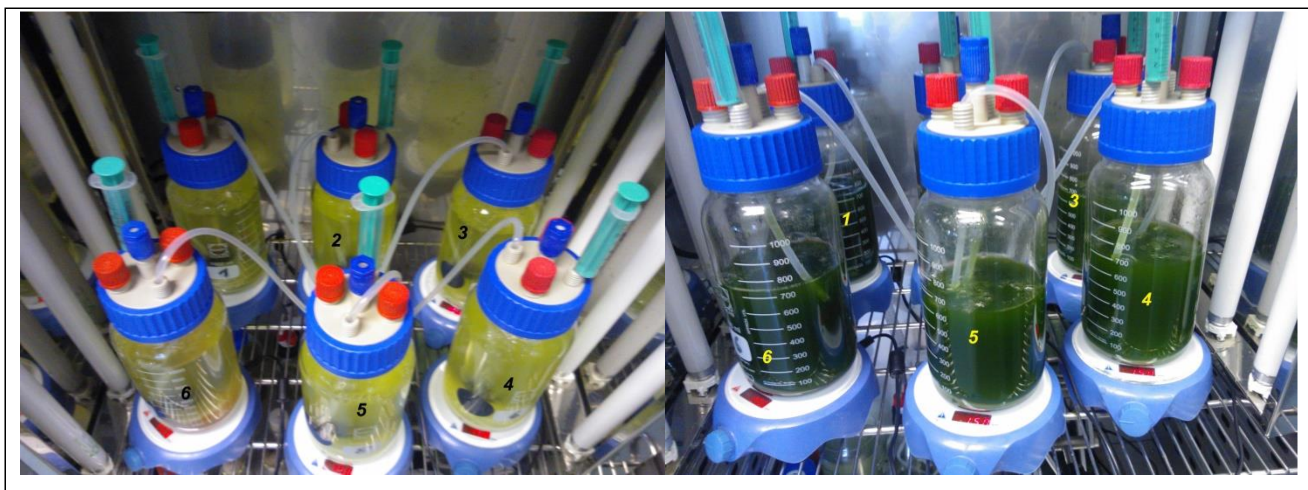


Figure 26: Fresh 1 day old cultures (on left) and same cultures after 14 days (on right). The order of the reactors is the same on both pictures.

The TABLE 19 shows that the cultures adapted well the pH 3 conditions. It is important to mention that between day 7 and 12 there were 4 days break when no measurement was done. Even though the break between those days, the biggest change (+0.695λ) of optical density occurred between days 12 and 13. The highest OD was (3.208±0.568)λ, which means approximately 30 fold increase from the starting value and it was achieved by the last day of measurement (14 days old cultures). The SD of OD (see TABLE 19. and also Figure 27) showed increasing tendency. The biomass on the last measurement day (14 days old culture) was also 30 fold higher than of the starting cultures. The highest value of cell count ($30.2*10^6$ cells/ml) was achieved on the last day. Concerning the Chlorophyll a, the biggest change (+0.106 mg/l) was between day 2 and 3 and the highest value (0.283 mg/l) was achieved on the last day. The pH of the cultures was already above 7 on the first day and after that the pH stayed between 8.9-10.3.

TABLE 19: Mean values of daily measurements of the samples inside the light incubator (OD±SD, n=6, pH3, duration =14 days)

Days	1	2	5	6	7	12	13	14
OD (λ)	0.191	0.334	1.009	1.331	1.591	2.234	2.929	3.208
SD (OD)	0.013	0.043	0.186	0.232	0.339	0.513	0.501	0.568
Biomass (*10^6 cell/ml)	1.6	2.9	6.6	8.1	11.0	25.2	27.9	30.2
Chlorophyll a (mg/l)	0.001	0.013	0.119	0.128	0.160	0.271	0.271	0.283
pH	7.33	8.93	10.17	10.26	10.24	9.60	9.69	9.70

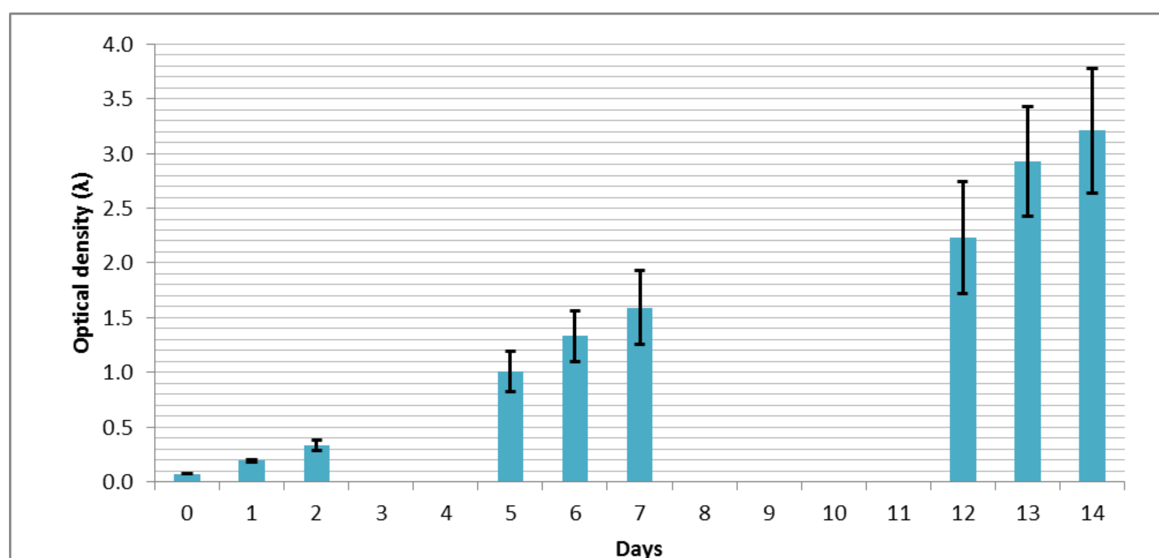


Figure 27: Optical densities of the control cultures with their SD (error bars) on the measurement days (n=6, starting pH = 3, duration = 14 days)

All the measured nutrient concentrations (NH_4, NO_2, NO_3, PO_4) stayed above zero (see TABLE 20). There was positive change of NH_4, NO_2 and PO_4 between the starting and end concentrations. The Culture 5 had the highest NO_2 (17.31 mg/l), NO_3 (4.64 mg/l) and PO_4 (11.09 mg/l) concentration. The highest amount of NH_4 was of Culture 1 (0.40 mg/l).

TABLE 20: Nutrient (NH_4, NO_2, NO_3, PO_4) content of the cultures inside the light incubator at the beginning and end of control experiment in pH 3

Culture	Start value	Culture 1	Culture 2	Culture 3	Culture 4	Culture 5	Culture 6	Mean
NH4-N (mg/l)	0.11	0.40	0.20	0.29	0.30	0.36	0.33	0.28
NO2-N (mg/l)	0.00	0.23	0.27	0.18	0.18	17.31	0.23	2.63
NO3-N mg/l)	18.56	3.48	3.42	3.50	3.57	4.64	3.77	5.85
PO4-P (mg/l)	-2.21	7.68	6.41	6.68	7.70	11.09	6.76	6.30

3.4.2 Heavy Metal (As, Pb, Cd, Hg) contaminated cultures at pH 3

Even though the cultures could adapt to the acidic (pH 3) conditions the heavy metal content was toxic to them. All the parameters (OD, cell counts, pH) were falling right away and the experiment needed to be stopped in three days. The color change as an indicator of not viable cultures is visualized on Figure 28.

Figure 28: Starting (on left) and 2 days old cultures (on right). 18 ml heavy metal solution (300µg/L Pb, 30µg/L As, 30µg/L Cd and 30µg/L Hg) was added to each cultures (n=6, pH3, t= 3 days). The color change of death cultures is visible on the right picture.

The TABLE 21 shows well that all the parameters fell compared to the starting values. From the second day, the Chlorophyll a content was already negative. The pH became even more acidic so the conditions for the cultures became even more toxic.

TABLE 21: Mean values of daily measurements of the cultures with heavy metal contents inside the light incubator (OD±SD, n=6, pH3, duration = 3 days)

Measurement Day	0	1	2	3
OD (λ)	0.115	0.103	0.082	0.064
SD (OD)	0.000	0.009	0.005	0.018
Biomass (*10^6 cell/ml)	1.3	1.0	0.8	0.6
Chlorophyll a (mg/l)	0.103	0.012	-0.002	-0.005
pH	3.00	2.72	2.75	2.77

The Figure 29 visualizes the fall of OD of the different cultures. There was some small increase of the OD of Culture 5 between the day 2 and 3 so the Culture was saved for further investigations.

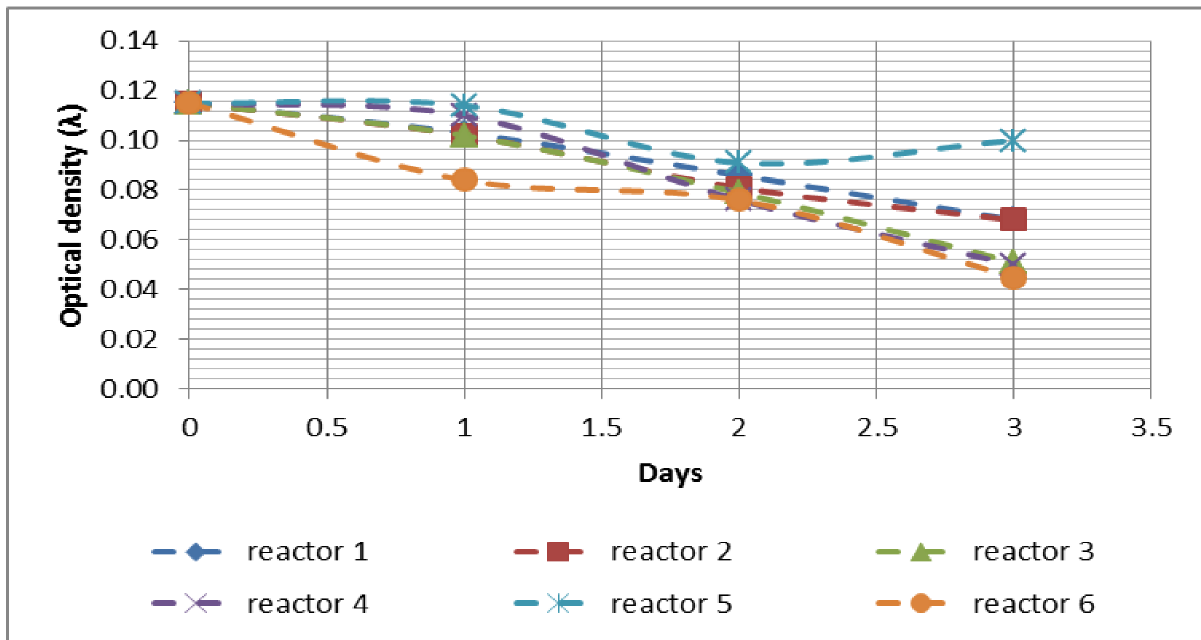


Figure 29: Optical densities of the different days old cultures (n=6, starting pH 3, duration = 3 days)

3.4.2.1 Development of Culture 5 (HM contaminated culture from pH 3)

The HM contaminated culture (Culture 5) saved from the previous experimental phase was put to near to the window of the laboratory. The culture was slightly stirred, no additional nutrient solution, artificial light or heat was given to the culture. The temperature of the laboratory was around 21˚C.

However the development of the culture was not as expected but a life inside the culture was visible. After 1 week, the culture was a bit greenish and approximately in 3 weeks, a kind of funnel appeared at the top middle of the culture (see Figure 30, picture A).

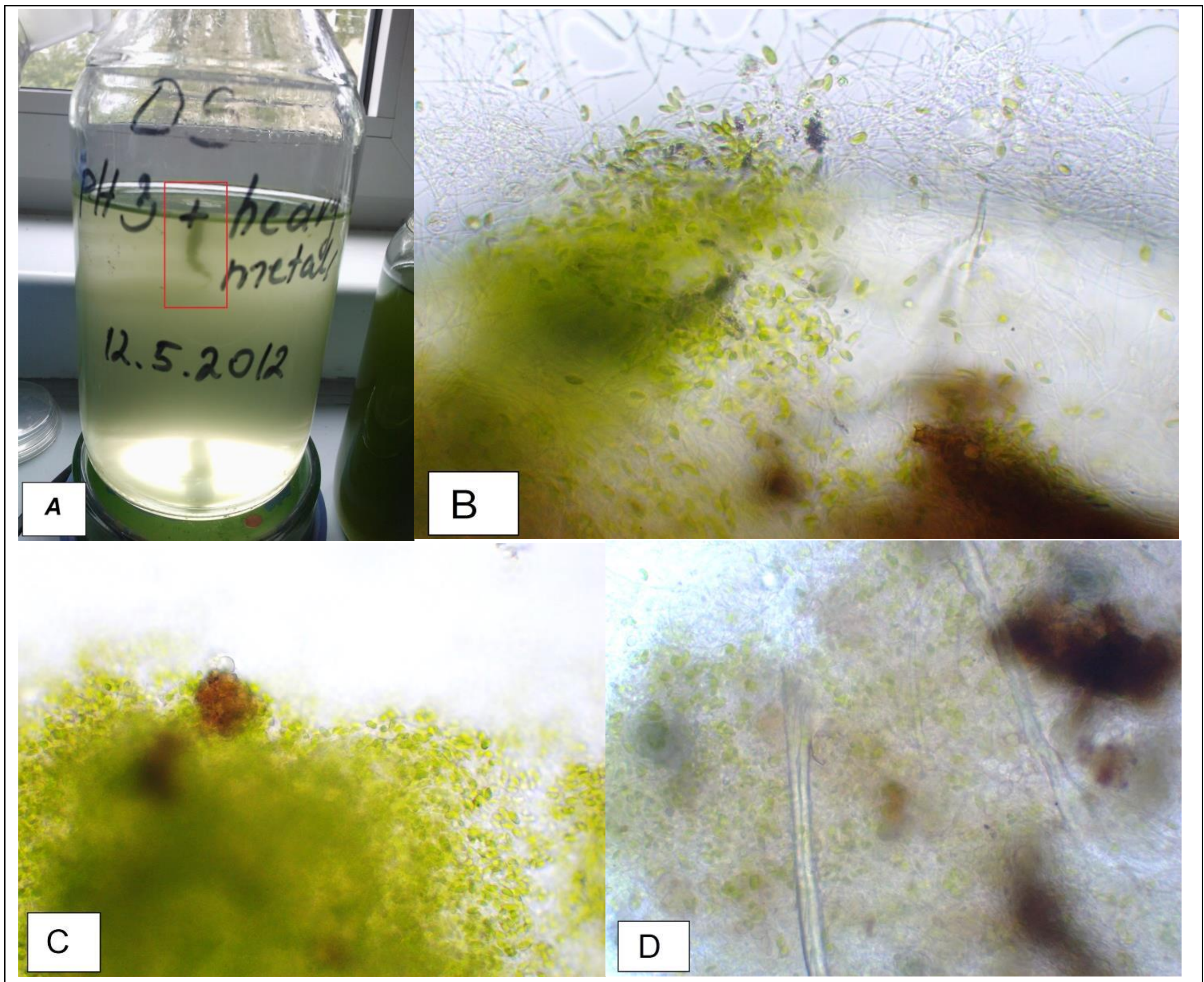


Figure 30: Culture 34 days (37 days old) after removal from the light incubator (A). Picture B, C and D are microscopic pictures about the sample taken from the funnel (seen at the middle of the Culture on picture A). Picture B shows the healthy *Desmodesmus subspicatus* cells, C is about the density of the DS culture and the D shows the fungal contamination of the culture. The microscopic pictures were taken with 1000x magnification (oil) of Motic (Type: BA310) microscope.

A sample was taken from the funnel of the 5 weeks old culture and analyzed with 1000x magnification. The DS cells seemed to be healthy and the culture had high density (see Figure 30, pictures B and C). The culture was infected by a fungus which is shown on picture B. The last picture (D) gives a closer view of the fungal filaments.

3.4.2.2 Testing the heavy metal resistance of the Culture 5

The new culture cultivated from the HM resistant Culture 5 was visually viable after 11 days (see Figure 31, picture B). The HM resistant *Desmodesmus subspicatus* culture seemed to keep its property even though the contamination again with the same concentration of heavy metals as before (Laboratory phase: Contamination study).

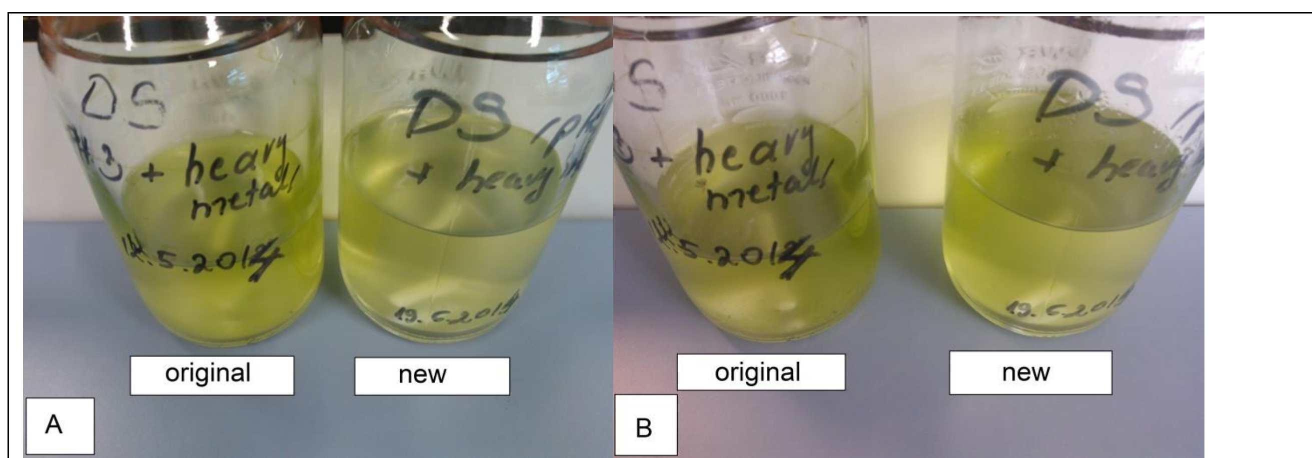


Figure 31: Culture 5 from the HM contamination phase with pH 3 (on the left side of pictures A and B) and the *Desmodesmus subspicatus* culture cultivated from the Culture 5 and contaminated again with the same concentration of HM (As, Cd, Hg, Pb) on the right side of the pictures. The new culture was 0 on picture A and 11 days old on picture B. 2xES medium was given to the new culture.

3.4.3 Control cultures with pH5

The *Desmodesmus subspicatus* cultures behaved similarly in the light incubator as outside in the greenhouse. The Figure 32 visualizes the color change of the cultures in 14 days. By the end of the experiment, the cultures were so dense that they turned from bright green to dark.

Figure 32: 1 day old (on left) and 14 days old cultures (right). The starting pH was 5, n=6 and the duration 14 days.

The TABLE 22 and Figure 33 show that the ODs of the cultures were well increasing during the control period with pH 5. There was 26 fold increases between the starting and 15 days cultures. The highest OD achieved was (2.642±0.259)λ on the last day of experiment. The growth of the biomass showed same tendency as the OD and its peak was 27.3*10^6 cells/ml (30 fold increase) on day 15. As it can be seen from TABLE 22 there was slow growth of biomass between days 9 and 11. The change of Chlorophyll a was highest (0.032mg/l) of the 2-3 days old fresh cultures. The highest Chlorophyll a was achieved on day 14 which was followed by a fall (-22 mg/l). The pH of the cultures was above already on day 2 and remained between 9.12 and 9.66 until the end of experiment.

TABLE 22: Mean values of daily measurements of the control cultures inside the light incubator (OD±SD, n=6, pH5, duration = 15 days)

Days	**0**	**1**	**2**	**3**	**4**	**7**
OD (λ)	0.097	0.166	0.358	0.537	0.592	1.343
SD (OD)	0.000	0.011	0.017	0.015	0.265	0.123
Biomass (*10^6 cell/ml)	0.9	1.9	2.6	4.8	5.2	12.4
Chlorophyll a (mg/l)		0.009	0.034	0.046	0.072	0.148
pH	5.00	8.14	9.59	9.66	9.61	9.34
Days	**8**	**9**	**10**	**11**	**14**	**15**
OD (λ)	1.557	1.747	1.931	1.937	2.491	2.642
SD (OD)	0.104	0.145	0.112	0.134	0.227	0.259
Biomass (*10^6 cell/ml)	12.8	14.5	14.5	15.6	24.5	27.3
Chlorophyll a (mg/l)	0.173	0.202	0.231	0.225	0.302	0.280
pH	9.12	9.59	9.54	9.41	9.23	9.00

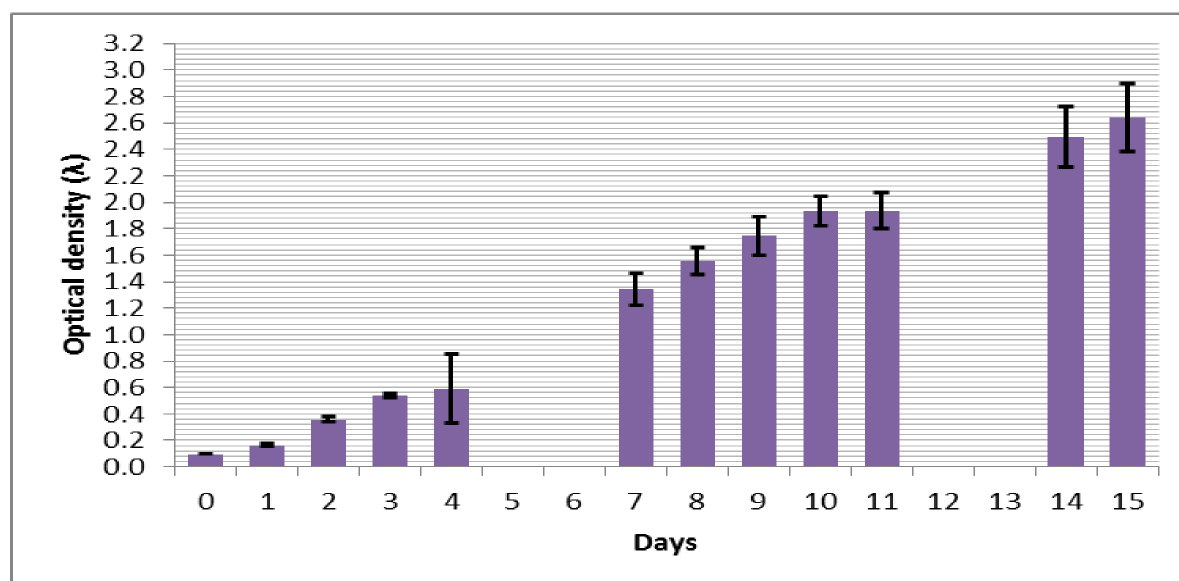


Figure 33: Optical densities of the control cultures with their SD (error bars) on the measurement days (n=6, starting pH 5, duration = 15 days)

All the nutrient concentrations of the samples stayed above zero (see TABLE 23). The results of Culture 6 differed from the other cultures. It had the highest amount of NH_4 (0.09 mg/l), NO_3 (17.4 mg/l) and PO_4 (19.27 mg/l). The NH_4 concentrations slightly increased of all cultures except of the Culture 4 which had no change. The NO_2 was showing

decreasing tendency except of Culture 4 (+23.29 mg/l). Culture 1, 3 and 4 lost and 2, 5 and 6 gained NO_3 content compared to the starting value. Culture 1 and 6 had much bigger change of PO_4 than the rest of the cultures. The Culture 1 had +4.88 mg/l (final value was 5.01 mg/l) and Culture 6 had +19.14 mg/l (final value was 19.27 mg/l) more concentration of PO_4 at the end.

TABLE 23: Nutrient (NH_4, NO_2, NO_3, PO_4) content of the cultures inside the light incubator at the beginning and end of control experiment in pH 5

Culture	Start value	Culture 1	Culture 2	Culture 3	Culture 4	Culture 5	Culture 6	Mean
NH4-N (mg/l)	0.01	0.02	0.02	0.02	0.01	0.08	0.09	0.04
NO2-N (mg/l)	0.42	0.13	23.71	0.08	0.08	0.08	0.08	4.03
NO3-N mg/l)	4.41	4.35	4.44	3.58	3.74	13.39	17.40	7.82
PO4-P (mg/l)	0.13	5.01	0.03	0.13	0.00	0.25	19.27	4.12

3.4.4 Heavy Metal (As, Pb, Cd, Hg) contaminated cultures at pH5

At this point of the Laboratory phase, all the cultures (n=6, pH5) were contaminated with heavy metals (HM). The concentration of HM of each culture was 30 µg/l As, Cd and Hg and 300µg/l Pb. The cultures were well adapting the concentration which can be seen from Figure 34. On the left hand side there are the bright green starting cultures (0 days) and on the right hand side the 14 days old dark green cultures. Turning into dark green from bright visualizes the high growth of biomass.

Figure 34: Fresh 0 day old cultures (on left) and same cultures after 14 days (on right). 18 ml heavy metal solution (300µg/L Pb, 30µg/L As, 30µg/L Cd and 30µg/L Hg) was added to each cultures (n=6, pH5, t= 14 days).

The TABLE 24 and Figure 35 show the mean changes of OD of the cultures through the 14 days experiment. The highest OD was (2.649±0.253)λ of the 14 days old cultures, which means 34 fold increase from the starting value. In general it can be told that the OD was increasing linearly. The SD was similar for the 8-10 days old cultures and was the highest (0.253λ) for the 14 days old cultures. With a final value of $23.1*10^6$ cells/ml (14 days) the biomass had been growing 21 fold compared to the starting cultures. It can be seen also from TABLE 24 that the biomass showed still increasing tendency between days 11-14. On the days 2-3 and 10-11 there was small or no change of biomass. Concerning the Chlorophyll a, the highest change (+0.044 mg/l) occurred between days 9 and 10 and the fresh cultures (1-2 days) increased by +0.037 mg/l, which was bigger change than on the other days in general. The Chlorophyll a content of the 14 days old cultures (0.317

mg/l) was 63 fold higher than of the 1 day old cultures. The mean pH of the cultures was already above by the 1st day and remained around 9 (pH 8.99-9.25) from the 2nd day.

TABLE 24: Mean values of daily measurements of the control cultures inside the light incubator with heavy metal content (OD±SD, n=6, pH5, duration = 14 days)

Days	0	1	2	3	4	7
OD (λ)	0.078	0.126	0.297	0.494	0.654	1.319
SD (OD)	0.000	0.003	0.036	0.040	0.057	0.121
Biomass (*10^6 cell/ml)	1.1	1.7	3.1	3.1	5.3	9.6
Chlorophyll a (mg/l)		0.005	0.042	0.043	0.071	0.151
pH	5.68	8.08	8.99	9.05	8.97	9.24
Days	8	9	10	11	14	
OD (λ)	1.580	1.732	1.910	1.937	2.649	
SD (OD)	0.168	0.163	0.162	0.102	0.253	
Biomass (*10^6 cell/ml)	10.7	11.6	14.1	14.9	23.1	
Chlorophyll a (mg/l)	0.180	0.180	0.224	0.246	0.317	
pH	9.13	9.25	9.12	9.09	9.11	

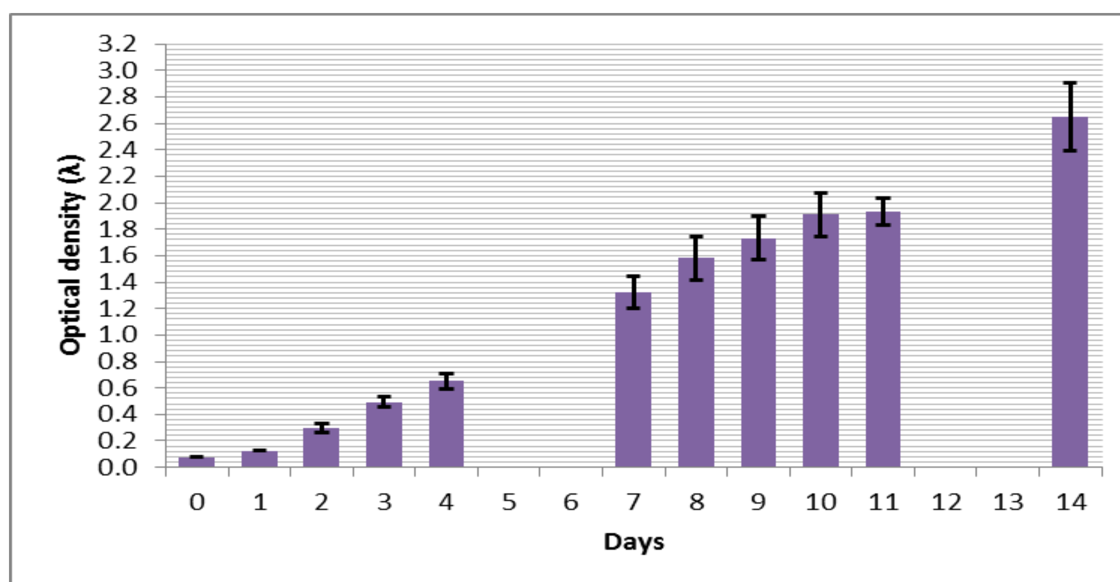


Figure 35: Optical densities of the HM contaminated cultures and their SD (error bars) on the measurement days (n=6, starting pH 5, duration = 14 days)

The end NH_4 concentration was falling with 0.07 mg/l and it was nearby the same of all cultures (see TABLE 25). The highest NO_2 concentration was of Culture 5 (27.43 mg/l) but in general the concentrations of cultures were similarly increasing and they had an average 25.94 mg/l NO_2 in them. All cultures lost high amount of their starting NO_3 content. The highest fall (-57.9 mg/l) was of the Culture 2 and the least (16 mg/l) was of Culture 1. Also the concentration of PO_4 showed decreasing tendency and the average concentration remained in the cultures was 0.06 mg/l.

TABLE 25: Nutrient (NH_4, NO_2, NO_3, PO_4) content of the HM contaminated cultures inside the light incubator at the beginning and end of control experiment (pH 5, n=6)

Culture	Start value	Culture 1	Culture 2	Culture 3	Culture 4	Culture 5	Culture 6	Mean
NH4-N (mg/l)	0.05	-0.02	-0.02	-0.02	-0.02	0.01	-0.02	-0.02
NO2-N (mg/l)	0.91	24.05	26.60	26.38	23.97	27.43	27.18	25.94
NO3-N mg/l)	63.62	15.88	5.72	5.92	13.07	10.71	10.36	10.28
PO4-P (mg/l)	5.17	0.16	0.06	0.02	0.02	0.04	0.04	0.06

3.4.4.1 Effect of HM (As, Pb, Cd, Hg) on the structure of the cells

Most of the DS cells adapted well the pH 5 with heavy metal conditions. On the Figure 36 (picture B) can be seen that there were many viable cells in the HM contaminated sample and visually no modification occurred except a bit swelling compared to the cells from not contaminated cultures (picture A). Besides the existing healthy cells, also dead, disrupted cells were in the HM contaminated sample. The high amounts of disrupted cells are maybe a sign of toxic conditions to some of the cells and/or the Culture achieved its stationary phase.

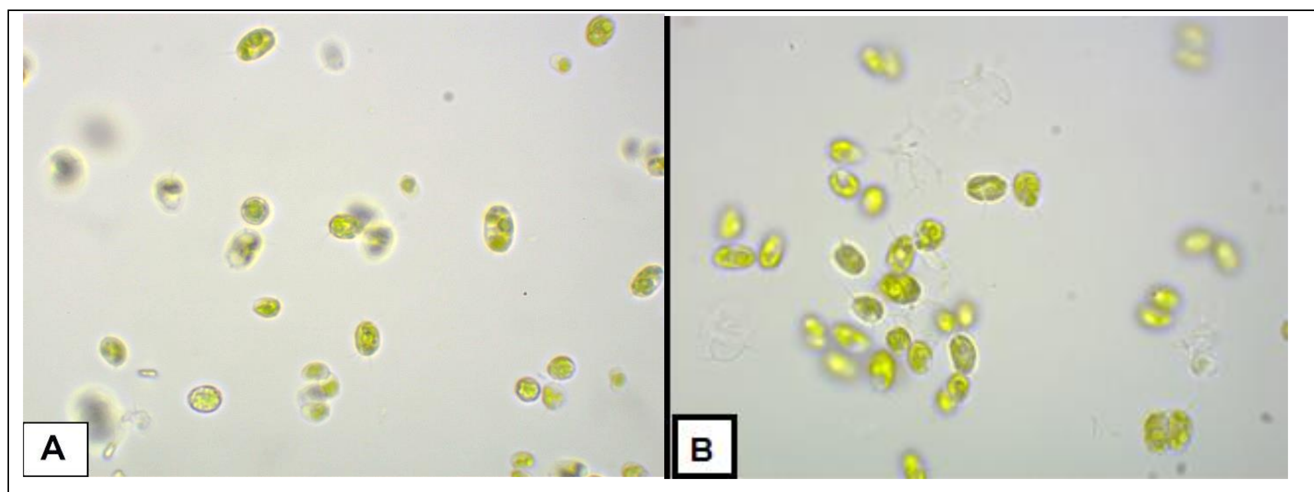


Figure 36: *Desmodesmus subspicatus* cells from the control culture (picture A) and from HM (Pb, As, Cd, Hg) contaminated culture (picture B) under 1000x magnification of Motic (Type: BA310) microscope (n=6 for each experimental runs, pH 5, t= 14-15 days).

3.4.4.2 Biosorption and bioaccumulation

The individual cultures had different efficiencies of uptake of metal ions in 14 days (see Figure, TABLE 26-27). Some cultures were more efficient with certain metals while the others could uptake more from the rest (see the uptakes in mg/kg TM from Appendix 13-14). Concerning the efficiencies of all cultures, the best uptake was of Cadmium (24.45±6.04%). Some individual cultures (Culture 1 and 4, 31.70% and 32.71% efficiencies) worked better than the others. The best uptake of Cd was 9.81μg/l and the worst performing culture was the number 6, it was only able to uptake 4.28μg/l (14.27%).

The second most efficient removal was of Mercury. The cultures were able to uptake (20.83±3.95) % of the initial Hg concentration. It can be seen from Figure 37 and TABLE 26-27 that the individual cultures performed similarly except the Culture 3, which was less efficient (12.09%). The highest uptake of Hg was 7.62μg/l.

On the third place of removed concentration was of Arsenic. The efficiency of the cultures was (15.00±2.07) %. It can be seen from Figure, TABLE 26-27 that the performance of individual cultures differed only slightly and the highest concentration uptake was 18.37μg/l.

The least amount of concentration uptake by biomass was of Lead. The efficiency of uptake (see TABLE 27) was only (7.03±0.89) %. The individual cultures performed similarly (SD=0.89%) and the best uptake of Pb was 8.38µg/l.

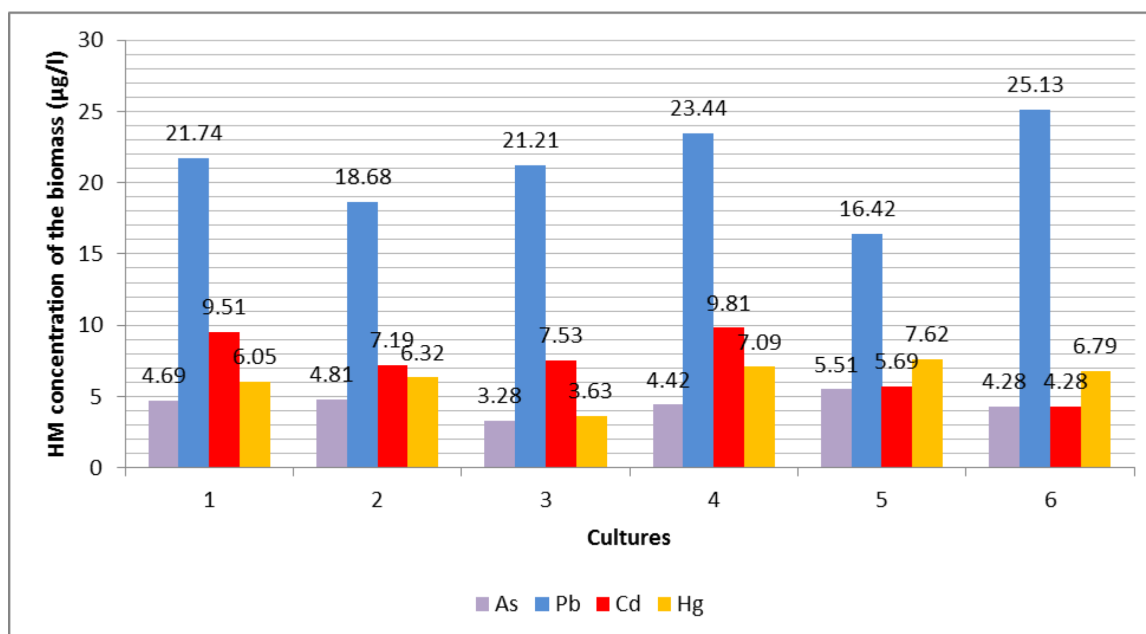


Figure 37: HM (As, Pb, Cd, Hg) concentration of the *Desmodesmus subspicatus* biomass of individual cultures in µg/l

TABLE 26: Heavy metal concentrations of the individual cultures (x̄±SD, n=6, pH5, t=14)

(µg/l)	As	Pb	Cd	Hg
C1	4.69	21.74	9.51	6.05
C2	4.81	18.68	7.19	6.32
C3	3.28	21.21	7.53	3.63
C4	4.42	23.44	9.81	7.09
C5	5.51	16.42	5.69	7.62
C6	4.28	25.13	4.28	6.79
Enrichment	30	300	30	30
x̄	4.50	21.10	7.33	6.25
SD	0.67	2.89	1.96	1.28
Enrichment	30	300	30	30

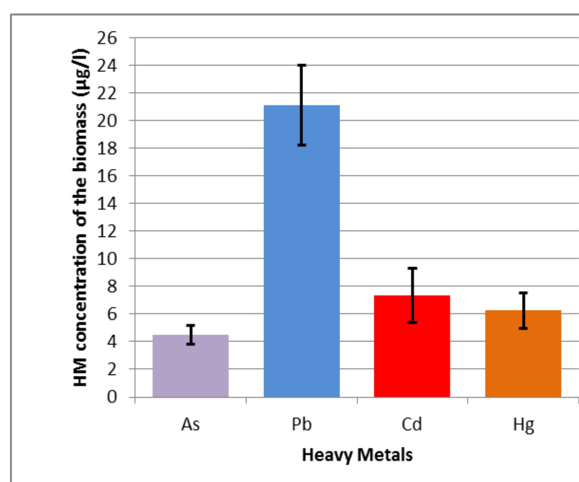


Figure 38: Average HM concentrations of the biomasses (error bars are the SD)

On the Figure 37 can be seen that the average biosorption + biocaccumulation of Cd and Hg were similar. Concerning the efficiencies of individual cultures seen on TALBE 27, the Culture 1 and 2 were middle category, Culture 3 was in general middle category but also

the worst with Hg, Culture 4 was good with Cd, Culture 5 was good with As and Hg and Culture 6 was better with Pb but the worst with Cd. The order of uptake capacity of *Desmodesmus subspicatus* of the heavy metals added to the cultures was:

Cd>Hg>As>Pb

TABLE 27: Efficiency of heavy metal removal by *Desmodesmus subspicatus* (x̄±SD, n=6, pH5, t=14)

%	Arsenic	Lead	Cadmium	Mercury
Culture 1	15.62	7.25	31.70	20.15
Culture 2	16.05	6.23	23.95	21.08
Culture 3	10.95	7.07	25.09	12.09
Culture 4	14.72	7.81	32.71	23.63
Culture 5	18.37	5.47	18.96	25.41
Culture 6	14.27	8.38	14.27	22.64
x̄	15.00	7.03	24.45	20.83
SD	2.07	0.89	6.04	3.95

At the end of the experimental run, most of (70-77%) the initial (enrichment) concentration of As stayed inside the medium (see TABLE 29). The highest concentration (23µg/l) of As stayed inside the medium of Culture 5 and 6 (visualized on Figure 39, A). The concentration in the individual media slightly differed (SD=2.49µg/l). However the enrichment of Mercury was 30µg/l the media contained extremely small portion (0.75±0.34) µg/l of it (see Figure 39, A). Concerning the values of the starting medium (Figure 39, A and B) it is visual that the concentrations were falling compared to the enrichment. The Lead concentration of media were high at the end (14 days) but on picture B can be seen that there was huge change (-79%) of its concentration at the beginning of experiment (Figure 39, B). In 14 days, approximately 67% (x̄=201.67±26.72 µg/l) of the Pb concentration (see TABLE 28-29) was again present inside the media. As the Figure Nr., picture A and TABLE 29 shows, approximately 22% which is (6.58±1.37)µg/l of the initial concentration of Cadmium stayed inside the media. The concentrations of the individual media were similar (SD=1.37µg/l).

The order of heavy metals stayed inside the cultures was the following:

As>Pb>Cd>Hg

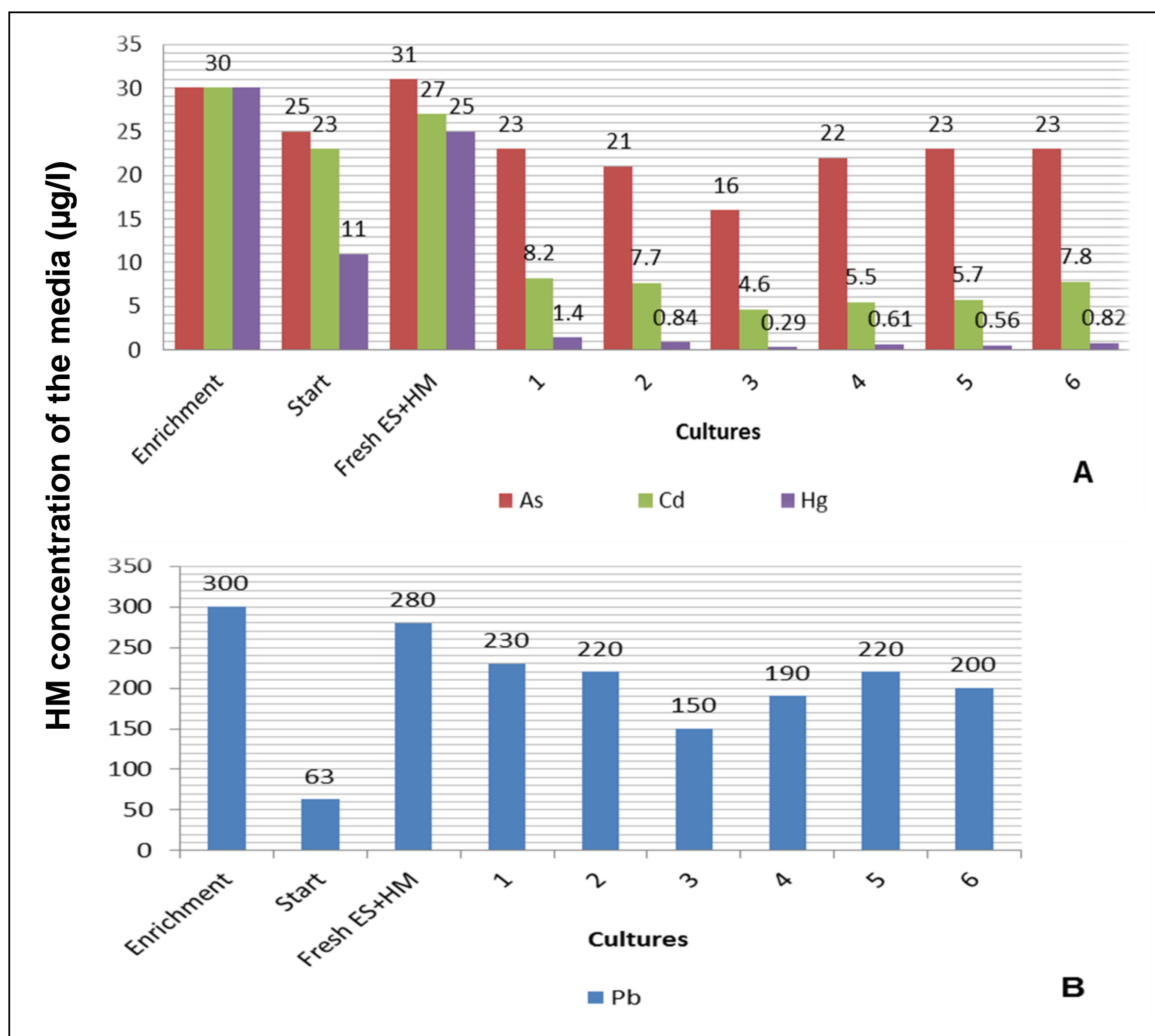


Figure 39: Concentration of HMs (As, Cd, Hg, Pb) inside the different media. On picture A are shown the concentrations of As, Cd and Hg; and on picture B are the different Pb concentrations of the media. The Start value can be associated with the biosorption by *Desmodesmus subspicatus* and small portion of adsorption by the glassware.

TABLE 28: Concentration of different HMs (As, Pb, Cd, Hg) in the different media. The concentration of cultures were measured after day 14 (n=6, pH5)

µg/l	**Arsenic**	**Lead**	**Cadmium**	**Mercury**
Start	25.00	63.00	23.00	11.00
Fresh ES+HM	31.00	280.00	27.00	25.00
Culture 1	23.00	230.00	8.20	1.40
Culture 2	21.00	220.00	7.70	0.84
Culture 3	16.00	150.00	4.60	0.29
Culture 4	22.00	190.00	5.50	0.61
Culture 5	23.00	220.00	5.70	0.56
Culture 6	23.00	200.00	7.80	0.82
x̄	21.33	201.67	6.58	0.75
SD	2.49	26.72	1.37	0.34
Enrichment	30	300	30	30

TABLE 29: Remaining percentages of the heavy metal (As, Pb, Cd, Hg) enrichment in the different media. The percentages inside the cultures were measured after day 14 (n=6, pH5)

%	**Arsenic**	**Lead**	**Cadmium**	**Mercury**
Start	83.33	21.00	76.67	36.67
Fresh ES+HM	103.33	93.33	90.00	83.33
Culture 1	76.67	76.67	27.33	4.67
Culture 2	70.00	73.33	25.67	2.80
Culture 3	53.33	50.00	15.33	0.97
Culture 4	73.33	63.33	18.33	2.03
Culture 5	76.67	73.33	19.00	1.87
Culture 6	76.67	66.67	26.00	2.73
x̄	71.11	67.22	21.94	2.51
SD	8.31	8.91	4.56	1.14

Concerning the heavy metal concentrations of the cultures (biomass + medium), there were missing concentrations (see TABLE 30-31). The missing concentration of different heavy metals in the Start culture possibly means the biosorption by the biomass and also small adsorption by the glassware. The highest biosorption of Lead (<79%) and Hg (<63%) were right at the beginning. The uptake of As (17%) and Cd (23%) were much smaller.

The missing values are shown in TABLE 30 and their shares in TABLE 31. Average 77% ((23.00±1.41)µg/l) of Hg was not detected inside the cultures after 14 days. As it was mentioned before, most of the As stayed inside the medium, small part in the biomass and

as an average 10% was missing from the enrichment. Similar concentrations were missing from the different cultures except of Culture 3, which lost 10.72µg/l (35%). It is also shown in the same tables that already 17% (5µg/l) was missing from the starting cultures. A considerable amount of Pb ((77.23±25.92) µg/l) was not detected inside the media (14 days). From the enrichment concentration 53% of Cd was missing of the end cultures.

TABLE 30: Missing HM (As, Pb, Cd, Hg) concentrations of the different cultures (x̄±SD, n=7, age of Culture 1-6 was 14 days)

µg/l	**Arsenic**	**Lead**	**Cadmium**	**Mercury**
Culture 1	2.31	48.26	12.29	22.55
Culture 2	4.19	61.32	15.11	22.84
Culture 3	10.72	128.79	17.87	26.08
Culture 4	3.58	86.56	14.69	22.30
Culture 5	1.49	63.58	18.61	21.82
Culture 6	2.72	74.87	17.92	22.39
x̄	4.17	77.23	16.08	23.00
SD	3.05	25.92	2.25	1.41
Start	5.00	237.00	7.00	19.00

TABLE 31: Missing HM (As, Pb, Cd, Hg) percentages of the different cultures (x̄±SD, n=7, age of Culture 1-6 was 14 days)

%	**Arsenic**	**Lead**	**Cadmium**	**Mercury**
Culture 1	7.71	16.09	40.97	75.18
Culture 2	13.95	20.44	50.38	76.12
Culture 3	35.72	42.93	59.58	86.95
Culture 4	11.95	28.85	48.95	74.34
Culture 5	4.96	21.19	62.04	72.73
Culture 6	9.07	24.96	59.73	74.62
x̄	13.89	25.74	53.61	76.66
SD	10.18	8.64	7.49	4.71
Start	16.67	79.00	23.33	63.33

3.5 Comparison of the performance of *Desmodesmus subspicatus* in different light, temperature and pH conditions (HM contamination phases excluded).

However the cultures performed well but there were derivations between the developments in the different conditions. Concerning the ODs of the cultures through 14 days (see Figure 40) of experiment, it can be told that the fastest growth was achieved inside the photobioreactor. The cultures in the greenhouse worked similarly and also the 2 cultures inside the light incubator. It can be also seen that the ODs of the control cultures inside the light incubator was higher than in the greenhouse but the difference was not enormous. It is also seen from the Figure 40 that the decay of the culture inside the photobioreactor started early (approximately day 3). The growth of this culture on the day 6 was because of addition of extra nutrient solution.

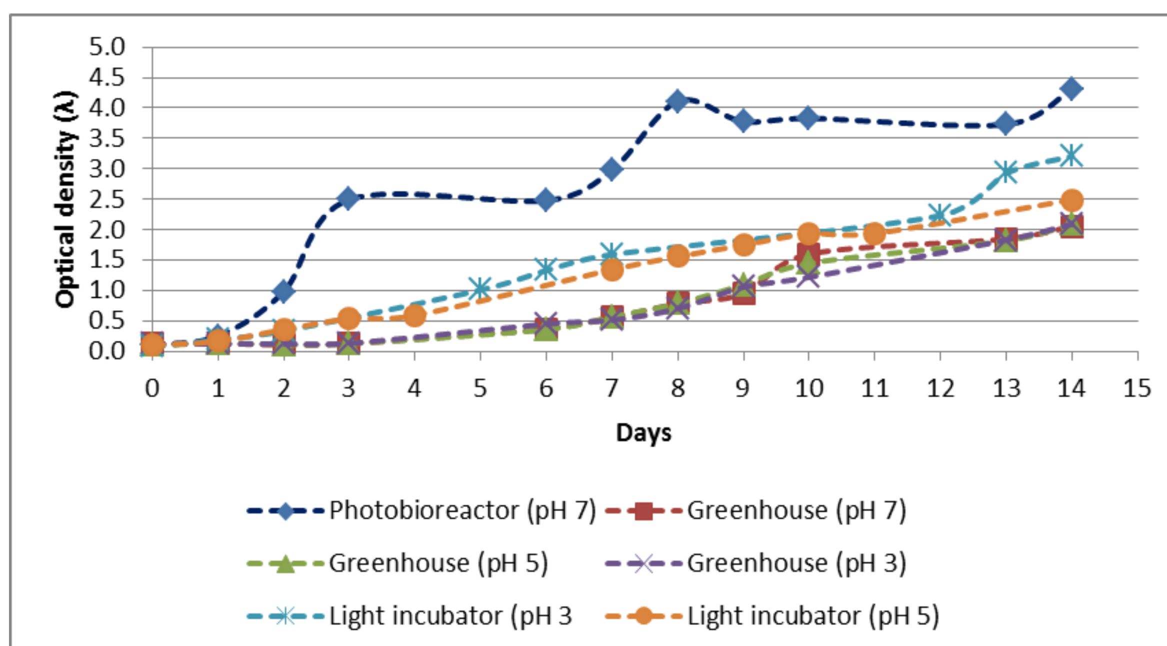


Figure 40: Comparison of optical densities of the *Desmodesmus subspicatus* cultures inside the photobioreactor (n=1, pH7), greenhouse (pH 3, 5, 7, n=2 to each pH levels) and light incubator (pH 3, 5, n=6 for each pH levels)

4. Discussion

In this chapter the efficiency of heavy metal removal and adaptation to different environmental conditions of *Desmodesmus subspicatus* (DS) is evaluated.

4.1 Evaluation of biosorption and bioaccumulation properties

The DS culture contaminated with a heavy metal (HM) mix (As, Pb, Cd, Hg) in pH 5 was able to remove different concentrations of the individual metals. Comparing the results of bioaccumulation (13-14 days) with the earliest studies made at Ostfalia, the *Desmodesmus subspicatus* culture performed not as well compared with Pb, Cd and Hg than *Chlorella vulgaris* and *Scenedesmus quadricauda* (see the efficiencies from TABLE 32). However low the efficiency, the DS culture could remove more (+2.0 µg/l, which is +6%) As than *Chlorella vulgaris*. The possible biosorption of Pb at the beginning of the experiment (at start) was greater of DS (<79%) than of *Scenedesmus quadricauda* (<68%). Surprisingly most of the initially biosorbed amount of Pb was released back from the biomass. This could happen because of three reasons. One of the reasons could be infection by some bacteria or fungi, which results disruption of bonds between the heavy metals and algal cell. The second reason is the equilibrium heavy metal content between the inner and outer cell (Shanab, Essa, Shalaby, 2012). Probably there also could be disruption of the decaying cells, which process breaks the bonds on the cell wall, and releases metals back to the medium. The initial As uptake was small and surprisingly the value of it was the similar of DS and *Scenedesmus quadricauda*. There were small derivation between the initial Cd and Hg biosorptions by the two species.

TABLE 32: Efficiencies of heavy metal removal of *Desmodesmus subspicatus, Chlorella vulgaris* and *Scenedesmus quadricauda* microalgal cultures tested at Ostfalia University of Applied Sciences

Microalgal species	Efficiency of heavy metal uptake (%)			
	Arsenic	Lead	Cadmium	Mercury
Desmodesmus subspicatus (at start)	<17	<79	<23	<63
Desmodesmus subspicatus (14 days)	15	7	24.5	21
Chlorella vulgaris (14 days) (1)	9	19	65	37
Scenedesmus quadricauda (*SQ)* (at start) (2)	<17	<68	<27	<81
Scenedesmus quadricauda (13 days) (2)	21	17	41	61

Sources: Rolfes, 2014 (1); Valdivia, M., 2013 (2)

The highest final heavy metal uptake (consists of biocaccumulation and biosorption at day 14) achieved by DS was with the removal of Cadmium (24.5%). This result was much lower than of *Ascophyllum nodusum* (99.8%), *Chlorella vulgaris* (98%), *Pseudochlorococcum typicum* (70%), *Phormidium ambiguum* (70%) and *Scenedesmus obliquus* (58.5%) *Desmodesmus pleiomorphus* (43%) (Sandau et al., 1996, Shanab, Essa and Shalaby, 2012; Monteiro et al., 2011; Mehta and Gaur, 2005; Zhou, Huang and Lin 1998; Monteiro et al, 2011; Mallick 2002)). The second most efficient final uptake was of Mercury (21%). This result was far behind the values achieved by *Scenedesmus quadricauda var quadrispina* (70%), *Pseudochlorococcum typicum* (70%) and *Spirulina spirulinoides* (60%) (Metha and Gaur, 2005; Chojnacka, Chojnacki and Go´recka, 2004) contaminated with Hg. The initial biosorption and final heavy metal uptake of Arsenic by DS was similar (≤17%). However the final uptake of Lead was minor but the initial biosorption showed good efficiency (<79%). This value is similar with the initial 70% biosorption by *Pseudochlorococum typicum* in 0.5 hour (Shanab, Essa and Shalaby, 2012) and with the 75% efficiency of *Laminaria hiperborea, Bifurcaria bifurcate, Sargassum muticum and Fucus spiralis* in 10 minutes (Nessim et. al., 2011).

High amounts of the initial heavy metal concentrations were missing from the final cultures. These concentrations were not found from the media or biomasses either so probably they were adsorbed by the glassware of the reactors and/or vaporized.

4.2 Evaluation of best pH condition for cultivation and for uptake of heavy metals (As, Pb, Cd, Hg)

The control cultures of *Desmodesmus subspicatus* were tested in pH 3 and 5. The cultures developed similarly in the two different pH levels, but there was some deviation between the densities of the end cultures (see the changes of OD on Figure 41). The results showed that the cultures in pH 3 could achieve higher density than in pH 5. A pH as low as 3 is lethal to most of the algal cultures so this property of *Desmodesmus subspicatus* is kind of unique. Even though the better performance with pH 3, the addition of the HM mix (300µg/l Pb, 30µg/l As, Cd, Hg) obviously caused high stress and the DS culture died off or its development slowed down. This happened because the heavy metals are highly soluble in pH 3. Thus the DS cells could probably take up high concentrations of heavy metals right from the beginning of the experiment. The pH 5 was right for the development of the culture in the medium with the heavy metals, but only low efficiency of the HM removals was achieved. The heavy metals are less soluble when the pH conditions are getting closer to neutral conditions so at pH 5 probably not all the heavy metal concentration was available for the cellular uptake.

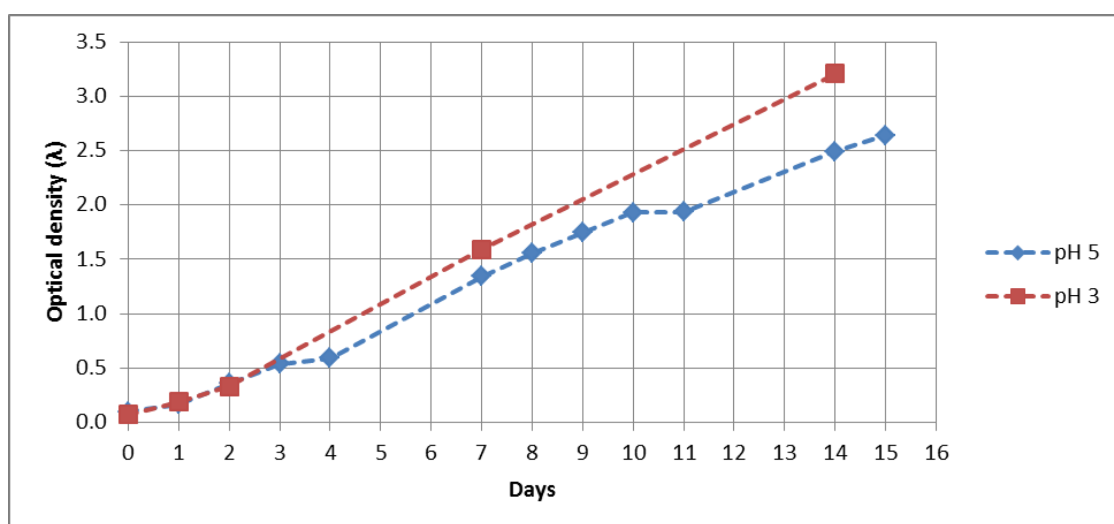


Figure 41: Average optical densities of the *Desmodesmus subspicatus* cultures cultivated in pH 3 and 5 without HM contamination (n=6 for each pH levels, duration = 14 days)

4.3 Evaluation of cultivation in controlled environment (light incubator) and natural conditions (greenhouse)

The overall optical density of the culture in the greenhouse was smaller than in the light incubator. As the Figure 42 shows the OD inside the greenhouse was high also, this means that the culture adapted well to the natural light and temperature changes. It can also be seen that the log-phase of the culture inside the greenhouse was longer lasting than in the light incubator. It can be told, that the daylight and nighttime ratio and the temperature inside a greenhouse are satisfactory for the healthy cultivation of *Desmodesmus subspicatus* in March and April.

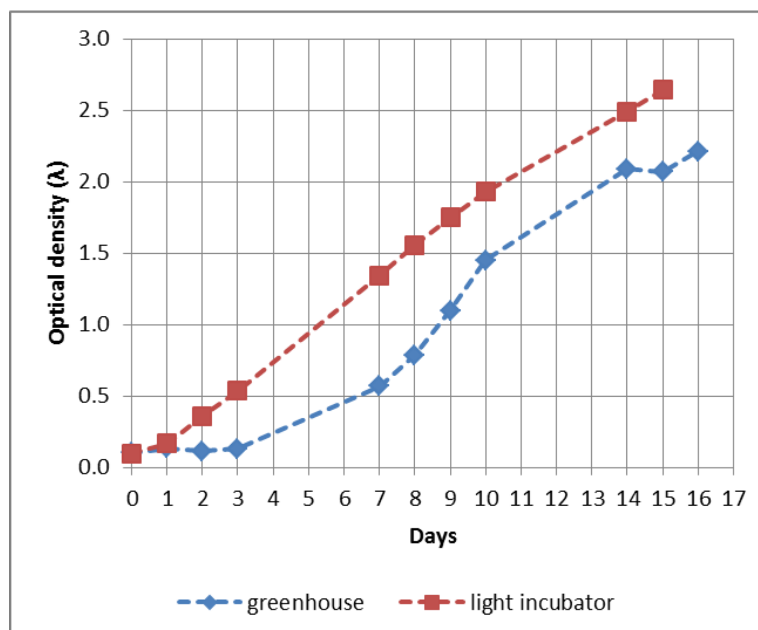


Figure 42: Development of OD of *Desmodesmus subspicatus* cultures inside the greenhouse and light incubator (control samples with pH 5, n=6 for each)

The results of nutrient analyses show also that the life cycle of the DS culture was faster in the light incubator than in the greenhouse. There was increase of NO_3 and PO_4 of the cultures inside the light incubator, which is a sign of the decaying culture (Fields, 2004). Probably the cultures started to die off and NO_3 and PO_4 were released back to the media by the decomposition of death biomass. The same nutrients were still consumed by the cultures inside the greenhouse after 16 days.

4.4 Error estimation

cell counting: Several errors could occurred during the counting of cells with Thoma-chamber. These could come from wrong pipetting; a not accurate amount of sample under the cover glass, wrong counting of individual cells of a cell aggregate and also statistical error could exist. The overall error is estimated to be 20-30%, which is (200-300)*10^3 cells/ml of a starting culture with 1*10^6 cells/ml (Bastidas, 2014).

pH: In a measurement period of 10 minutes, approximately ±0.05-0.5 units of error could occur. This error is can be due to several reasons like temperature difference between the reactor and the laboratory, composition of the glassware, effect of concentrated acids and interaction of buffer with pH electrode (Advanced Sensors Technology).

optical density: Inaccurate vortexing (mixing) of the samples before measuring their OD with Spectrometer could cause error of measured values. Also unclear wall of the cuvette can increase the final results of OD. The sedimentation of the sample could be minimized by running the optical density measurement right after sample taking. The error from the transparency of the cuvette could be avoided by cleaning its wall with lens paper before inserting to the spectrometer.

DM: There could be some small amount of evaporation during the weighting of the samples, which could affect the DM results.

digestion: Some amount of the sample might have remained in the glass tubes after the digestion.

volatilization of heavy metals: Mercury is highly volatile so big amount of it was probably volatized during the contamination phase, which would explain the Hg concentration loss from the culture (Wilkinson, Goulding and Robinson, 1990). The same phenomena might have happened with some amounts of the Arsenic, Lead and Cadmium.

biosorption of the glassware: Through the contamination phase, some concentration of the heavy metal solution was possibly adsorbed by the glassware of the bioreactors. The reason for this is that the charge of the glass material in aqueous solution is negative so it attracts the positively charged heavy metal ions (Stas et al., 2004). This adsorption by the glassware is reducing the available HM concentration for uptake by the biomass.

5. Conclusion

The *Desmodesmus subspicatus* (DS) cultures had good initial biosorption of Pb and Hg, but the long term uptake of heavy metals (As, Pb, Cd, Hg) was not efficient at pH 5. Hence it is recommended to use the DS culture only for short term biosorption of heavy metals.

However there could be options to increase the efficiency of the biomass. As the results showed the best cultivation of DS were in pH 3 and 5 but only the latter one was liveable for the heavy metal contaminated cells. Even though in general the HM contaminated cultures with pH 3 were decaying, one culture survived. There can be a pH level between 3 and 5, which would result higher solubility of the HMs and thus better availability for uptake but less toxicity than in pH 3 would be expected. Also an option could be to add only 1 or 2 heavy metals to the solution at a time to decrease the toxic conditions. It can be that the DS culture would have better efficiency and could survive also in lower pH with that HM content. The culture survived from the HM contamination with pH 3 was infected with a fungus, which is a good example for survival of a multi culture. *Desmodesmus subspicatus* (DM) could be applied together with another microalga or microorganism like fungus. In case of short term application, this second microorganism should have the property of good biosorption of Arsenic and Cadmium, the heavy metals that DS cannot uptake efficiently. For the short term biosorption of heavy metals, a filter material made of the biomass of alive or death DS cells could be applied. To remove all the heavy metals (As, Pb, Cd, Hg) wanted, application of a multi culture (previously mentioned) filter would be a more efficient solution. Another way to increase the efficiency would be application of immobilization and/or pretreatment. Natural immobilization materials like Calcium alginate, agar or carrageenan beds could be used (see the immobilization materials in chapter 1.3.4.2 Immobilization). In case of short term application (only biosorption) Calcium alginate bed and for longer term (bioaccumulation) the agar and carrageenan could be used. Good example for the protection given by the immobilization beds is the funnel, which appeared in the surviving HM contaminated culture with pH 3. This funnel behaved like an immobilization material, kept the culture together and hence gave protection against toxic conditions. The most economical pretreatments to increase the efficiency of Cd and Pb would be by $CaCl_2$ and only for Cd the 1M KOH pretreatment could be applied (Feng and Aldich, 2004; Hao, Zhao and Ramelow 2001). Increasing the population could give also an option to improve the efficiency of HM uptake. As an example the initial

biomass of *Rhizochlonium* was increased and it resulted higher efficiency of As uptake (Mallik, 2001).

The heavy metal contamination experiments were only run inside controlled environment (light incubator). Because of the good cultivation results with different pH levels in the greenhouse, the contamination phase could be also tried out in natural conditions. The cultivation inside e.g. in a greenhouse would prolong the log-phase of the culture, which could increase the heavy metal removal capacity. Furthermore, it is also recommended to do further investigations on the HM resistant cultures with pH 3.

It is also important to mention that the contamination study was only short term (14 days) so the bioaccumulation property of *Desmodesmus subspicatus* culture should be tried out longer term. Also the glassware could be replaced with some other material, which is positively charged in aquatic solution, so it adsorbs less heavy metals especially Mercury.

The cultivation of *Desmodesmus subspicatus* is rather easy and high density can be achieved in a short while. For its cultivation pH levels between 3 and 7 can be applied but to obtain the highest density in as short time as possible, pH 3-5 should be adjusted.

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APPENDICES

Appendix 1: Double concentrated ES media applied for the enrichment of the DS culture during all the different phases

Name of the compound	**Concentration of compound (g/l)**	**Used concentration in 1 liter of media (ml)**	**Used concentration in 3 liters of media (ml)**	**Used concentration in 6 liters of media (ml)**	**Used concentration in 10 liters of media (ml)**
KNO3	10	40	120	240	400
K2HPO4 (KH2PO4 with M=138.09 g/mol)	1	40	120	240	400
MgSO4*7H2O	1	40	120	240	400
extract of ground soil		60	180	360	600
Trace elements		10	30	60	100
ZnSO4*4H2O	1				
MnSO4*4H2O	1				
H3BO3	2				
Co(NO3)2*6H2O	0.2				
Na2MoO4*2H2O	0.2				
CuSO4*5H2O	0.005				
deionized water					
FeSO4*7H2O					
EDTA (Triplex III., Merk)					
Distilled water		810	2430	4860	8100

Appendix 2: Concentrations of the different reagents used for determination of NH_4, NO_2, NO_3 and PO_4

Name of the compound	Contents
PO_4 mix	14 ml Reagent mix 1 + 6 ml Ascorbic acid (18g=l)
Reagent mix 1 (500 ml)	75 ml Ammonium molybdate + 250 ml Sulfuric acid + 25 ml Antimony potassium tartrate + 150 ml distilled water
NH_4-1 reagent (50 ml)	6.5 g Na-salicylate ($C_7H_5O_3Na$) + 6.5 g Na-citrate ($C_6H_5Na_3O_7$*$2H_2O$) + 0.0486 g Na-nitroprussid + distilled water
NH_4-2 reagent (50 ml)	1.6 g NaOH + 0.5 g Na-dichlorisocyanurat * $2H_2O$ + distilled water
TON 1 (100 ml)	0.8 g NaOH+ distilled water
TON2 (100 ml)	0.1304 g Hydrazine sulfate + 0.150 ml Copper sulfate (3.9 g/l) + 1 ml Zink sulfate (45 g/l)
Farbreagent (100 ml)	5 ml Phosphoric acid + 0.5 g Sulfanilamid ($C_6H_8N_2O_2S$) + 0.025 g N-(1-Naphthyl)-ethylene diamindihydrochlorid

Appendix 3: Amount of HCl and HNO_3 added to the dried biomasses before digestion

Culture	1	2	3	4	5	6
DM (g)	0.6793	0.7186	0.6842	0.5452	0.5863	0.6793
HCl (ml)	4.8	5.0	4.8	3.8	4.1	4.6
HNO_3 (ml)	1.6	1.7	1.6	1.3	1.4	1.6

Appendix 4: Result of *Desmodesmus subspicatus* cultures cultivated inside the photobioreactor for 23 days (n=1, pH7)

OD	n=1	n=1	n=1	n=1	n=1	n=1	n=1	n=1	n=1	n=1
Days	0	1	2	3	6	7	8	9	10	13
Culture	3.3	4.3	5.3	6.3	7.3	10.3	11.3	12.3	13.3	14.3
1	0.10	0.25	0.99	2.50	2.48	2.98	4.11	3.77	3.83	3.73
Coulter Count (10^6 cells/ml)					4					9
1	1.020	1.960	6.357	10.960	19.610	20.185	28.000	30.770	37.456	41.820
Cell Count (10^6 cells/ml)										
1	1.031	1.813	7.563	14.625	30.375	34.875	37.125	28.500	29.456	31.563
Chlorophyll- a (mg/l)										
1		0.009	0.067	0.158	0.286	0.185	0.206	0.211	0.177	0.145

OD	n=1	n=1	n=1	n=1	n=1	n=1	n=1	n=1	n=1	n=1
Days	14	15	16	17	18	19	20	21	22	23
Culture	17.3	18.3	19.3	20.3	21.3	24.3	25.3	26.3	27.3	28.3
1	4.30	7.05	7.02	7.61	7.67	9.540	7.865	11.089	11.089	11.193
Coulter Count (10^6 cells/ml)										
1	45.010	47.340	50.700	55.600	59.550	88.640	92.560	100.600	87.810	82.050
Cell Count (10^6 cells/ml)										
1	46.850	66.533	67.813	49.375	62.813	101.250	107.250	114.969	117.813	110.500
Chlorophyll- a (mg/l)										
1	0.133	0.161	0.151	0.159	0.181	0.205	0.215	0.186	0.206	0.185

Appendix 5: Result of Greenhouse phase I: Growth study (pH7, n=6, duration 8 days)

OD	n=6	n=6	n=6	n=6	n=6	n=6	n=6
Days	0	1	2	5	6	7	8
Culture	12.3	13.3	14.3	17.3	18.3	19.3	20.3
1	0.137	0.169	0.211	0.397	0.467	0.529	0.738
2	0.137	0.137	0.189	0.317	0.361	0.420	0.604
3	0.135	0.159	0.211	0.393	0.465	0.527	0.739
4	0.135	0.166	0.230	0.479	0.587	0.671	
5	0.133	0.151	0.178	0.319	0.363	0.401	0.474
6	0.133	0.133	0.182	0.319	0.349	0.349	0.399
Mean	0.135	0.153	0.200	0.371	0.432	0.483	0.591
SD	0.002	0.014	0.019	0.059	0.085	0.106	0.137
Coulter Count (10^6 cells/ml)							
1		1.184	3.334	4.299	3.713	4.055	5.301
2		1.156	1.364	2.588	2.166	2.685	3.366
3		1.374	1.497	2.748	3.167	2.906	4.198
4		1.584	1.497	3.787	4.007	4.525	
5		1.834	1.388	2.468	2.613	2.270	2.428
6		1.247	1.397	2.152	2.564	2.032	1.899
Mean		1.397	1.746	3.007	3.038	3.079	3.438
SD		0.242	0.712	0.768	0.655	0.911	1.220
Cell count (10^6 cells/ml)							
1	1.159	1.063	1.469	2.219	2.219	2.344	3.938
2	1.159	1.375	1.031	1.750	2.000	2.125	3.219
3	1.275	0.906	1.094	2.438	2.281	1.656	4.688
4	1.275	1.063	1.219	3.281	4.000	1.438	
5	1.260	1.125	1.406	2.563	1.563	2.625	4.031
6	1.260	1.656	1.000	1.750	1.469	1.750	1.188
Mean	1.231	1.198	1.203	2.333	2.255	1.990	3.413
SD	0.052	0.248	0.180	0.525	0.838	0.412	1.206
Chlorophyll- a (mg/l)							
1	0.001	0.006	0.019	0.009	0.043	0.072	0.078
2	0.001	0.002	0.009	0.002	0.034	0.062	0.078
3	0.002	0.009	0.010	0.008	0.044	0.068	0.048
4	0.002	0.004	0.032	0.013	0.049	0.083	-
5	0.029	0.002	0.007	0.004	0.040	0.046	0.029
6	0.029	- 0.001	0.009	0.004	0.035	0.055	0.026
Mean	0.011	0.004	0.015	0.007	0.041	0.064	0.043
SD	0.013	0.003	0.009	0.004	0.005	0.012	0.028

Appendix 6: Result of Greenhouse phase II: pH study (pH 7, n=2, duration 16 days)

OD	n=2	n=2	n=2	n=2	n=2	n=2	n=2	n=2	n=2	n=2	n=2	n=2	n=2
Days	25.3	26.3	27.3	28.3	31.3	1.4	2.4	3.4	4.4	7.3	8.4	9.4	10.4
Culture	0	1	2	3	6	7	8	9	10	13	14	15	16
1	0.124	0.119	0.098	0.129	0.371	0.554	0.797	0.964	1.656	1.830	2.016	2.058	2.094
2	0.124	0.136	0.119	0.127	0.367	0.558	0.772	0.932	1.540	1.866	2.088	2.046	2.178
Mean	0.124	0.128	0.109	0.128	0.369	0.556	0.785	0.948	1.598	1.848	2.052	2.052	2.136
SD	-	0.009	0.011	0.001	0.002	0.002	0.013	0.016	0.058	0.018	0.036	0.006	0.042
Coulter Count (10^6 cells/ml)													
1		1.350	2.020	2.156	3.637	4.842	7.357	9.084	10.870	11.650	12.720	11.860	13.180
2		1.300	2.378	1.249	2.977	4.762	6.862	9.485	11.720	11.830	13.100	11.560	13.350
Mean		1.325	2.199	1.703	3.307	4.802	7.110	9.285	11.295	11.740	12.910	11.710	13.265
SD		0.025	0.179	0.454	0.330	0.040	0.248	0.201	0.425	0.090	0.190	0.150	0.085
Cell count (10^6 cells/ml)													
1	0.875	1.063	0.688	0.656	2.625	3.781	4.000	8.094	11.625	17.813	15.938	14.813	14.063
2	0.875	0.875	0.500	0.438	2.781	4.000	5.781	5.125	13.313	9.375	11.813	16.125	12.938
Mean	0.875	0.969	0.594	0.547	2.703	3.891	4.891	6.609	12.469	13.594	13.875	15.469	13.500
SD	-	0.094	0.094	0.109	0.078	0.109	0.891	1.484	0.844	4.219	2.063	0.656	0.563
pH													
1	7.00	9.20	9.28	9.27	9.38	9.48	9.59	9.64	9.56	9.43	9.49	9.43	9.44
2	7.00	9.17	9.3	9.29	9.32	9.4	9.46	9.5	9.43	9.37	9.46	9.35	9.38
Mean	7.00	9.19	9.29	9.28	9.35	9.44	9.53	9.57	9.50	9.40	9.48	9.39	9.41
SD	-	0.015	0.010	0.010	0.030	0.040	0.065	0.070	0.065	0.030	0.015	0.040	0.030
Chlorophyll- a (mg/l)													
1		0.002	0.002	0.008	0.021	0.041	0.056	0.080	0.118	0.157	0.159	0.166	0.184
2		0.006	-	0.008	0.020	0.042	0.053	0.080	0.106	0.147	0.160	0.168	0.185
Mean		0.004	0.001	0.008	0.021	0.042	0.055	0.080	0.112	0.152	0.160	0.167	0.185
SD		0.002	0.001	0.000	0.001	0.001	0.002	-	0.006	0.005	0.001	0.001	0.001

Appendix 7: Result of Greenhouse phase II: pH study (pH 5, n=2, duration 16 days)

OD	n=2	n=2	n=2	n=2	n=2	n=2	n=2	n=2	n=2	n=2	n=2	n=2	n=2
Days	25.3	26.3	27.3	28.3	31.3	1.4	2.4	3.4	4.4	7.3	8.4	9.4	10.4
Culture	0	1	2	3	6	7	8	9	10	13	14	15	16
1	0.063	0.133	0.109	0.109	0.328	0.529	0.751	0.920	1.332	1.740	1.992	1.968	2.100
2	0.063	0.132	0.121	0.148	0.389	0.610	0.820	1.272	1.566	1.896	2.184	2.166	2.328
Mean	0.063	0.133	0.115	0.129	0.359	0.570	0.786	1.096	1.449	1.818	2.088	2.067	2.214
SD	-	0.001	0.006	0.020	0.031	0.041	0.035	0.176	0.117	0.078	0.096	0.099	0.114
Coulter Count (10^6 cells/ml)													
1		1.273	1.403	1.029	3.602	6.406	7.036	11.630	13.080	13.460	15.200	16.700	17.130
2		1.249	1.714	1.244	4.063	7.938	9.637	11.160	13.520	18.540	18.690	19.230	18.710
Mean		1.261	1.559	1.137	3.833	7.172	8.337	11.395	13.300	16.000	16.945	17.965	17.920
SD		0.012	0.156	0.108	0.231	0.766	1.301	0.235	0.220	2.540	1.745	1.265	0.790
Cell count (10^6 cells/ml)													
1	1.500	0.563	0.469	0.438	2.594	3.156	4.156	8.406	6.938	9.563	11.250	13.125	18.188
2	1.500	0.719	1.031	0.844	3.125	3.688	6.844	8.625	8.625	18.563	17.625	14.813	18.375
Mean	1.500	0.641	0.750	0.641	2.859	3.422	5.500	8.516	7.781	14.063	14.438	13.969	18.281
SD	0.000	0.078	0.281	0.203	0.266	0.266	1.344	0.109	0.844	4.500	3.188	0.844	0.094
pH													
1	5.00	7.88	8.21	8.3	8.59	8.88	9.51	9.82	9.48	8.81	8.89	8.83	8.72
2	5.00	7.79	8.24	8.18	8.47	8.62	9.04	9.08	8.67	8.73	8.75	8.66	8.66
Mean	5.00	7.84	8.23	8.24	8.53	8.75	9.28	9.45	9.08	8.77	8.82	8.75	8.69
SD	-	0.045	0.015	0.060	0.060	0.130	0.235	0.370	0.405	0.040	0.070	0.085	0.030
Chlorophyll- a (mg/l)													
1		0.002	0.000	0.007	0.017	0.039	0.055	0.081	0.109	0.232	0.170	0.175	0.217
2		0.003	0.003	0.008	0.022	0.045	0.061	0.020	0.133	0.175	0.194	0.203	0.219
Mean		0.003	0.002	0.008	0.020	0.042	0.058	0.051	0.121	0.204	0.182	0.189	0.218
SD		0.001	0.002	0.001	0.003	0.003	0.003	0.031	0.012	0.028	0.012	0.014	0.001

Appendix 8: Appendix: Result of Greenhouse phase II: pH study (pH 3, n=2, duration 16 days)

OD	n=2	n=2	n=2	n=2	n=2	n=2	n=2	n=2	n=2	n=2	n=2	n=2	n=2
Days	25.3	26.3	27.3	28.3	31.3	1.4	2.4	3.4	4.4	7.3	8.4	9.4	10.4
Culture	0	1	2	3	6	7	8	9	10	13	14	15	16
1	0.123	0.105	0.127	0.138	0.429	0.656	0.899	1.482	1.728	2.412	2.754	2.868	3.054
2	0.123	0.134	0.126	0.125	0.468	0.370	0.505	0.634	0.710	1.248	1.428	1.540	1.628
Mean	0.123	0.120	0.127	0.132	0.449	0.513	0.702	1.058	1.219	1.830	2.091	2.204	2.341
SD	0.000	0.015	0.001	0.007	0.020	0.143	0.197	0.424	0.509	0.582	0.663	0.664	0.713
Coulter Count (10^6 cells/ml)													
1		1.123	2.718	1.245	4.523	8.293	10.570	13.250	21.500	23.460	26.720	25.580	27.860
2		1.144	2.768	1.188	2.884	3.001	4.526	7.219	8.644		12.550	11.170	12.280
Mean		1.134	2.743	1.217	3.704	5.647	7.548	10.235	15.072	23.460	19.635	18.375	20.070
SD		0.011	0.025	0.029	0.819	2.646	3.022	3.016	6.428	0.000	7.085	7.205	7.790
Cell count (10^6 cells/ml)													
1	1.219	0.688	0.688	0.656	2.875	4.188	6.250	10.688	12.188	24.563	21.188	21.938	23.813
2	1.219	0.906	0.719	0.906	3.375	2.781	3.656	4.594	6.063	10.500	10.875	13.875	13.375
Mean	1.219	0.797	0.703	0.781	3.125	3.484	4.953	7.641	9.125	17.531	16.031	17.906	18.594
SD	0.000	0.109	0.016	0.125	0.250	0.703	1.297	3.047	3.063	7.031	5.156	4.031	5.219
pH													
1	3.00	5.87	6.75	7.02	7.97	8.55	9.4	9.35	8.36	8.71	8.59	8.68	8.57
2	3.00	5.43	6.22	6.62	7.75	8.46	9.75	9.41	8.37	8.94	9.28	8.77	8.36
Mean	3.00	5.65	6.49	6.82	7.86	8.51	9.58	9.38	8.37	8.83	8.94	8.73	8.47
SD	-	0.220	0.265	0.200	0.110	0.045	0.175	0.030	0.005	0.115	0.345	0.045	0.105
Chlorophyll- a (mg/l)													
1		-0.002	0.004	0.008	0.024	0.051	0.066	0.103	0.137	0.202	0.222	0.221	0.239
2		0.001	0.000	0.008	0.015	0.084	0.032	0.039	0.066	0.091	0.111	0.120	0.137
Mean		-0.001	0.002	0.008	0.020	0.068	0.049	0.071	0.102	0.147	0.167	0.171	0.188
SD		0.002	0.002	0.000	0.005	0.017	0.017	0.032	0.036	0.056	0.056	0.051	0.051

Appendix 9: Results of control cultures of Laboratory phase: Contamination study (pH 3, n=6, duration 14 days)

OD	n=6	n=6	n=6	n=6	n=6	n=6	n=6	n=6	n=6
Days	0	1	2	5	6	7	12	13	14
Culture	23.4	24.4	25.4	28.4	29.4	30.4	5.5	6.5	7.5
1	0.073	0.197	0.360	1.152	1.466	1.824	2.265	3.057	2.708
2	0.073	0.168	0.305	0.831	1.142	1.304	1.986	2.664	3.048
3	0.073	0.204	0.384	1.178	1.602	1.998	3.126	3.416	3.880
4	0.073	0.202	0.373	1.168	1.574	1.890	2.583	3.636	4.020
5	0.073	0.178	0.260	0.691	0.978	1.056	1.533	2.154	2.472
6	0.073	0.198	0.322	1.036	1.222	1.476	1.908	2.646	3.120
Mean	0.073	0.191	0.334	1.009	1.331	1.591	2.234	2.929	3.208
SD	0.000	0.013	0.043	0.186	0.232	0.339	0.513	0.501	0.568
Coulter Count (10^6 cells/ml)									
1	0.8676	2.3490	3.4500	11.2000	14.2600	15.2900	34.7800	42.4700	48.7500
2	0.8676	2.2390	3.9590	8.0770	8.8760	8.9650	23.4800	27.6200	33.1500
3	0.8676	2.3930	5.2880	10.9400	17.5000	20.7600	51.2700	53.7700	57.5300
4	0.8676	2.6180	4.9260	9.3110	17.4400	19.5500	51.1400	53.9000	57.2100
5	0.8676	2.0660	2.9410	7.7680	7.0980	8.2360	20.0400	24.2300	29.5500
6	0.8676	2.3870	2.7220	10.3900	11.5600	12.4000	23.3800	29.1900	41.4700
Mean	0.868	2.342	3.881	9.614	12.789	14.200	34.015	38.530	44.610
SD	0.000	0.167	0.957	1.338	3.983	4.814	12.981	12.212	10.884
Cell count (10^6 cells/ml)									
1	1.47	1.81	3.34	9.00	12.06	11.34	28.97	33.00	23.00
2	1.47	1.94	3.13	5.94	6.88	8.94	11.72	22.50	28.88
3	1.47	1.81	3.81	8.63	6.44	14.06	37.38	39.75	38.13
4	1.47	1.81	3.94	7.31	11.31	14.16	35.63	34.88	36.41
5	1.47	1.00	1.00	2.22	4.88	7.75	18.84	20.72	23.16
6	1.47	1.41	2.09	6.56	7.25	9.56	18.47	16.69	31.88
Mean	1.47	1.63	2.89	6.61	8.14	10.97	25.17	27.92	30.24
SD	0.00	0.33	1.03	2.24	2.63	2.46	9.47	8.38	5.88
pH									
1	3.00	7.23	8.24	10.42	9.33	10.04	9.11	9.81	9.87
2	3.00	7.38	9.33	10.54	10.68	10.70	10.13	10.51	10.49
3	3.00	7.43	8.54	9.52	10.27	10.15	9.31	9.12	9.10
4	3.00	7.37	8.72	9.69	10.35	9.59	9.51	9.23	9.10
5	3.00	7.31	9.62	10.46	10.50	10.45	10.16	9.91	9.96
6	3.00	7.26	9.12	10.39	10.40	10.53	9.36	9.58	9.65
Mean	3.00	7.33	8.93	10.17	10.26	10.24	9.60	9.69	9.70
SD	-	0.070	0.473	0.405	0.433	0.367	0.405	0.463	0.490
Chlorophyll- a (mg/l)									
1		0.002	0.010	0.134	0.088	0.193	0.290	0.290	0.290
2		0.002	0.014	0.105	0.117	0.124	0.249	0.236	0.239
3		0.002	0.008	0.139	0.174	0.210	0.309	0.314	0.313
4		0.002	0.015	0.131	0.166	0.198	0.311	0.307	0.320
5		0.002	0.013	0.080	0.090	0.097	0.188	0.208	0.245
6		- 0.001	0.018	0.124	0.133	0.141	0.280	0.274	0.289

Appendix 10: Results of Heavy Metal (As, Pb, Cd, Hg) contaminated cultures (pH 3, n=6, duration 3 days)

OD	n=6	n=6	n=6	n=6
Days	0	1	2	3
Culture	7.5	8.5	9.5	10.5
1	0.115	0.103	0.086	0.068
2	0.115	0.102	0.081	0.068
3	0.115	0.102	0.079	0.051
4	0.115	0.110	0.076	0.050
5	0.115	0.114	0.091	0.100
6	0.115	0.084	0.076	0.045
Mean	0.115	0.103	0.082	0.064
SD	0.000	0.009	0.005	0.018
Coulter Count (10^6 cells/ml)				
1	1.664	1.270	1.135	0.897
2	1.664	1.202	0.930	0.746
3	1.664	1.202	0.920	0.654
4	1.664	1.213	0.832	0.680
5	1.664	1.330	1.133	1.364
6	1.664	1.066	0.903	0.551
Mean	1.664	1.214	0.976	0.815
SD	0.000	0.080	0.116	0.267
Cell count (10^6 cells/ml)				
1	1.313	0.844	0.781	0.781
2	1.313	1.094	1.063	0.656
3	1.313	1.000	0.625	0.563
4	1.313	1.094	0.656	0.250
5	1.313	1.063	0.938	1.031
6	1.313	0.625	0.719	0.281
Mean	1.313	0.953	0.797	0.594
SD	0.000	0.170	0.156	0.273
pH				
1	3.00	2.9	2.87	2.92
2	3.00	2.77	2.79	2.83
3	3.00	2.75	2.78	2.75
4	3.00	2.66	2.72	2.78
5	3.00	2.63	2.69	2.68
6	3.00	2.59	2.64	2.66
Mean	3.000	2.717	2.748	2.770
SD	-	0.104	0.075	0.088
Chlorophyll- a (mg/l)				
1	0.103	0.016	0.004	-0.002
2	0.102	0.010	-0.005	-0.006
3	0.102	0.010	-0.002	-0.002
4	0.110	0.014	-0.002	-0.002
5	0.114	0.010	-0.004	-0.007
6	0.084	0.011	-0.003	-0.009
Mean	0.103	0.012	- 0.002	-0.005
SD	0.009	0.002	0.003	0.003

Appendix 11: Results of control cultures of Laboratory phase: Contamination study (pH 5, n=6, duration 15 days)

OD	n=6	n=6	n=6	n=6	n=6	n=6	n=6	n=6	n=6	n=6	n=6	n=6
Days	0	1	2	3	4	7	8	9	10	11	14	15
Culture	12.5	13.5	14.5	15.5	16.5	19.5	20.5	21.5	22.5	23.5	26.5	27.5
1	0.097	0.175	0.348	0.552	0.718	1.448	1.640	1.920	2.004	2.036	2.620	2.800
2	0.097	0.148	0.343	0.535	0.674	1.104	1.328	1.452	1.692	1.680	2.015	2.155
3	0.097	0.160	0.384	0.544	0.736	1.428	1.592	1.800	2.008	2.068	2.700	2.940
4	0.097	0.174	0.351	0.507	0.703	1.328	1.592	1.736	1.920	1.852	2.490	2.605
5	0.097	0.160	0.343	0.547	0.000	1.292	1.588	1.824	2.008	2.024	2.640	2.830
6	0.097	0.178	0.378	0.535	0.718	1.456	1.604	1.752	1.952	1.964	2.480	2.520
Mean	0.097	0.166	0.358	0.537	0.592	1.343	1.557	1.747	1.931	1.937	2.491	2.642
SD	0.000	0.011	0.017	0.015	0.265	0.123	0.104	0.145	0.112	0.134	0.227	0.259
Coulter Count (10^6 cells/ml)												
1	1.162	2.180	2.779	4.929	8.187	17.120	16.160	21.430	22.520	25.130	38.190	39.560
2	1.162	1.596	2.998	5.297	5.923	13.350	13.430	14.400	15.920	20.240	23.270	24.020
3	1.162	1.951	3.409	6.436	7.539	18.660	18.780	15.330	20.360	23.180	34.630	42.650
4	1.162	2.047	2.967	3.858	7.743	15.490	16.570	19.130	21.120	24.530	27.970	31.010
5	1.162	1.630	4.602	6.116	7.149	15.630	15.200	17.010	18.540	23.290	29.680	31.380
6	1.162	2.412	3.799	5.929	7.828	18.750	18.500	18.920		25.290	31.380	34.500
Mean	1.162	1.969	3.426	5.428	7.395	16.500	16.440	17.703	19.692	23.610	30.853	33.853
SD	0.000	0.289	0.624	0.863	0.728	1.907	1.844	2.395	2.282	1.714	4.753	6.073
Cell count (10^6 cells/ml)												
1	0.906	1.969	2.094	5.188	6.719	12.250	11.750	19.125	16.500	15.375	30.781	36.719
2	0.906	1.844	2.156	4.469	3.844	9.750	9.378	12.000	10.125	12.875	19.219	17.969
3	0.906	2.531	3.031	4.625	6.094	15.375	13.875	17.500	15.250	18.375	25.625	35.781
4	0.906	1.719	2.250	4.094	4.781	9.875	14.875	12.375	17.875	17.000	28.125	22.656
5	0.906	1.625	3.063	5.061	5.406	10.500	11.875	14.000	12.625	14.875	24.219	26.145
6	0.906	1.750	3.094	5.219	4.188	16.625	15.000	11.750	14.750	15.250	19.219	24.688
Mean	0.906	1.906	2.615	4.776	5.172	12.396	12.792	14.458	14.521	15.625	24.531	27.326
SD	0.000	0.299	0.451	0.414	1.015	2.700	1.999	2.857	2.539	1.721	4.276	6.800

pH												
1	5.00	7.94	9.32	9.64	9.08	9.11	8.97	9.55	9.14	9.1	9.04	9.52
2	5.00	8.31	10.28	10.46	10.39	9.47	9.62	10.16	10.03	10	9.49	6.67
3	5.00	8.16	9.77	9.42	9.68	9.21	8.85	9.26	9.52	9.53	9.23	9.6
4	5.00	8.21	9.62	10.14	9.64	9.35	9.25	9.66	9.29	9.27	9.52	9.65
5	5.00	8.07	9	8.88	9.28	9.56	8.82	9.39	9.65	8.96	8.81	9.08
6	5.00	8.13	9.57	9.41	9.57	9.31	9.2	9.53	9.6	9.62	9.26	9.49
Mean	5.00	8.14	9.59	9.66	9.61	9.34	9.12	9.59	9.54	9.41	9.23	9.00
SD	-	0.115	0.394	0.516	0.409	0.151	0.276	0.284	0.282	0.348	0.247	1.059
Chlorophyll- a (mg/l)												
1		0.009	0.028	0.052	0.074	0.159	0.186	0.222	0.236	0.230	0.300	0.273
2		0.010	0.093	0.041	0.063	0.117	0.138	0.162	0.193	0.178	0.265	0.246
3		0.008	0.022	0.046	0.074	0.154	0.178	0.205	0.242	0.238	0.309	0.283
4		0.010	0.022	0.044	0.070	0.154	0.177	0.203	0.237	0.219	0.302	0.279
5		0.011	0.020	0.046	0.073	0.151	0.181	0.220	0.252	0.246	0.329	0.328
6		0.005	0.020	0.048	0.076	0.156	0.177	0.200	0.229	0.242	0.306	0.269
Mean		0.01	0.03	0.05	0.07	0.15	0.17	0.20	0.23	0.23	0.30	0.28
SD		0.002	0.027	0.003	0.004	0.014	0.016	0.020	0.019	0.023	0.019	0.025

Appendix12: Data of digestion and heavy metal concentration of the biomass

Date	**30.6 for digestion**							
Reactor	1	2	3	4	5	6		
weight of cruicible	31.4596	31.7825	32.0560	34.2875	31.8707	32.7650		
wet weight	90.2763	82.6086	88.2393	76.7764	68.5507	74.5186		
wet weight without cruicible	58.8167	50.8261	56.1833	42.4889	36.6800	41.7536		
dry weight	90.9556	83.3272	88.9235	77.3216	69.1370	75.1979		
DM (g)	0.6793	0.7186	0.6842	0.5452	0.5863	0.6793		
DM (%)	0.0115	0.0141	0.0122	0.0128	0.0160	0.0163		
replacement volume (ml)	25	25	25	25	25	25		
(V/m)	36.803	34.790	36.539	45.855	42.640	36.803		
As (mg/kg)	6.9	6.7	4.8	8.1	9.4	6.3		
Pb (mg/kg)	32	26	31	43	28	37		
Cd (mg/kg)	14	10	11	18	9.7	6.3		
Hg (mg/kg)	8.9	8.8	5.3	13	13	10		
(µg/l)	1	2	3	4	5	6	Mean	SD
As	4.69	4.81	3.28	4.42	5.51	4.28	4.50	0.67
Pb	21.74	18.68	21.21	23.44	16.42	25.13	21.10	2.89
Cd	9.51	7.19	7.53	9.81	5.69	4.28	7.33	1.96
Hg	6.05	6.32	3.63	7.09	7.62	6.79	6.25	1.28

Appendix 13: Results of heavy metal analyzes made on the digested biomasses of the by GBA (Gebellschaft für Bioanalytik MBH)

GBA LABORGRUPPE – WISSEN WAS DRIN IST...

GBA GESELLSCHAFT FÜR BIOANALYTIK MBH
Daimlerring 37 • 31135 Hildesheim

Ostfalia Hochschule
Herr Genth
Salzdahlumer Str. 46/48

38302 Wolfenbüttel

Prüfbericht-Nr.: 2014P606075 / 1

Auftraggeber	Ostfalia Hochschule
Eingangsdatum	10.07.2014
Projekt	
Material	Köwaaufschluss
Kennzeichnung	siehe Tabelle
Auftrag	Analytik gem. Vorgabe des Auftraggebers
Verpackung	PE-Röhrchen
Probenmenge	25 mL
GBA-Nummer	**14603188**
Probenahme	durch den Auftraggeber
Probentransport	GBA
Labor	GBA Gesellschaft für Bioanalytik mbH
Beginn der Analysen	10.07.2014
Ende der Analysen	16.07.2014
Methoden	siehe Anlage
Unteraufträge	keine
Bemerkung	
Probenaufbewahrung	Wenn nicht anders vereinbart, werden Bodenproben drei Monate und Wasserproben vier Wochen aufbewahrt.

Hildesheim, 17.07.2014

I.A. W. Schlösser
Projektbearbeitung

Die Prüfergebnisse beziehen sich ausschließlich auf die genannten Prüfgegenstände. Ohne schriftliche Genehmigung der GBA darf der Bericht nicht auszugsweise vervielfältigt werden.

Seite 1 von 2 zu Prüfbericht-Nr.: 2014P606075 / 1

Standort: Hildesheim
Telefon: +49 5121 750 96-50
Fax: +49 5121 750 96-55
E-Mail: hildesheim@gba-laborgruppe.de
Homepage: www.gba-laborgruppe.de

HypoVereinsbank
IBAN: DE 45 2003 0000 0050 4043 92
BIC: HYVEDEMM300
Commerzbank Hamburg
IBAN: DE 67 2004 0000 0449 6444 00
BIC: COBADEHHXXX

USt.-Ident-Nr.: DE 118 554 138
St.-Nr.: 47/723/00196
Sitz der Gesellschaft: Hamburg
Handelsregister:
Hamburg HRB 42774

Geschäftsführer:
Manfred Giesecke
Ralf Murzen
Dr. Roland Bernerth
Carsten Schaffors
Dr. Hedwig Dölsfeld

Prüfbericht-Nr.: 2014P606075 / 1

GBA-Nummer		14603188	14603188	14603188	14603188
Probe-Nr.		001	002	003	004
Material		Köwaaufschluss	Köwaaufschluss	Köwaaufschluss	Köwaaufschluss
Probenbezeichnung		Köwa 1	Köwa 2	Köwa 3	Köwa 4
Probemenge		25 mL	25 mL	25 mL	25 mL
Probeneingang		10.07.2014	10.07.2014	10.07.2014	10.07.2014
Analysenergebnisse	*Einheit*				
Arsen	mg/kg TM	6,9	6,7	4,8	8,1
Blei	mg/kg TM	32	26	31	43
Cadmium	mg/kg TM	14	10	11	18
Quecksilber	mg/kg TM	8,9	8,8	5,3	13

Prüfbericht-Nr.: 2014P606075 / 1

GBA-Nummer		14603188	14603188	14603188
Probe-Nr.		005	006	007
Material		Köwaaufschluss	Köwaaufschluss	Köwaaufschluss
Probenbezeichnung		Köwa 5	Köwa 6	Köwa Blindwert
Probemenge		25 mL	25 mL	25 mL
Probeneingang		10.07.2014	10.07.2014	10.07.2014
Analysenergebnisse	*Einheit*			
Arsen	mg/kg TM	9,4	6,3	<1,0
Blei	mg/kg TM	28	37	<1,0
Cadmium	mg/kg TM	9,7	15	<0,10
Quecksilber	mg/kg TM	13	10	<0,10

Prüfberichtsnummer 2014P606075 / 1

Angewandte Verfahren und Bestimmungsgrenzen

Parameter	Bestimmungs-grenze	Einheit	Methode
Arsen	1,0	mg/kg TM	DIN EN ISO 16171[a] 5
Blei	1,0	mg/kg TM	DIN EN ISO 16171[a] 5
Cadmium	0,10	mg/kg TM	DIN EN ISO 16171[a] 5
Quecksilber	0,10	mg/kg TM	DIN EN ISO 16171[a] 5

Die mit [a] gekennzeichneten Verfahren sind akkreditierte Verfahren. Die Bestimmungsgrenzen können matrixbedingt variieren.
Untersuchungslabor: 5GBA Pinneberg

Appendix 14: Results of heavy metal analyzes made on the media by GBA (Gebellschaft für Bioanalytik MBH)

GBA LABORGRUPPE – WISSEN WAS DRIN IST...

GBA GESELLSCHAFT FÜR BIOANALYTIK MBH

Ostfalia Hochschule
Herr Genth
Salzdahlumer Str. 46/48

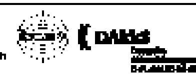
38302 Wolfenbüttel

Prüfbericht-Nr.: 2014P606076 / 1

Auftraggeber	Ostfalia Hochschule
Eingangsdatum	10.07.2014
Projekt	
Material	Wasser
Kennzeichnung	siehe Tabelle
Auftrag	Analytik gem. Vorgabe des Auftraggebers
Verpackung	PE-Röhrchen
Probenmenge	50 mL
GBA-Nummer	**14603188**
Probenahme	durch den Auftraggeber
Probentransport	GBA
Labor	GBA Gesellschaft für Bioanalytik mbH
Beginn der Analysen	10.07.2014
Ende der Analysen	17.07.2014
Methoden	siehe Anlage
Unteraufträge	keine
Bemerkung	Die Aufschlusslösungen wurden vom AG zur Verfügung gestellt
Probenaufbewahrung	Wenn nicht anders vereinbart, werden Bodenproben drei Monate und Wasserproben vier Wochen aufbewahrt.

Hildesheim, 17.07.2014

i.A. W. Schlösser
Projektbearbeitung



Prüfbericht-Nr.: 2014P606076 / 1

GBA-Nummer		14603188	14603188	14603188	14603188
Probe-Nr.		008	009	010	011
Material		Wasser	Wasser	Wasser	Wasser
Probenbezeichnung		**1.1**	**2.1**	**3.1**	**4.1**
Probemenge		50 mL	50 mL	50 mL	50 mL
Probeneingang		10.07.2014	10.07.2014	10.07.2014	10.07.2014
Analysenergebnisse	***Einheit***				
Arsen	µg/L	23	21	16	22
Blei	µg/L	230	220	150	190
Cadmium	µg/L	8,2	7,7	4,6	5,5
Quecksilber	µg/L	1,4	0,84	0,29	0,61

Prüfbericht-Nr.: 2014P606076 / 1

GBA-Nummer		14603188	14603188	14603188	14603188
Probe-Nr.		012	013	014	015
Material		Wasser	Wasser	Wasser	Wasser
Probenbezeichnung		**5.1**	**6.1**	**7.1**	**8.1**
Probemenge		50 mL	50 mL	50 mL	50 mL
Probeneingang		10.07.2014	10.07.2014	10.07.2014	10.07.2014
Analysenergebnisse	***Einheit***				
Arsen	µg/L	23	23	29	31
Blei	µg/L	220	200	98	280
Cadmium	µg/L	5,7	7,8	23	27
Quecksilber	µg/L	0,56	0,82	0,38	25

Prüfbericht-Nr.: 2014P606076 / 1

GBA-Nummer		14603188
Probe-Nr.		016
Material		Wasser
Probenbezeichnung		**9.1**
Probemenge		50 mL
Probeneingang		10.07.2014
Analysenergebnisse	***Einheit***	
Arsen	µg/L	25
Blei	µg/L	63
Cadmium	µg/L	23
Quecksilber	µg/L	11

Prüfbericht-Nr.: 2014P606076 / 1

Angewandte Verfahren und Bestimmungsgrenzen

Parameter	Bestimmungs-grenze	Einheit	Methode
Arsen	0,50	µg/L	DIN EN ISO 17294-2 (E29)ᵃ 5
Blei	1,0	µg/L	DIN EN ISO 17294-2 (E29)ᵃ 5
Cadmium	0,30	µg/L	DIN EN ISO 17294-2 (E29)ᵃ 5
Quecksilber	0,20	µg/L	DIN EN ISO 17294-2 (E29)ᵃ 5

Die mit ᵃ gekennzeichneten Verfahren sind akkreditierte Verfahren. Die Bestimmungsgrenzen können matrixbedingt variieren.

Untersuchungslabor: 5GBA Pinneberg