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A comparison of nutrient reduction between activated carbon and coconut fibre in wastewater treatment

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En jämförelse av näringsreduktion mellan aktivt kol och koksnötsfibrer i rening av avloppsvatten.

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A comparison of nutrient reduction between activated carbon and coconut fibre in wastewater treatment.

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Abstract
Two batch mesocosms were created on site in Da Nang, Vietnam to reduce nutrients in wastewater from fish processing factories. The mesocosms contained either activated carbon or coconut fibre which in earlier studies has shown promising results in wastewater treatment. Three aspects of the materials were compared; Chemical content, which measured levels of COD, total-nitrogen and total-phosphorus. Rate of biofilm formation, where biofilm were measured visually and through weight. The last aspect was microbiological presence where fours species of microorganisms were cultivated. The experiment showed no obvious difference between the materials but concludes that this is an experiment that could and should be developed further.

Keywords
Wastewater treatment, Activated Carbon, Coconut fibre.

Sammanfattning
Två mesokosmer skapades i Da Nang, Vietnam med målet att reducera näringsämnen i avloppsvatten från fabriker som processar fisk. Mesokosmerna innehöll antingen aktivt kol eller kokosnötsfibrer vilka har visat lovande resultat i tidigare studier på avloppsrening. Tre aspekter av materialen jämfördes, den första var kemiskt innehåll, vilket innebar att nivåer av COD, total-kväve och total-fosfor mättes. Den andra aspekten var bildning av biofilm, vilket innebar att biofilmsbildning mättes visuellt och genom vikt. Den sista aspekten var mikrobiologisk skillnad, vilket innebar att fyra arter av mikroorganismer odlades och förekomsten av dessa mikroorganismer jämfördes. Experimentet visade att det inte fanns någon tydlig skillnad mellan aktivt kol och kokosnötsfibrer men drar slutsatsen att experimentet kan och borde utvecklas.

Ämnesord
Rening av avloppsvatten, Aktivt kol, kokosfiber.

Content

Foreword

We all want to make a difference and contribute to a better world, in large way or a small way, our own way. When I got the possibility to visit Vietnam and maybe in a small way assist Dr Thao with his work regarding wastewater treatment I was so grateful. And now, a long time afterwards, I am even more so. There were many difficulties and lessons in this project, for instance to plan a project from across the world and then realize when in place that it simply will not work. But the rewards and lessons from it were all worth it. I want to thank Dr Thao for his great hospitability, brilliance and patience. I want to thank the staff of the College of Technology for their good advice and for putting up with an endless amount of questions. I want to thank all my friends I got there for taking care of me and making me feel at home. Lastly I want to thank Vietnam for all the lessons, all the memories and its incomparable beauty which I hope will remain with all of our combined efforts.

Thousands has lived without love, no one without water.

- H.W Auden

1. Introduction

Water is central to all life, from the smallest of microorganisms to the largest of animals. It is vital in many parts of human everyday life and is used for drinking, cooking and washing etc. However, using water may contaminate it and if there are no well-functioning wastewater treatment systems the contaminations eventually return to us. A straightforward example of this is when WHO stated on their factsheet that “at least 1.8 billion people use a drinking source contaminated with faeces” (WHO, 2015). Even so, the use of appropriate wastewater treatment systems is not always prioritised as they are costly, occupy large areas and might not give immediate visible or economic gain. The lack of appropriate wastewater treatment is most prominent in developing countries and according to UNEP’s (2010) “Sick Water”- report, 90 % of wastewater in developing countries is let out untreated into receiving waterbodies, negatively affecting the ecosystem. This problem seems to require another take on wastewater treatment, with inexpensive and novel systems as well as an innovative thinking in use of materials. A promising field of research is the use of different materials for reduction of pollutants in wastewater. Not only might this reduce very specific and difficult contaminants but depending on material it might be an inexpensive treatment. In Vietnam, at the College of Technology in the city of Da Nang, intense work is performed to combine the use of these sorts if materials with effective and novel wastewater treatment systems. Dr Thao Tran Minh, Head of Division of environmental engineering, is working with the development of a small prototype anaerobic baffled reactor/membrane bioreactor (ABR-MBR reactor) with a ceramic membrane. The goal is to treat highly nutritious wastewater from several fish processing factories. This is a potentially low-cost, effective wastewater treatment system which has shown promising results so far.

In the development of the prototype, problems with rapid formation of biofilm which clogs the membranes of the system, thereby reducing efficiency, was encountered. This rapid formation of biofilm is assumed to partly depend on the high levels of nutrients in the wastewater. To solve this problem an extra treatment step has been added, using activated carbon and coconut fibre. Activated carbon is known to adsorb different nutrients in wastewater through its large volume, porosity and active sites (Ferhan & Özgür, 2011). Coconut fibre has shown to be a well-functioning medium for microorganisms that reduces nutrients through attached growth process, also called

biofilm (Manoj & Vasudevan, 2012). If these materials are effective in reducing nutrients in the wastewater the concentration of these substances, and therefore biofilm production, might be lowered.

1.1. Aim

The aim of this study is to compare activated carbon (AC) and coconut fibre (CF) regarding their ability to reduce nutritional content in wastewater from the anaerobic membrane baffled reactor/membrane bioreactor (ABR/MBR-reactor). Three questions were formulated to make a comparison more focused;

• Was there any difference between AC and CF regarding impact on levels of nutrients (chemical oxygen demand, total-nitrogen and total-phosphorus) in wastewater from the ABR/MBR-reactor?

• Was there a difference between AC and CF regarding formation of biofilm on microscopic slides and in glass filters?

• Was there any difference between AC and CF regarding presence of the different bacterial genus *Clostridia*, *Desulfovibrio, Methanobacteria* and *Lactobacillus* between the materials?

These bacterial species were chosen because they can be producers and inhabitants of biofilms in wastewater treatment plants (Ji et al., 2015; Mohanakrishnan et al., 2011; Fernández et al., 2008; Eusébio et al., 2010).

1.2. Definition

In this study coconut fibre and activated carbon are called nutrient reduction materials. This is a term used on site in this experiment and refers to materials that has characteristics that can reduce amounts of nutrients in wastewater, for instance through adsorption or biofilm formation.

2. Basics of wastewater treatment

2.1. Wastewater

According to Bitton (1994) wastewater can be domestic or industrial. Domestic wastewater is generated from smaller households and businesses. Industrial wastewater originates from production processes and contamination differs largely depending on

the industry but can contain fats, pharmaceutical waste, metals or other contaminants. In order to well integrate wastewater into a recipient body of water there are three objectives of wastewater treatment;

1. Reduce the organic content of wastewater; this category also includes the removal/reduction of trace organics which are recalcitrant to biodegradation and may be toxic or carcinogenic.
2. Removal/reduction of nutrients (like nitrogen or phosphorus) to reduce pollution of receiving surface waters or groundwater.
3. Removal or inactivation of pathogenic microorganisms and parasites.

2.1.1. Pollutants

Organic residues are common pollutants in wastewater. The organic concentration can be measured as biological oxygen demand (BOD) or, chemical oxygen demand (COD) which indicates the amount of oxygen (mg/l) needed to degrade the organic material biologically or chemically. Biological and chemical degradation processes occurs naturally. In wastewater treatment plants the processes can be encouraged and the wastewater retained long enough to let the organic material be degraded, prior to entering the recipient. If large amounts of organic material exit the treatment plant the degradation processes may consume oxygen in the recipient leading to oxygen depletion (Bitton, 1994; Svenskt Vatten AB, 2007).

Nitrogen is essential to life and is often a limiting factor in plant growth. When large amounts of nitrogen enters recipients, usually as the accessible forms of organic nitrogen and ammonia, there are several potential hazards, such as eutrophication and oxygen depletion. When nitrogen enters the water as unionized ammonia it may be toxic to fish and high amounts of nitrate that merges to nitrite might have negative health impacts it if enters human drinking water as if affects haemoglobin in the red blood cells. (Bitton, 1994; Svenskt Vatten AB, 2007)

Phosphorus in wastewater may both be organically and inorganically bound phosphorus (polyphosphate, orthophosphate) and in wastewater treatment plants, it is generally transformed into the nutrient orthophosphate, which often is a limiting factor to algal growth in lakes. In high concentrations it can contribute to eutrophication. (Bitton, 1994).

2.2. Wastewater treatment

Wastewater treatment can be divided into four different categories and is described by Bitton (1994) as follows;

"Preliminary treatment- Removing debris and coarse material that may clog the equipment.

Primary treatment – Treatment brought about by physical forces like screening or floatation.

Secondary treatment – Biological and chemical unit processes are used to treat wastewater. Removal of nutrients usually takes place here.

Tertiary (or advanced) treatment – Unit operations (physical treatment) and chemical unit processes are used to further remove BOD, parasites, nutrients and pathogens. Sometimes toxic substances."

There are large variations in if and how these treatments are used in wastewater treatment plants around the world. The treatment plants normally claim large areas due to the basins used for each treatment step. Wastewater treatment is a relatively slow process as especially the biological steps needs time for desired effect (Bitton, 1994).

2.2.1. Chemical treatment

Chemical treatment aims to reduce several pollutants in wastewater and has been particularly useful reducing phosphorus and BOD. One way to chemically treat the wastewater is the use of different kinds of metal salts, usually based on aluminium or iron (for example Al^{3+}or Fe^{3+}). These soluable salts bind to ortophosphates (PO_4^{3-}) and creates insoluble flocks in the wastewater, for instance $AlPO_4$ or $FePO_4$. These flocks are allowed to sediment and may thereby be removed. These processes are highly dependent on the wastewater having the correct pH which can vary depending on the salt used as well as the composition of the wastewater. Generally however the pH is between 5 and 7. (Svenskt Vatten AB, 2007). This can be a somewhat costly method since the metal salts only may be used once before they are depleted.

2.2.2. Biological treatment

Biological treatment processes may be economical and efficient options if the wastewater contain biodegradable pollutants. Biological processes in wastewater treatment are performed by the present microorganisms which decomposes organic substances, such as nitrogen and phosphorus. There are several species of microorganisms in most wastewaters. The nutritional composition of wastewaters, temperature, oxygen level and pH will determine which species of microorganisms that are present (Svenskt Vatten AB, 2007). There are two categories of biological treatment; i) suspended-growth and ii) attached-growth processes (An, 2013). i) The suspended growth process is based on bacteria and protozoa floating freely in the wastewater in large basins, which usually is aerated to encourage decomposition processes. The microorganisms and particles will eventually flocculate and settle to the basin bottom. ii) The attached growth process is based on adding a medium, on which biofilm can grow, to the wastewater, and serves as a medium where decomposition processes may take place (Westerling, 2014). Biofilm is a thin membrane like structure created by a broad range of microorganisms as they attach to surfaces. Biofilm mainly consists of bacterial cells, extracellular polymeric substances (EPS) and inorganic materials. However, depending on its composition, molecular mechanisms varies greatly (López et al., 2010). According to Fernández et al. (2008), biofilm development can be roughly divided into three phases, the initial attachment phase (0-36 h), the consolidation phase (36 h to 2 weeks) and the maturation phase (2 weeks to 2 months). Biofilm in certain parts of the wastewater process may be of great use and is a common practise (Svenskt Vatten AB, 2007) and it has been determined that biofilm gives numerous benefits to the bacterial community. López et al. (2010) states that it gives protection against many antimicrobials, protozoan grazing and host defences. Biofilm may withstand a broad range of compositional variations in the wastewater and even temporary shocks of toxic substances (Svenskt Vatten AB,

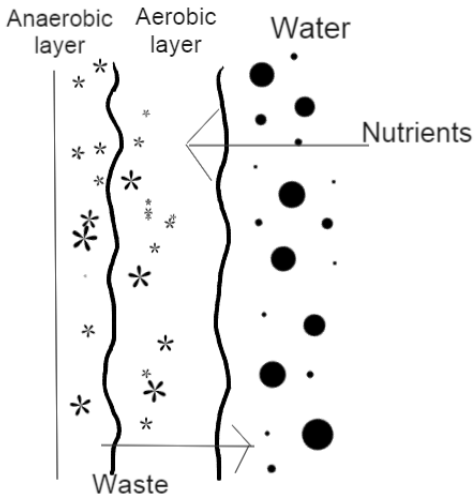

Figure 1. A schematic drawing of the layers and diffusion of biofilm. The microorganisms resides in both the anaerobic and aerobic layer of the biofilm.

2007). Madigan et al. (2012) state three additional reasons to the formation of biofilm. Firstly, it creates a favourable niche for microorganisms within the biofilm, as they could be fixed to a location with a fresh supply of nutrients. Secondly, it allows microorganisms to live in close association with each other, which can increase chances of survival as they favour each other through metabolic processes for instance. Lastly, it is stated that biofilms seems to be the "default" mode of growth for many prokaryotes.

A biofilm may absorb molecules necessary for growth and excretes its waste materials (Fig. 1). Most transportation of molecules in the biofilm is based on diffusion, which limits its thickness (Svenskt Vatten AB, 2007). According to Stewart and Franklin (2008), this is especially true for nascent biofilms that has not yet reached full maturity. A mature biofilm generally consists of different layers and/or zones which may be anaerobic or aerobic and within these biofilms transportation of nutrients and metabolic products occurs between different microorganisms as well. Transportation of nutrients and oxygen therefore goes in all directions within a mature biofilm. A biofilm may contain many different species of microorganisms and their different metabolisms may very well favour each other (Stewart & Franklin, 2008).

2.2.3. The Anaerobic Baffled Reactor (ABR) and the Membrane Bioreactor (MBR)

The ABR/MBR reactor combines two systems, the anaerobic baffled reactor (ABR) and the membrane bioreactor (MBR), and is relatively new system for wastewater treatment. The anaerobic baffled reactor is a version of the septic tank, but unlike the septic tank the ABR contains several anaerobic departments that the water passes. The water is in contact with the sludge inside the reactor for a long time, enabling the anaerobic microorganisms to process more nutrients in the wastewater. For each compartment there is less sludge and with less sludge there is a clearer effluent. This anaerobic process may generate methane gas which may be used as energy (Akvo Foundation, 2016; SSWM, 2014). The other component of the ARB/MBR reactor is the MBR-system, which is a version of the conventional activated sludge (CAS) system and uses an aerobic biological step to process the sludge. The CAS system uses a secondary clarifier or settlement tank in order to separate the sludge and the liquid while the MBR system uses a membrane inside the same tank as the biological process and as the water

is filtered through the membrane the effluent is usually quite clear and of good quality (Judd & Judd, 2013). Combining the anaerobic baffled reactor with the membrane bioreactor (ABR/MBR reactor) gives a system that contains both anaerobic and aerobic departments. The departments of the ABR system makes the water stay in contact with the microorganisms for a longer period of time and the membrane of the MBR system keeps the larger particles in the tank, creating a well-treated effluent (Ratanatamskul et al., 2015).

2.3. Nutrient reduction material

The materials added to the treatment process may work through adsorption of nutrients (Ferhan & Özgür, 2011) or by promoting development of biofilm (Manoj & Vasudevan, 2012) which in turn purifies the water through the metabolism of the microorganisms. Two potential materials are activated carbon, a well-known and widely used material in wastewater treatment and coconut fibre, a local, cheap and more novel product in wastewater treatment.

2.3.1. Activated carbon

Activated carbon is one of the most widely used absorbent materials in water and wastewater treatment. The material has been used in wastewater treatment, in less complex versions, for hundreds of years (Ferhan & Özgür, 2011). Activated carbon has been used in several different experiments and has proven to be able to adsorb nutrients from wastewater. In an experiment made by Dalahmeh et al. (2012) the COD was reduced with 94 %, total nitrogen about 98 % total phosphorus with about 91%. These results were obtained using activated carbon filters that were 0.6 m high with a 20 cm diameter. The filters received a total of 1 L of artificial grey water each day for 113 days. However, according to Lito et al. (2012) activated carbon is not as efficient in removing anionic pollutants like NO_3^-. Today, activated carbon is largely used in the environmental field to control pollution in both water and gases. Studies has shown that it is effective when reducing the amount of organic compounds in the wastewater as well as a range of other unwanted compounds such as phenolics, chlorinated solvents and herbicides. (Dalahmeh et al., 2012; Lito et al., 2012)

Activated carbon is broadly defined by Ferhan & Özgür (2011) as amorphous material (a material that lack ordered positions among its atoms (Vocabulary, 2017)) prepared to

exhibit a high degree of porosity and an extended surface area. Its ability to adsorb may be explained by that the non-carbon impurities are removed and the surface is oxidized which create active sites for molecules to bind. Carbon atoms forms hexagonal rings which are fused to each other and forms structures called microcrystallites. Between the microcrystallites there are spaces which are called pores and this is where most adsorption takes place. The “porewalls” are the microcrystallites planar surfaces which adsorb substances with van der Waals forces. Adsorption through chemical bonding also takes place with functional groups on the edges of the microcrystallites. There can be different functional groups based on material and treatment of the carbon, an example is carbonyl oxygens which can adsorb aromatic compounds (Ferhan & Özgür, 2011). The absorption capacity will eventually deteriorate and the activated carbon has to be regenerated or replaced. Regenerating the carbon may be costly and the adsorption capacity will not be quite as good as with virgin activated carbon (Ferhan & Özgür, 2011).

2.3.2. Coconut fibre

Coconut fibre has been less explored than activated carbon but some studies have shown that the material is quite efficient for adsorption of specific compounds. Kooczek et al. (2016) states that this is because the fibre consists of various matters in different stages of decomposition which may contain hydroxyl and weak acidic groups which can bond to different metals and polar organics. Kooczek et al. (2016) investigated this using atomic absorption spectroscopy with a Perkin Elmer spectrophotometer to measure amounts of chromium in wastewater. The adsorption is dependent on a donor-acceptor chemical covalent bond with hydroxyl groups as ligands. When performing their study Kooczek et al noticed that that there were two phases of reduction. The first phase, the chemical reduction phase, was quite short and started directly when the fibre was added to the wastewater. During this phase the levels of chromium decreases rapidly and quite extensively. The second phase started after about 18 minutes and ended 20 hours later and was believed by the authors to be the yeast-fungi enzymatic reduction phase. The presence of these organisms had been proven earlier by cultivation from the fibre. This phase shows a much slower and more planar reduction compared to the first phase. Coconut fibre had the ability to both reduce the metal chemically through adsorption but also enzymatically though microorganisms, overall the reduction

rate was 19.21 mg/g for Cr(III) and 9.54 mg/g for Cr(VI) (Kooczek et al., 2016). Manoj & Vasudevan (2012) also showed that coconut fibre may be used in wastewater treatment, not necessarily for its adsorption abilities but as a medium for attached growth (biofilm) for microorganisms. When attachable surface area for microorganisms increase, the amount of organisms increase as well as the performance level of the biological treatment. In an experiment performed by Manoj & Vasudevan (2012) it was shown that coconut fibre worked well as a synthetic alternative of support medium in an anoxic bioreactor by lowering levels of NO_3-N, COD and dissolved orthophosphate. The use of this material could then be two-fold, adsorption as well as a support medium for bacteria in a suspended growth reactor.

3. Method

3.1. Site description

This study was performed in the spring of 2015 at The College of Technology in the northern part of the city of Da Nang, in eastern Vietnam (Fig. 2). Several seafood processing factories treat their wastewater in their own treatment plants prior to being discharging it to the common treatment plant, the *Centre wastewater treatment plant*. The treatment is generally a conventional design of five steps, an equalization step, an anaerobic step, an aerobic step and sedimentation and disinfection. This information was received through personal communication with Dr Thao Tran Minh. At the *Centre wastewater treatment plant* further treatment takes place before effluent is let out into a recipient. In this study the prototype ABR/MBR-reactor is connected to the collection tank (Fig. 3).

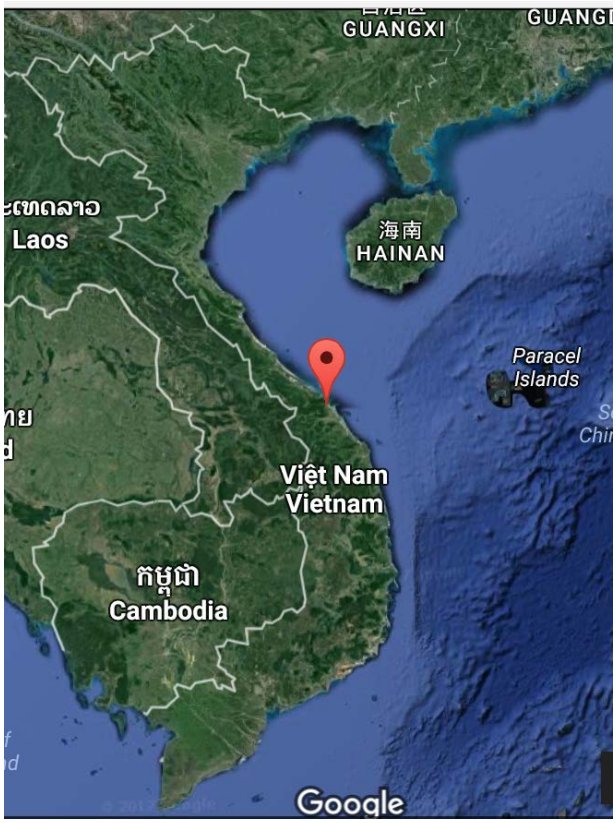

Figure 2. Satellite map of Vietnam. The red pin shows Da Nangs position (google.maps, 2017).

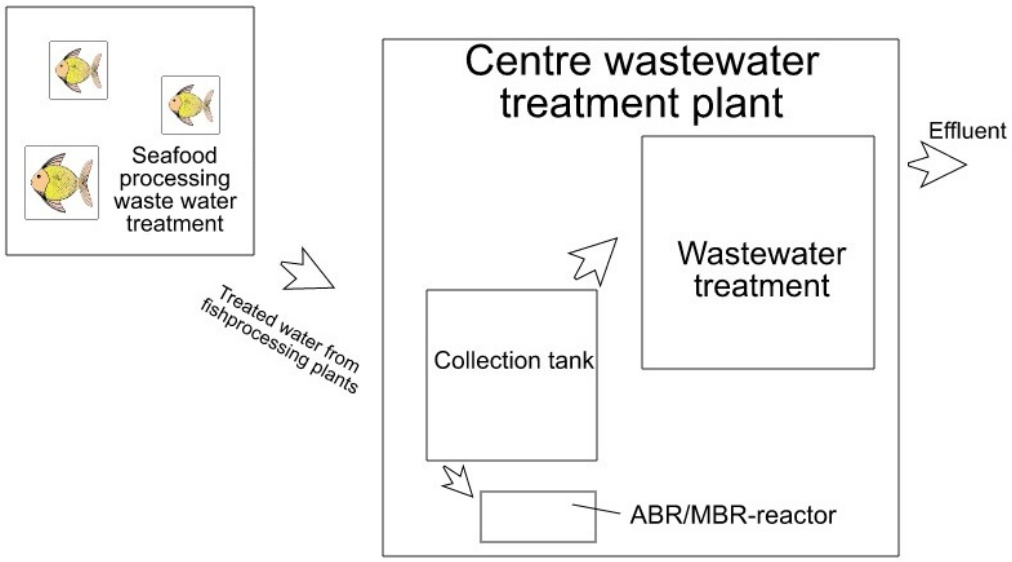

Figure 3. The flow of water from the seafood processing factories. The effluent enters the recipient which is the South China Sea.

3.2. Experimental design

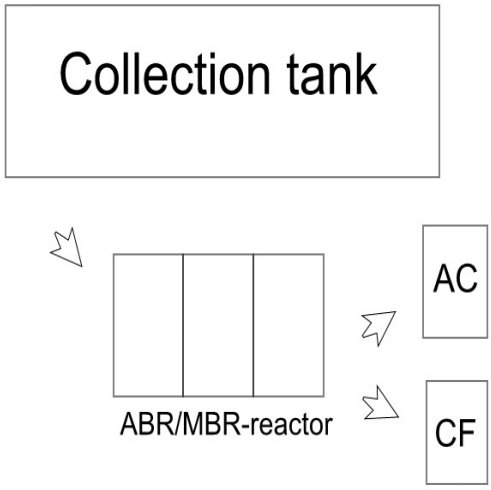

Figure 4. The experimental design. The filter is removed from the reactor and 20 liter wastewater that has passed through the reactor is taken from the last chamber to the mesocosms containing Activated coal (AC) respective Coconut fibres (CF).

This study was performed creating mesocosms in batches with two separate materials, activated carbon and coconut fibre. The mesocosms were made of two reused plastic tanks for cooking oil and could each hold 10 litre of fluid. Water from the collection tank in the Centre wastewater treatment plant entered and was treated in the prototype ABR/MBR-reactor. A total of 20 liter of treated wastewater was then discharged from the reactor to the two tanks containing nutrient reduction materials, 10 liters to each, via two hoses from the reactor connected to the bottom of the mesocosms. At this point one sample of wastewater was collected. To the first mesocosm 4.3 kg of activated carbon (AC) was added to the second 3.3 kg of coconut fiber (CF) (Fig. 4). No more water was added throughout the study. The material was allowed to sediment for 1 hour and 45 minutes prior to the second chemical measurements. At this point in the process the microscopic slides and glass filters were placed deep inside the mesocosms. Each filter and slide was labelled and attached to the external sides of the mesocosms using string and tape. Samples were collected during seven days. The mesocosms had outlets which were closed in this experiment and the top of the tanks were covered with aluminium foil (Fig. 5).

Figure 5. The actual prototype ABR/MBR-system together with the mesocosms. The picture shows how the hose connects the ABR/MBR-system to the bottom of the tanks containing nutrient reduction materials.

3.3. Analyses

Three aspects of the materials were compared, biofilm formation was tested in two separate ways.

- Measuring levels of total nitrogen (tot-N), total phosphorus (tot-P) and chemical oxygen demand (COD) in the mesocosms containing nutrient reduction materials and wastewater at 13 different occasions.
- Estimating formation of biofilm at six different occasions by observation of biofilm growth on microscopic slides that has been placed in the mesocosms containing nutrient reduction materials and wastewater.
- Determining growth of biofilm at five different occasions by change of weight caused by biofilm accumulated in glass filters that had been placed in the mesocosms containing nutrient reduction materials and wastewater.
- Cultivation of *Clostridia, Desulfovibrio, Methanobacteria* and *Lactobacillus* at three different occasions to confirm their presence.

3.3.1. Chemical analyses

For the tot-N, tot-P and COD analysis, samples of wastewater was collected in 100 ml glass bottles from the laboratory in the College of Technology. Samples were collected at 13 different occasions (Table 1). Because of budget restrictions no replicas were collected. The samples were generally collected from the surface water in order to avoid stirring of the materials. The first samples had to be filtered to separate the largest debris from the water. The samples were placed with ice packs during transportation to the College of Technology where they were placed in a refrigerator. When all samples for that particular day had been collected the samples were transported to the University of Technology. At the university they were placed in a refrigerator for at most 5 hours until analysis were performed by Mr An Hoang Ngocan at the University of Technology using the following methods according to the National Standards of Viet Nam (Ministry of Science and Technology of the Socialist Republic of Vietnam, 2017);

Table 1. A table representing when samples were collected.

Occasion	Time (h)
T_0	0
T_1	1,45
T_2	3
T_3	4
T_4	23,3
T_5	24,45
T_6	26
T_7	27
T_8	48
T_9	72
T_{10}	96
T_{11}	120
T_{12}	144

COD: SMEWW 5220C:2012

TN: SMEWW 4500-N: 2012

TP: TCVN: 6202: 2008 (ISO 6878:2004) (*TCVN = National Technical Regulation of VIET NAM). Those days when only one sample was collected it was immediately taken to the University of Technology. During the weekend there were no staff available and the samples were then stored in the refrigerator at the College of Technology until Monday.

3.3.2. Biofilm analysis

Filters

A total of 42 glass filters were placed deep in the mesocosms containing each nutrient reduction material at the start of the experiment, 21 filters in each mesocosm, for each sampling n=3. Results are based on average values of the three filters from each sampling. The filters used were GF/C-filters (Whatman), 47 mm diameter circles with a pore size of 1.2 micron. The filters were marked with small cuts on the sides in order to be identified later and dried for one hour in about 105° C in small aluminium tins (Fig. 6). The filters were then placed in individual own made steel net cages and lowered into the wastewater. The net cages were attached to strings, labelled with tank and sampling number. At sampling occasions, the filters were collected and taken to the laboratory at the College of Technology, and dried again without the steel net in their tins. The dry weight of the filters was calculated by determining the difference in between the fresh dry weight and the current dry weight. To determine the ash dry weight the filters were burned in a muffle furnace for 1 h in 500°C and weighted again.

Figure 6. Aluminium tins used when drying the glass filter in the oven and the net cages, still with filters inside.

Figure 7. The microscopic slides stained with Coomassie Brilliant Blue.

Microscopic slides

In total 28 standard microscopic glass slides from VWR were lowered into the tanks with nutrient reduction material at the start of the experiment, 14 in each mesocosm. The slides were attached to clippers which were tied to strings with a label stating the mesocosm and sampling number, for each sampling n=2. The slides were carefully placed in plastic bags and taken to the laboratory of the College of Technology where one side of the slide was cleaned with alcohol. They were then placed in UV-light in order to kill any microorganisms still alive. A few drops of Coomassie Brilliant Blue for staining of proteins were added to the side with microbial growth (Fig. 7). After about 20 minutes the colour was carefully rinsed off with distilled water and was then allowed to dry for 30±5 minutes. The microscopic slides were then observed in an LW scientific microscope at 100 X magnification and photos on areas showing biofilm growth were taken with an LW scientific camera attached to the microscope.

3.3.3. Microbial cultivation

Selective agar were used in order to cultivate four genera of microorganisms; *Clostridia, Desulfovibrio, Methanobacteria* and *Lactobacillus* at three different occasions. The three occasions were decided upon depending on where there were time in the schedule for the cultivation process. A make-up applier from Trang Diem Beauty Accessories, was used as a swab to wipe the inside of the mesocosms in order to get some biofilm. New make-up appliers were used at every sampling and for the different mesocosms. The samples were then placed in test tubes filled with a phosphate buffer solution (Lennox, 2003). Two samples out of three were collected like this. A third sample was collected with a syringe connected with a plastic tube in order to make the samples come in as little contact with oxygen as possible. This sample was taken from deep inside the ABR-MBR reactor. Collected samples were placed with some ice and cloth during the 20-30 min transportation to the lab, but the temperature might have increased a few degrees. Although there are no exact measurements of temperature, based on temperature in Da Nang May 2015 (average 27 C°) it can be assumed that the temperature was at least 20+ C° (WeatherUnderground, 2017). The test tubes were kept in a refrigerator for two-three hours while the agars were prepared. The samples were diluted in five steps of 0.85% NaCl. Each step diluted the sample by 10x. Samples of

0.1 ml were then swabbed on the surface of the agar with a swab alternatively mixed with the agar before it was poured into Petri dishes. The Petri dishes were incubated at 37°C for 24 ± 3 hours. Some of them were incubated in a homemade anaerobic chamber.

All recipes except for one of the agars were from the Handbook of Microbiological Organisms (Atlas, 2010). The recipe for Clostridia, the Tryptone Sulfite Cycloserine Agar came from Thermo Fisher Scientific Inc (2015).

The Desulfovibrio Medium Composition (for *Desulfovibrio*):

15g agar, 5g glucose, 5g peptone, 3g beef extract, 1.5g $MgSO_4$, 1.5g Na_2SO_4, 0.2g of Yeast extract and 0.1g of $Fe(NH_4)2(SO_4)_2$ per liter of tap water.

The Methanobacterium Enrichment Medium Composition (for *Methanobacterium*):

100 g $CaCO_3$, 5g K_2HPO_4, 0.3g of $(NH_4)_2SO_4$, 0.1 g of $MgSO_4 \cdot 7H_2O$, 0.02g of $FeSO_4 \cdot 7H2O$, 10 ml Na_2CO_3 solution (0,5g $NaHCO_3$ in 10 ml DI-water), 10 ml $Na_2S \cdot 9H_2O$ solution (0.1g $Na_2S \cdot 9H_2O$ in 10 ml DI-water), 10 ml Ethanol and 5 ml Yeast autolysate per 1000mL water. Filter sterilisation was not available and UV-light was used for sterilization and the broth was put in the autoclave before addition of the Na_2CO_3 solution and $Na_2S \cdot 9H_2O$ solution.

Tomato Juice Agar Special Composition (for *Lactobacillus*):

20g of Agar, 10 g Pancreatic digest of casein, 10 g peptonized milk and 400 grams of tomato juice per 1000mL water.

The Tryptone Sulfite Cycloserine Agar (for *Clostridia*):

15g of Tryptone, 5g of Yeast Extract, 5g of Soybean peptone, 1g iron (ammonium) citrate, 20g Na_2SO_4 and 20g agar per 1000mL water. The Soybean peptone was not available and instead a mixture of soybean flour and peptone called soyton was used where 4g of soybean flour and 1g of peptone was used per 5 g of soyton.

The bacterial cultivation was evaluated through qualitative observations.

3.4. Pilot study

Prior to the experiments the methods was pre-tested on site. These test were performed inside the last chamber of the ABR/MBR-reactor as the mesocosms were not yet completed.

3.4.1. Staining

The original experimental design included two types of staining dyes for detection of microorganisms; Sudan Yellow and Coomassie Brilliant Blue. Sudan Yellow is a fat-soluble dye which binds to triglycerides and stains them yellow (Santa Cruz Biotechnology, 2016) and Coomassie Brilliant Blue stains proteins in a shade of blue, mainly by binding to the amino acids arginine, lysine and histidine (ThermoFisher Scientific, 2015). Both of these dyes were prepared at the laboratory at the College of Technology in Da Nang and pre-tested on microscopic slides with biofilm growth that had been placed in the prototype ABR/MBR-reactor. This revealed that Coomassie Brilliant Blue worked well but that Sudan Yellow left artefacts that made the results difficult to analyse. Therefore only Coomassie Brilliant Blue was used.

3.4.2. Filters

The glass filters which were used to measure change in weight were also pre-tested in the prototype ABR/MBR-reactor. This involved creating a system of identifying the different filters and pretesting the equipment for drying the filters to get dry weight and ash dry weight. During the pre-testing period, small individual net cages that were created to protect the filters from falling apart due to the exposure to wastewater. It also facilitated the collection of filters.

3.4.3. Microorganisms

There were some changes in recipes for the different microorganisms. For *Clostridia* a recipe for Clostridia Agar Composition from the handbook of microbiological media (Atlas, 2010) was chosen at first, however as some chemicals were not available and/or expensive the receipt was changed to Trypton Sulfite Cycloserine from Thermo Scientific (2015). As there were no Soya Peptone an alternative was created on site, soyton, a mixture of soyabean flour and peptone. For *Lactobacillus* the LBS™ Agar (Lactobacillus Selection Agar) Composition was chosen to begin with but because of similar reasons as for *Clostridia*, the recipes was replaced. The replacement was the Tomato Juice Agar Special Composition, also from The Handbook of Microbiological Media (Atlas, 2010).

There were no anaerobic chamber available so an alternative homemade one was created on site using an aquarium, candles, aluminium foil and tape.

4. Results

4.1. Chemical analyses

Figure 8, 9 and 10 shows the values for COD, total-nitrogen and total-phosphorus respectively for 13 sampling occasions spread out during 144 hours. All three graphs show similar patterns with a fast, steep increase in the beginning as well as a quite steep reduction the first 24 hours which then slows down. The sample representing 0 hours was taken from the wastewater *before* the nutrient reduction materials were added. The next sample shows a steep increase in levels of nutrients in both mesocosms in all three graphs and is collected 1,45 hours *after* the nutrient reduction materials has been added.

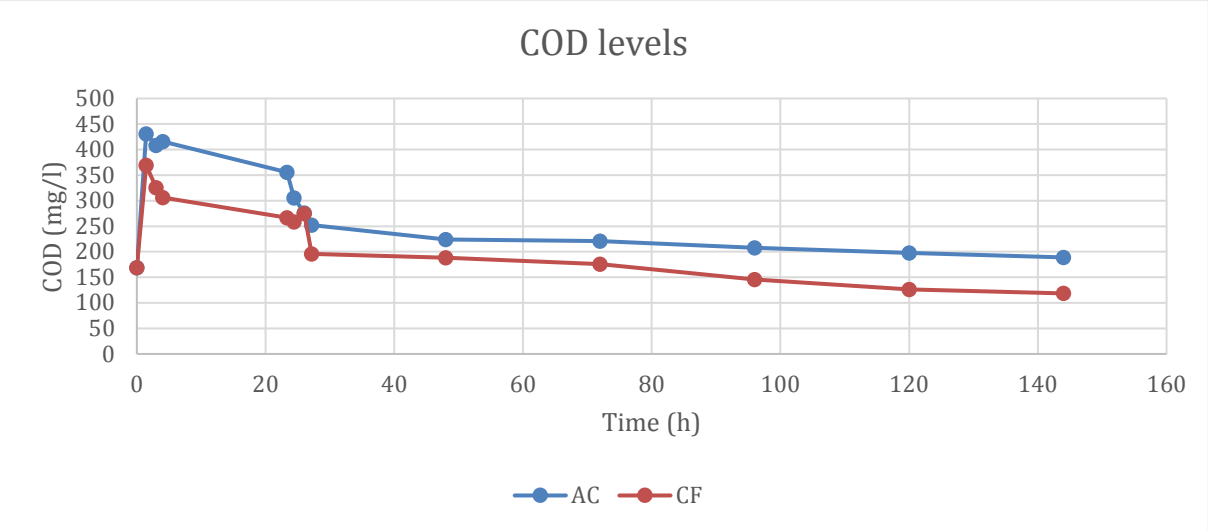

Figure 8. Levels of COD at 13 sampling occasions for both mesocosms during 144 hours. Spearman's rank correlation between time and COD level gave $r_s = -0.56$ (P=0.044) for AC and $r_s = -0.73$ (P=0.0041) for CF. N=13.

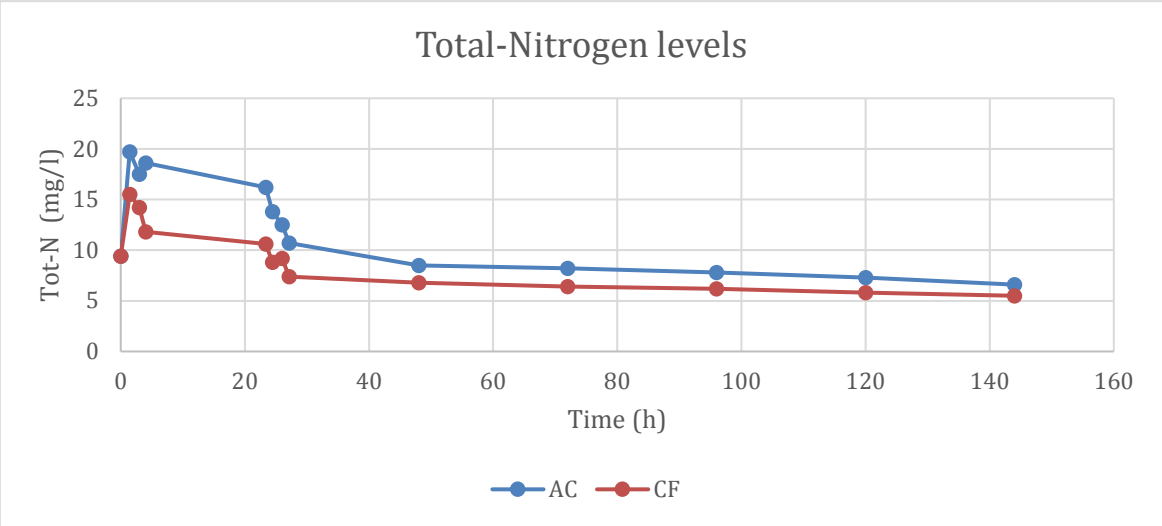

Figure 9. L*evels of Total-Nitrogen at 13 sampling occasions for both mesocosms during 144 hours. Spearman's rank correlation between time and Total-Nitrogen level gave* r_s *= –0.84 (P=0.00032) for AC and* r_s *= –0.93 (P= 0) for CF. N=13.*

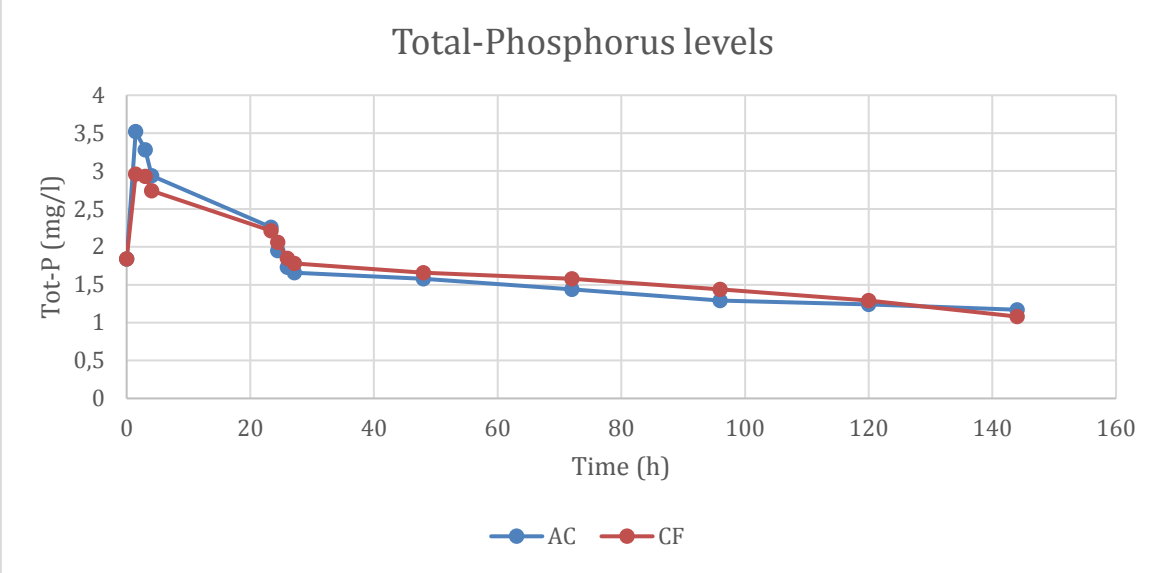

Figure 10. Levels of Total-Phosphorus at 13 sampling occasions for both mesocosms during 144 hours. Spearman's rank correlation between time and Total-Phosphorous level gave r_s *= –0.91 (P= 1E-05) for AC and* r_s *= –0.88 (P=6E-05) for CF. N=13.*

In order to analyse if there was a consistent change in chemical levels over time, the non-parametric Spearman's rank correlation was used. This test shows that there is a statistically significant correlation between the between the values of the x-axis and the y-axis for each series of the materials in all three mesocosms. There are no large differences between the correlation values (r_s) of the series in each of the graphs.

The graphs shows that there is some reduction of nutrients in the two mesocosms but

when comparing the first and last value as in table 2, it is not necessarily a large decrease. For COD-levels in the AC-mesocosm the last measurement even showed an increase of COD compared to the first measurement. In general the AC-mesocosm shows a smaller rate of reduction then the CF-mesocosm based on these measurements.

Table 2. Showing the difference in percent between the first and last measurement for each nutrient in each of the materials.

Nutrient	Nutrient reduction material	Difference between first and last value (%)	Difference in levels of nutrient (mg/l) per gram of nutrient reduction material
COD	AC	11%	+0.006
	CF	-29%	-0.011
Tot-N	AC	-29%	-0.0008
	CF	-41%	-0.0009
Tot-P	AC	-36%	-0.0002
	CF	-41%	-0.0001

In order to make the comparison of the actual reduction of nutrients more apparent there are some changes in figure 11, 12 and 13. The first value, represented by 0 in the earlier graphs (figure 8, 9 and 10) has been removed which means that the first value in figure 11, 12 and 13 is 1,45 h after addition of nutrient reduction materials. The values has been normalised (all values in the series has been divided by the first value) as well so that both of the series start on 1.

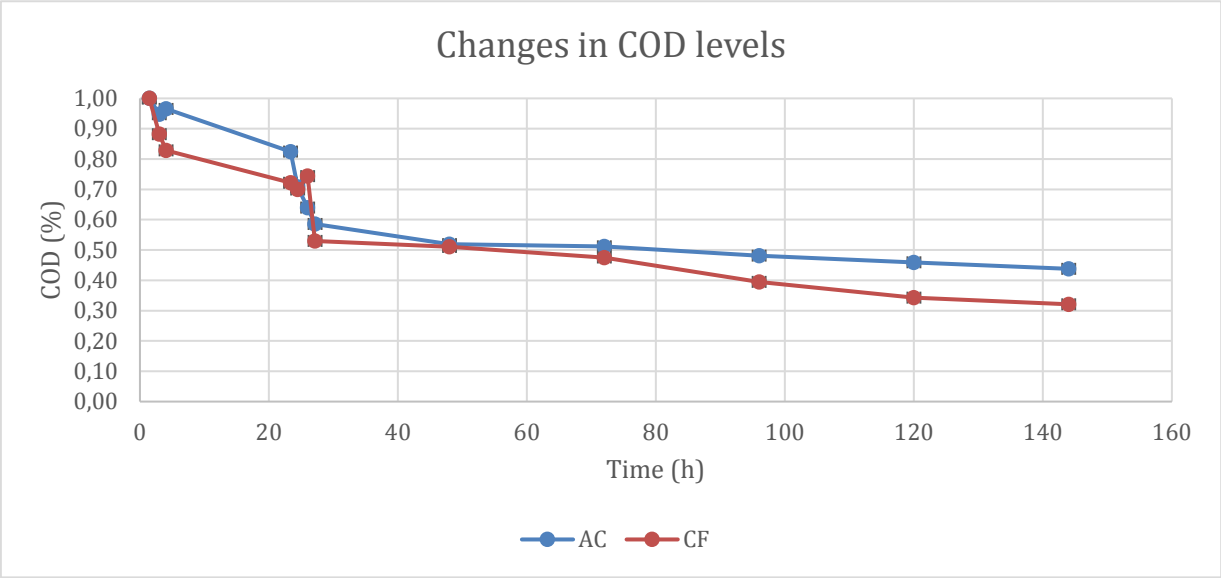

Figure 11. COD levels (%) in the wastewater for AC respective CF mesocosms during the course of 142,5 hours. All values are normalized. N=12.

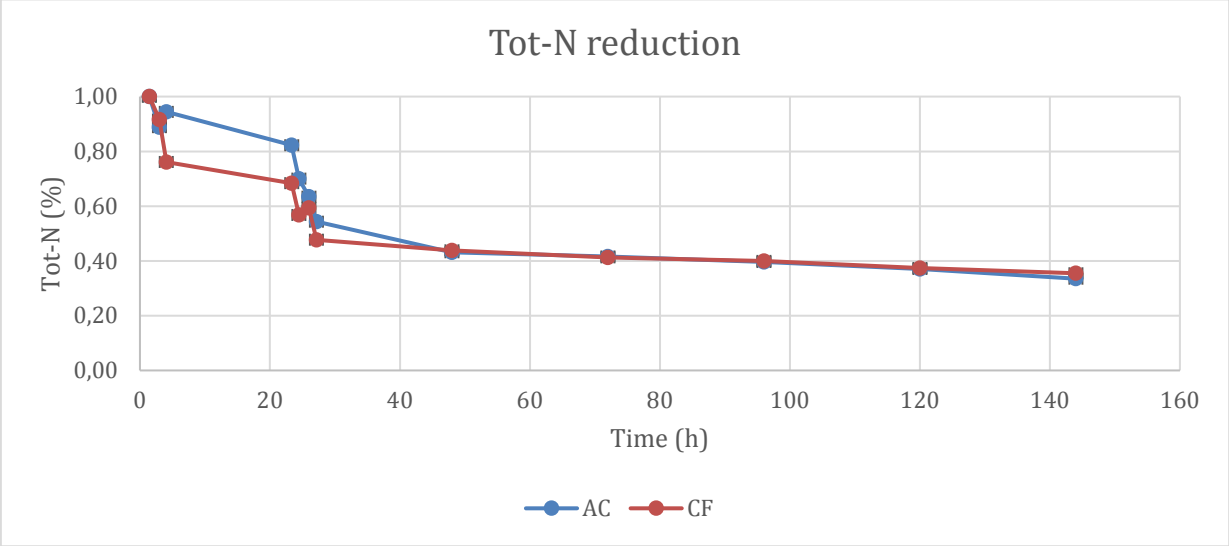

Figure 12. Tot-N levels (%) in the wastewater for AC respective CF mesocosms during the course of 142,5 hours. All values are normalized. N=12.

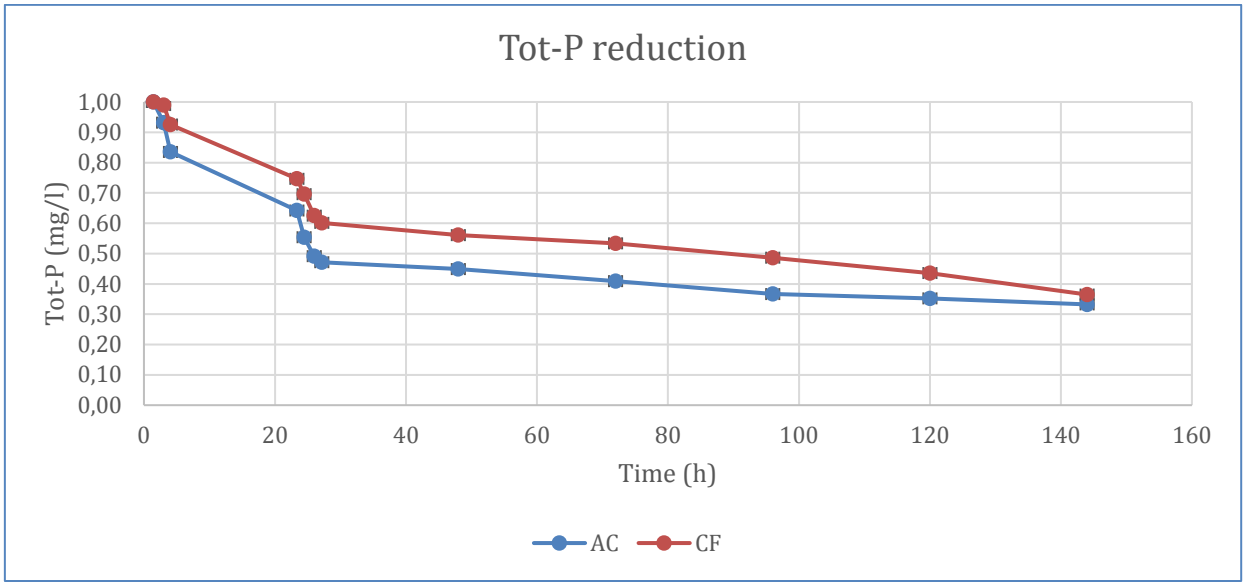

Figure 13. Tot-p levels (%) in the wastewater of AC respective CF mesocosms during the course of 144,5 hours. All values are normalized. N=12.

In all three figures (11, 12 and 13) there is a pattern that shows that the greatest reduction occurs in the first 24 hours and then slows down. The graphs indicated other trends although these cannot be confirmed due to the lack of replicates. There does seems however that the CF-mesocosm has a slightly greater rate of reduction regarding COD (Fig. 11). Regarding tot-N there is slightly more reduction for CF than AC but they then become almost identical (Fig. 12). In the graphs for tot-P values for AC are continuously slightly lower than for CF until the last measurement (Fig. 13). These trends cannot be confirmed due to lack of replicates.

4.2. Microscopic slides

Observation of the microscopic slides indicates that there is an increase of biofilm on the slides, shown in figure 14. The slides from the CF-mesocosm is generally more cluttered and contains more filament growing microorganisms than the AC-tank.

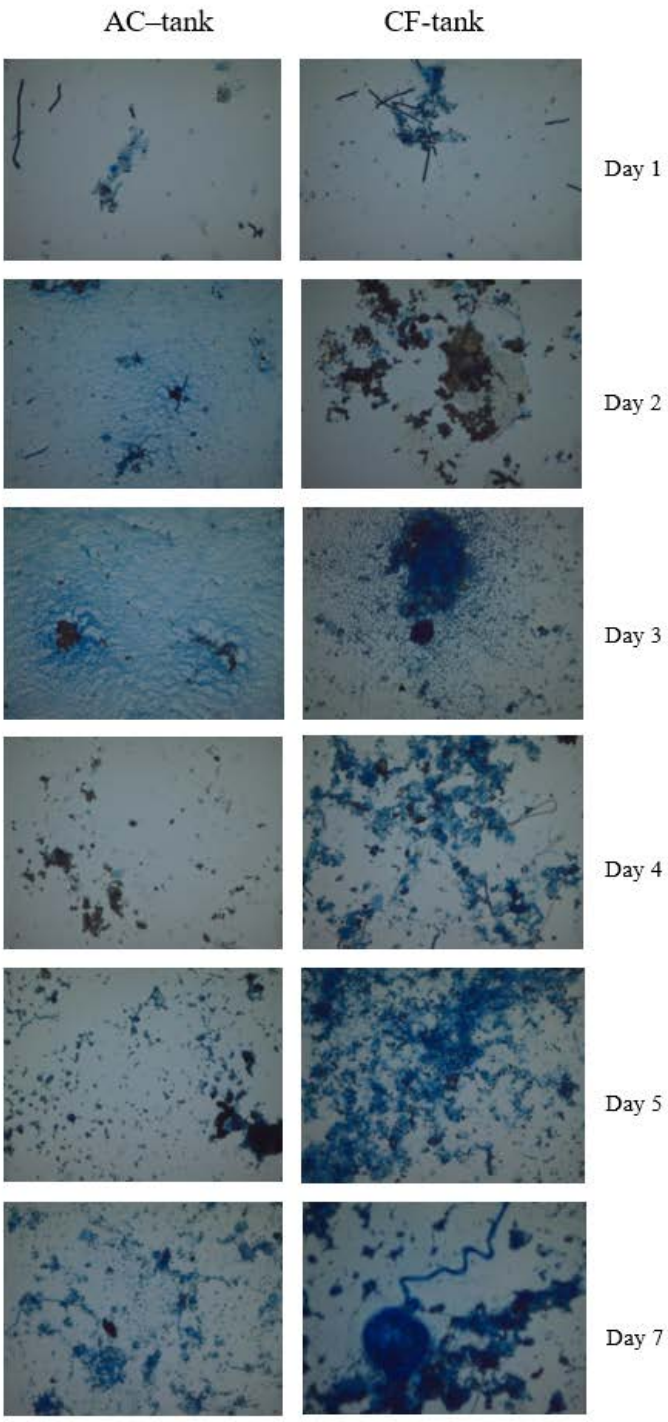

Figure 14. The pictures represents a timeline of seven days and reveals that there is formation of biofilm early on. Day six is missing as there were no slides collected that day. Coomassie blue has been used to stain the proteins blue.

4.3. Filters

Three filters were collected from each mesocosm at each sampling occasion and the values used in figure 15 and 16 are the average value of the three. When subtracting the original weight from the dry weight after incubation there was an increase in weight of the filters (Fig. 15) which broadly correlates with time placed in the wastewater. According to the graph there is a slightly larger increase in filter weight in the AC-mesocosm as well as a larger standard deviation, this especially true for the last value. However, Spearman's rank correlation analyses did not reveal any significant associations between time and the measured change in filter weight for AC or CF.

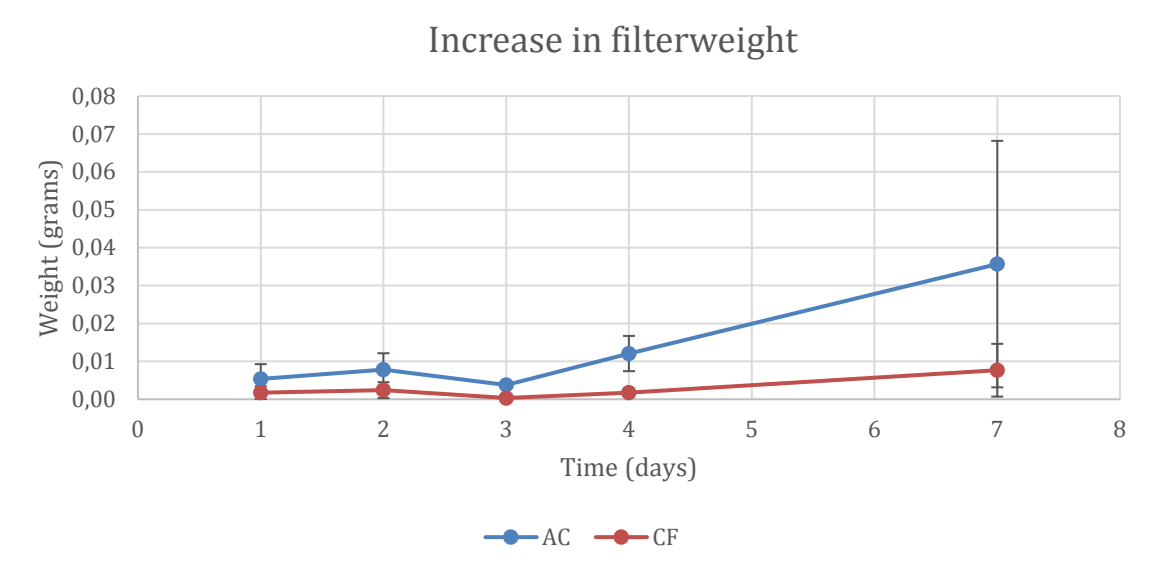

Figure 15. Change in filter weight during five days. The graph shows difference in weight before and after being in the mesocosms. Each node in the graph represents the average weight of three filters. Spearman's rank correlation between time and weight gave $r_s = 0.7$ (P=0.19) for AC, and $r_s = 0.5$ (P=0.39) for CF. N=15.

After being weighted the filters were burned in the muffled furnace to remove the carbon, turning it to CO_2 and was then weighted again. Subtracting the weight of the burned filters from the values for the dry weight (the weight after the filters has been in the mesocosm) gives the weight of the organic material burned off from the filters (Fig. 16). The graphs shows a small increase in organic matter over time. The graph in figure 15 broadly correlates with the contents in figure 16. The filters incubated in the AC-mesocosm contain more organic material and shows a larger increase in weight. There is an issue with these graphs, which is that the organic weight in figure 16 sometimes is larger than the corresponding change in filter weight in figure 15, this especially true for the filters in the CF-mesocosm. This would suggest that more material was burned off than was actually accumulated on the filters in the tank. Spearman's correlation was

applied to the values of figure 16 with an r_s-value of 0.7 for AC showing a non-significant relationship between x- and y-axis. For CF however it got a r_s-value of 0.9 which indicated a significant relationship between the x- and y-axis for these values.

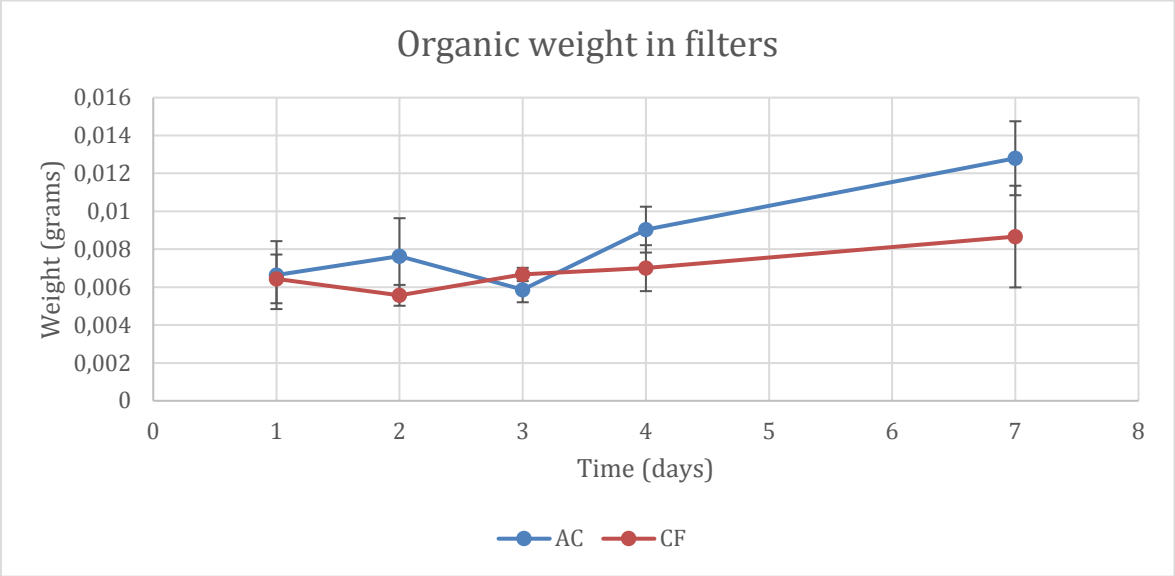

Figure 16. Organic weight at five measurements over the first seven days from the start of the experiment. The graph shows the difference in weight before and after the filters was burned in the muffled oven. r_s = 0.7 for AC (P=0.19) and r_s = 0.9 for CF (P=0.037). N=15.

The increase of the values has been calculated in table 3 which shows the increase in percentage between the first and last measurement for each mesocosm.

Table 3. The percentage increase in filter and organic weight and between the first and last measurement.

	AC	CF
Increase in filter weight (%)	564	342
Increase in organic weight (%)	92	34

4.4. Microbial analysis

During cultivation there were growth on the selective agars for *Clostridia* and *Desulfovibrio*. There were no growth on the selective agars for *Methanobacteria* or

Lactobacillus. Based on qualitative visual assessment there was no difference between the two nutrient reduction materials and no difference between the samples gathered at different times.

5. Discussion

During this experiment two materials, activated carbon and coconut fibre, were compared using four different methods and overall there are no large differences between the materials. It has become evident that there are high amounts of nutrients in both mesocosms and quick biofilm formation which could be seen on the objective slides.

5.1. Chemical analysis

The aim of the chemical analyses was to investigate if there were any differences between activated carbon and coconut fibre regarding reduction of nutrients. The results show similar rates of reduction with some small variances. However, as there were no replicates the results are somewhat questionable and can only be considered as indications at this point. This is especially true for the small variances observed. Using Spearman's correlation analysis does not show if there is any significant difference between the materials but one can see the r_s-values as indications, and if they were very different then there might be a difference between the materials. However, there were no large differences in the r_s-values of the series in each of the graphs which indicate similarity between the series. This is not in any way proven however.

It may be noticed that for all the chemical aspects there is a fast and quite large reduction in the first 24 hours which later stabilises to a much slower reduction. This could be because of adsorption of nutrients or perhaps just settlement of particles. Then, the slower reduction is more likely from microorganisms. Since the wastewater has already been treated for some time there is most likely already some favored microorganisms in the water which will continue to reduce nutrients.

Although there is no proven statistical difference between the materials one can speculate on some indications in the graphs. Looking at the graph for COD reduction, after about 70 hours the values for CF continues to decrease while the values for AC does so to a lesser extent, indicating that there could be a slight tendency for a larger rate of reduction of COD with coconut fibre. This could be because of a greater

microbial activity since the fibre has proven to work well as a support medium in earlier studies (Manoj & Vasudevan, 2012). Continued measurements over a longer period of time would be interesting if this experiment was to be developed. In the figure showing tot-P reduction there could be a small tendency for a larger adsorption of phosphorus in the AC-mesocosm in the first hours. After about 144 hours the CF-mesocosm has about the same values as the AC-mesocosm which could indicate that a greater microbial activity has reduced amounts of phosphorus.

There was an increase of COD, Tot-N and Tot-P in both mesocosms directly after adding the two nutrient reduction materials in the mesocosms. The high increase in levels of nutrients after addition of AC and CF may be explained by the fact that the materials themselves contain organic materials and that these were suspended in the water when added to the tanks. The increase was especially large in the AC-mesocosm. This shows that in further experiments, it is crucial to treat the materials in some way that will reduce the amount of nutrients already present in these materials. Other studies have shown that the nutrient reduction materials usually are used with filters which may remove the larger particles. (Dalahmeh et al., 2012; Lito et al., 2012; Manoj & Vasudevan, 2012). Kolozcek (2016) had also washed the fibres used and also treated them 0.1 M NaCl solution to increase efficiency and remove unwanted debris and pollutants.

Overall there are quite large amounts of nutrients left in the mesocosms even though there have been some reductions, much of this is because of the sharp increase after the addition of the materials. Since there was a lot of material added, the rate of reduction based on the values for this current experiment cannot be considered very effective. If using these materials again there needs to be some changes in the design of the experiment.

5.2. Microscopic slides

The microscopic slides showed that there was microbial growth in both mesocosms. After seven days, large areas of the microscopic slides were covered with biofilm and bacteria. According to Fernández et al. (2008) the biofilm in this experiment was still in the consolidation phase (36 h to 2 weeks) indicating that the biofilm was still growing and developing, which might have affected the pattern of nutrient reduction. There was more growth of biofilm on the microscopic slides from the CF-mesocosm which might

indicate that this material is well suited for formation of biofilm. Shown in the picture of the microscopic slides (fig 11), there were stalked ciliates growing in the CF-mesocosm which indicates that the water had become oligotrophic (O'Sullivan & Reynolds, 2003). Further observations and species identification of the ciliates observed might in another experiment give more insight into the status of the wastewater. However, these organisms were also found in the AC-mesocosm but not to the same extent as in the CF-mesocosm. This is not proven empirically but was noticed through personal observation. Being able to measure the formation of biofilm could be quite useful when trying to find links between levels of nutrients and biofilm. It could also be useful for studying the clogging of membranes as was the original problem of ABR/MBR-reactor. Larimer et al. (2016) has developed a method to measure the formation of biofilm. They scanned a small area with a microscope detecting contrasts between non-covered areas and areas covered with biofilm. These areas were measured and the coverage is calculated with an image analysis algorithm that was developed by Larimer et al. (2016) using a Matlab script. In the current experiment, however there were a lot of debris in the wastewater and therefore on the slides, which would make a method based on calculations of coverage difficult. Using filter or washing the nutrient reduction materials before usage could be a solution.

The use of Coomassie Brilliant Blue worked well in this experiment and enhanced differences of biofilm growth between the two materials. The original idea of this experiment however was to use two dyes when observing the microscopic slides. By using different colours in combination there is a possibility to characterize the biofilm, since its different contents and topography will become more evident. This makes it possible to determine for instance the age of different parts of the biofilm (Larimer et al., 2016; Amr, 2012). Larimer et al. (2016) used erythrosine B, KeyAcid Rhodamine and Coomassie Brilliant Blue G-250 for staining before using their script to analyse images taken of the stained biofilm. Amr (2012) used f 5-cyano-2, 3-ditolyl tetrazolium chloride (CTC) as a fluorescent indicator of respiratory activity to find if the samples suspected to be early stages of biofilm were abiotic or biotic. These are interesting possibilities for future possibly more profound comparisons.

5.3. Filters

There is an evident increase of biofilm growth in the waste water. This is shown by the microscopic slides but also by the increase of weight in the filters. The larger increase for AC is shown in both increase of filter weight and organic material. However, when looking at filter weight there is a large standard deviation which is a reflection of the scattered values. This indicated that the increase in filter weight might not be quite as great as is shown in the diagrams and the table with increase in percent. Repeating the experiment might results in more clarity in how much the weights increases.

For increase in filter weight, no correlation between weight and time was found for neither of the materials. This is not very strange as the values are somewhat scattered and because there were only five sampling occasions. Also the r_s-values are not very far from each other which could suggest that there is not a very large difference between the two series. For the figure showing change in organic weight however there was a significant correlation for CF but not for AC which could indicate that there is larger difference between these although the actual r_s-values (0.7 and 0.9) are not that far apart. Considering how scattered the values for AC are it is not surprising that the R-value was lower but this could still suggest that there is more valid difference between AC and CF in this graph. Like many other issues this could be settled by repeating the experiment with more samples.

When comparing the time aspect of the increase in filter weight and organic material it is about the same time that the biofilm formation is increasing, especially in the CF-mesocosm, seen in figure 8 and 11. At this point the COD starts decreasing more in the CF-mesocosm. Although only speculation, this might indicate that there is biofilm formation in the CF-mesocosm, which gives the larger reduction in COD. This leads to lower levels of nutrients, which promotes growth of ciliates. However, this should make the filter heavier, as for now the filter from the AC-mesocosm is heavier and contains more organic material. This might however be because of the biofilm growth in the CF-mesocosm that takes place elsewhere, like on the material itself. In the AC-mesocosm the biofilm might grow more in the filters as there are not many other places to grow. There should then be more growth on the microscopic slides from the AC-mesocosm as well which is not as evident as for the CF-mesocosm. Since the results from the objective slides are based on observation and the filter samples are quite few they are only indications but are also certainly an interesting stepping stone for further studies. If

a similar study with filters were to be conducted it should be during a longer period of time with more samples and more numerous measuring points to see if the weight continues to increase.

According to other studies the time in the oven could be prolonged for an accurate value. In an article by Horn and De la Vega (2016) when measuring the ash free dry weight from various birds they left 0.1 ml of the already dried sample in the muffled furnace for 5 hours. In this study, the materials were only left in the oven for 1 hour. Moreno (2001) who used the ash-drying technique on different development stages of *Patiriella*, dried the samples for 6 hours. This might be because of the nature of their samples. In the current study, we followed a method description the WOW-website (2004) where filters in the muffled furnace were dried for 1 hour. If the experiment was to be repeated then the samples should be weighted and then dried again until there were no more changes in weight, indicating that there was no more organic material left. The same method should be considered for the dry weight. This might change the fact that the graphs showed how the weight of the organic material was sometimes heavier than the actual accumulated weight recorded for the filters. Although this might have been due to some error in the treatment of the samples or the equipment used. The WOW (2004) website also mentions in their method description that there is no way of determining the source of the ash free dry weight. The material might come from bacteria, fungus and/or alga which is also true for this experiment.

5.4. Microbial analysis

There was growth on the agars for *Clostridia* and *Desulfovibrio* but none for the agars for *Methanobacteria* and *Lactobacillus*. The results did not differ between AC respective CF indicating that there was the same condition for growth and no difference between the nutrient reduction materials in these aspects. The results of this experiment was qualitatively measured but with small changes in the design of the experiment the results could easily be made quantitative in order to get more information.

Problems with equipment makes the anaerobic results somewhat doubtful. There was no proof that the homemade anaerobic chamber had an anaerobic environment the whole incubation time. There are some potential ways to improve this method and make a more profound comparison. For instance, it would be useful with equipment specific for anaerobic cultivation or use other techniques than cultivation. In some papers on similar

experiments FISH, pyrosequencing or some similar method has been used instead (Fernández et al., 2008; Botchkova et al., 2014). This could more definitely confirm species present both aerobic and anaerobic. An approach like this is more costly and demands more equipment. It might also be preferable to look at other microorganisms involved in aerobic rapid biofilm formation which is easier to cultivate. Larimer et al., (2016) for instance cultivated *Pseudomonas* which is a common present microorganism in biofilm formation.

One other difficulty in this study was the composition of the agars. Recipes for cultivation of specific species had to be changed due to some ingredient that were difficult to find or too expensive.

The first two times samples of wastewater and biofilm were collected from the mesocosms but no *Methanobacteria* could be cultivated from these samples. Because of this the third sampling was from the ABR-part of the prototype ABR/MBR-reactor. This was to be done as anaerobically as possible by using a syringe to collect the waste water. This did not show any different results, which might be because there are no *Methanobacteria* present but most probably because of wrong cultivation condition.

5.5. Limitations

Because of limited time and difficulties regarding materials such as ceramic membrane filters the study could only be performed once. A repetition and development of this study would have been interesting as it could have confirmed results and maybe narrowed the focus of the study.

5.6. Conclusion

There were few differences between the two materials regarding the reduction of nutrients, also there was not much reduction of nutrients overall. However, the study indicates that there is indeed a rapid development of biofilm. In three to four days, the organic material started to increase in the filters and there is visible formation of biofilm. Although there is no strong evidence there seems to be more growth of biofilm and more reduction based on microbial activity in the CF-mesocosm and perhaps a slightly larger adsorption in the AC-mesocosm. This could be an interesting stepping stone for further studies. There are several improvements that could be made for further comparisons of the materials. The three most important possible improvements is firstly

some kind of treatment of the materials before using them which may prevent levels of nutrients from rising when the materials are added to the waste water. Secondly there needs to be more replicas and test pints. Thirdly there should be a zero tank (a tank containing no nutrient reduction material) to compare the effects from the materials and the effects occurring from natural processes.
As the purpose of the ABR/MBR-reactor mentioned in the introduction is to find a low-cost and effective solution for wastewater treatment that has be taken into account in these conclusions. Considering that there has been a very small difference between the two materials in most aspects in this experiment there is no need for investment of the more expensive activated carbon at this point.

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