Valorisation of waste streams from recirculating aquaculture systems by photoautotrophic microalgae

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A B S T R A C T

The low water consumption in Recirculating Aquaculture Systems (RAS) can result in relatively high concentrations of polluting nutrients in effluent waste streams (WSs) compared to traditional Flow-through systems (FTS). These potentially valuable WSs are currently poorly exploited. It is known that photoautotrophic microalgae could be an option for WS valorisation as they can utilise these nutrients (mainly nitrogen (N) and phosphorous (P)). This thesis examined the suitability of various WSs (freshwater (FW) outlet water, sludge, and concentrated sludge and seawater (SW) outlet water) as nutrient sources for FW *Chlorella vulgaris* and SW *Microchloropsis gaditana* and the effect of different manipulations (autoclaving, sterile filtration, the addition of micro/macronutrients). Some samples underwent an enzymatic/chemical hydrolysis treatment conducted externally by LEITAT to liberate N and P and enhance microalgae nutrient availability. In this thesis, experiments were conducted with three different unit volumes: 2 mL in the well plate (WP), 300 mL in the bubble column, and 20 L in the photobioreactor (PBR). All WS samples were tested in the WP experiment with various manipulations. The results from the WP experiment were binary (growth (G) or no growth/fail (F)). One WS sample was selected for the bubble column experiment (FW, *C. vulgaris*), where the effect of micro/macronutrient addition was tested. Another WS sample was chosen for the PBR experiment (SW, *M. gaditana*), where it was tested unmanipulated. In the bubble column and PBR experiment, biomass development was assessed by OD, quantum yield (QY), nutrient analyses (N and P), dry weight (DW), total fatty acid (TFA) % of DW, and TFA composition. All the unmanipulated and untreated outlet sample media allowed algae growth in the WP experiment, while only 50% of the LEITAT-treated samples did. Generally, the binary results for most WS samples were unaffected by manipulation. Autoclaving had a growth-promoting effect in one sludge sample, as a G result only was registered after manipulation. Two LEITAT-treated samples were affected differently by micronutrient addition (inhibiting and algae growth promoting). The Bubble column and PBR experiment demonstrated that *C. vulgaris* and *M. gaditana* could utilise the nutrients in axenic FW and non-axenic SW waste streams, respectively. The macronutrient additions lacking effect on final OD and DW in the Bubble column experiment were questionable as the initial concentration of P in the WS medium was low, and intracellular P storage of the algae inoculum was not taken into account. However, earlier stress indications (elevated TFA % DW, changing TFA composition, and declining QY) were registered in cultures with lower initial P concentrations. This indicates that a repeated batch experiment could have uncovered P-limitations in an unmanipulated WS medium. The results from this thesis support previous findings that polluting nutrients in WSs from RAS can be utilised by photosynthetic microalgae but that algae growth in some WS might be inhibited or limited without further manipulations (autoclaving, micronutrient addition). The original N:P ratio of WSs will vary, but the results suggest that, generally, P will be the growth limiting element.

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1 List of abbreviations

AAR – *Aller Aqua Research (partner in iFishIENCi project, aquaculture test facility)*

ABT – *AquaBioTech (partner in iFishIENCi project, aquaculture test facility)*

N – *nitrogen*

P – *phosphorus*

RAS – *recirculating aquaculture system*

RO - *reverse osmosis water*

TAN – *total ammonia nitrogen (NH_4+ and NO_3-)*

TFA – *total fatty acids*

TN – *total nitrogen*

TP – *total phosphate*

WP – *well plate*

WS – *Waste stream from aquaculture*

2 Introduction

Aquaculture is the fastest-growing sector in the food production industry (FAO, 2020). Norway has an extensive production of salmonids (Atlantic salmon, *Salmo Salar* and Rainbow trout, *Oncorhynchus mykiss*) and is the dominant European aquaculture producer (Hough, 2022). The country aims to increase the national seafood production to 5 million tonnes by 2050 (Sjømat Norge, 2018). Considering the annual sales in 2020, this equals a production growth of roughly 3.5 million tonnes (Fiskeridirektoratet, 2021). As the intensity and scale of seafood production increase, the amount and concentration of polluting nutrients in the effluents from these systems also increase. Proper waste management is, therefore, crucial to substantiate an industry expansion that complies with the UN Sustainable Development Goals (Meriac, 2019). The emerging demand for increased circularity within the European economy is a further incentive to work with the polluting nutrients in effluents from aquaculture.

The polluting nutrients in aquacultural production water come from feed spills and by-products from fish metabolisms, such as CO_2, TAN (Total ammonia nitrogen, NH_3- NH_4^+), urea, and faeces. Of the total nitrogen (TN), approximately 87 % is excreted as dissolved N-compounds (TAN and urea), and the rest is discharged as particulate matter in faeces. Phosphorus (P) is excreted in faeces only and will appear in both particulate (67%) and dissolved/orthophosphate (33%) forms (Bregnballe, 2015).

The practicability of effluent containment for the subsequent valorisation of polluting nutrients in waste streams (WS) strongly depends on the system layout. As salmonid fish are anadromous, the production cycle traditionally consists of a land-based freshwater phase (production of smolts) and a sea-based saltwater phase. At sea, the fish are commonly kept in open cages. Currently, no commercial technologies allow nutrient containment in sea-based systems (Aas and Åsgård, 2017). The emission limit depends on the recipient (i.e., bottom conditions and water currents), and regular environmental inspections are required (Akvakulturdriftsforskriften, 2008). Pilot projects like the *LiftUP sludge collector* and innovations like semi-closed containment systems can bring about new WSs in the future (LiftUP, no date; Øvrebø *et al.*, 2022).

Flow-through systems (FTS) are the most common land-based systems. As the name implies, the production water flows through these systems and is discharged into the recipient (most often coastal waters). FTSs have a relatively high average specific water consumption rate of around 30 m^3 / kg produced fish (Lomnes, Senneset and Tevasvold, 2019). The legislative

requirements regarding pollution limits for land-based aquaculture facilities in Norway vary as they are county and case-specific (Forurensningsloven, § 11; Lomnes, Senneset and Tevasvold, 2019). Often, imposed emission requirements will be based on Forurensningsforskriften with demands of primary cleansing of effluents (Forurensningsforskriften, § 14-2). FTSs usually filtrate the production water to meet these requirements before releasing it to the recipient, offering some degree of effluent containment (Lomnes, Senneset and Tevasvold, 2019).

Norway has had notable success with salmonid smolt production in land-based Recirculating Aquaculture Systems (RAS) (Hough, 2022). Different water treatment steps are incorporated in RASs to minimise specific water consumption while maintaining good fish health. These steps involve but are not limited to: the removal of insoluble particles from circulating waters and conversion of TAN to less toxic nitrate in biofilters (Bregnballe, 2015). The continuous filtration and cleaning of production water enable a low specific water consumption (approximately 1.3 m^3 / kg produced fish/ day) and a high degree of effluent containment. Therefore, the WSs from RASs can potentially contain large quantities of nitrogen (N) and P (Lomnes, Senneset and Tevasvold, 2019).

The first step in any RAS is to separate suspended solids (faeces and feed spill) from the polluted water, and a mechanical filter (40-100 µm) is always involved in this process. The solids trapped in the filter elements are transferred to a collecting unit where it is dewatered to ≤25% dry weight (Lomnes, Senneset and Tevasvold, 2019). The remaining substance is commonly known as sludge. Some aquaculture facilities include one or more steps to produce concentrated sludge. The scope and techniques of the sludge treatment vary, and the final product can consist of >90% dry weight (Lomnes, Senneset and Tevasvold, 2019). The reject water from sludge treatments, usually rich in N, can continue through the recirculating process but is most often discharged to the recipient (coastal waters) (Bregnballe, 2015). After the mechanical filter, the system water continues to the biofilter.

The biofilter consists of nitrifying bacteria that convert TAN to less toxic nitrate via nitrite (Bregnballe, 2015). To balance the nitrate concentration, RASs generally have a water exchange rate of 300-400 L/ kg feed per day, resulting in an approximate nitrate level of 7 – 11 mM (Bregnballe, 2015; Fjellheim *et al.*, 2016). The exchange rate, however, depends on the system setup and the cultivated species' sensitivity towards nitrate. A study by Davidson *et al.* found that post-smolt Atlantic salmon displayed no long-term adverse effects when exposed to nitrate concentrations up to 7 mM (2017). However, NO_3- levels of 6 -7 mM have been related

to chronic health and welfare impacts in Rainbow trout (*Oncorhynchus mykiss)*, and an upper limit of approximately 5 mM NO_3- has been advised (Davidson *et al.*, 2014). Further reduction of water exchange is possible by introducing an anaerobic denitrifying biofilter (30-40 L /kg feed) (Fjellheim *et al.*, 2016). This is unusual as denitrification units are expensive and energy-consuming, and the end-product, N_2 gas, is a non-readily useful form of N (Ramli *et al.*, 2020).

The amounts and nutritional content of sludge and outlet water are poorly documented, and the nutritional composition and quantities of these WSs will vary (Aas and Åsgård, 2017; Lomnes, Senneset and Tevasvold, 2019). The variation depends, among other factors, on the cultivated species and size, feeding regime and rate, stock density, system layout, and degree of recirculation. Therefore, the nutritional composition and quantities of WSs will not only vary between RASs but also in the same RAS over time (Ytrestøyl *et al.*, 2013; Bregnballe, 2015). As P is mainly particulate and N is mainly dissolved (NO_3- and NH_4-), sludge will generally contain a large fraction of the total phosphate (TP) and a smaller part of the total nitrogen (TN); naturally, the opposite will be true for outlet water (Bregnballe, 2015). In 2017 it was estimated that the annual amount of phosphorus in sludge from land-based smolt production in Norway was 225 tons P (Aas and Åsgård, 2017). The concentration of orthophosphate in sludge is unknown. One report by Nofima estimated that roughly 10% of the TP in sludge from one particular land-based RAS was in the form of dissolved PO_4- (Aas, 2016). Given the estimation by Aas and Åsgård, this amounts to a total of roughly 22.5 tons of orthophosphate-P in sludge annually (2017). Although this is a rough estimate, it does give the impression that there are great amounts of accessible phosphorus in sludge. Outlet water will also contain nutrients, mainly dissolved. Containment of the potentially nutritious outlet water is not common, as it is considered “clean” after the sludge is removed and can therefore be released directly to the recipient (Lomnes, Senneset and Tevasvold, 2019). Sludge, concentrated sludge, and outlet water are WSs that should be considered as resources, but this could require adaptions of the aquacultural value chain. As the mentioned regulations (Forurensningsforskriften, § 14-2) have prohibited emission from most facilities of water with high concentrations of particulate matter, now known as sludge - solutions for their disposal have been developed. These solutions have, however, offered little valorisation of polluting nutrients. Sludge is a waste product for aquaculture companies, and they will often be charged by biogas or fertiliser producers for sludge disposal (Ytrestøyl *et al.*, 2013; Aas and Åsgård, 2017). Only the dry matter in sludge is utilised in biogas and fertiliser production (Lanari and Franci, 1998; Ytrestøyl *et al.*, 2013).

Primary producers can utilise dissolved nutrients, and photosynthetic organisms offer the potential for sustainable valorisation as they can use energy from sunlight and carbon from CO_2 (Kruse, 2015). Recently, photoautotroph microalgae have been recognised as a remedy for wastewater treatment as they can utilise dissolved nutrients to produce biomass without requiring arable land (Pires *et al.*, 2013; Milhazes-Cunha and Otero, 2017; Li *et al.*, 2019). Further, photosynthetic microalgae have higher photosynthetic efficiency, growth rate, and CO_2 fixation rate than other photoautotrophic organisms (Huntley and Redalje, 2006; Vasudevan and Briggs, 2008). There has therefore been much interest in the possible usage and production setups of microalgae in recent years (Pires *et al.*, 2013).

Microalgae can synthesise protein, lipid, and natural pigments from dissolved nutrients and CO_2 (Halfhide et al., 2014; Sirakov et al., 2015; Leng et al., 2018). Algae biomass can be processed in biorefineries and converted into many high-value products. Microalgal lipids (30-50% of dry weight) can be converted into biofuel, protein (50-70% of dry weight) can be used in feedstock or humane nutrition, pigments can be utilised as colourants or pharmaceuticals, starch (<60% of dry weight) can be used in bioethanol and bioplastics, and the residual biomass can be applied in biogas generation or feedstock production etc. (Pulz and Gross, 2004; Brányiková et al., 2011; Chew et al., 2017).

The aquaculture industry is in increasing need of new environmentally sustainable sources of feed ingredients (mainly protein and lipids), and nutritional evaluations have demonstrated that microalgae have the potential to cover these needs (Becker, 2007; Nagappan *et al.*, 2021). Further, some algae can accumulate high concentrations of carotenoids like astaxanthin, a high-valued pigmentation source in aquaculture (Pulz and Gross, 2004). Gouveia *et al.* (1998) found that including *Chlorella vulgaris* in fish feed yielded higher retention of astaxanthin in muscle tissue of Rainbow trout (*Oncorhynchus mykiss)* than synthetic pigments.

Currently, photoautotrophic microalgae products are mainly present in niche markets such as supplements, cosmetics, and some animal feed (Chandrasekhar *et al.*, 2022). Although substantial technological advances have been made since the commercial cultivation of microalgae emerged over 50 years ago (Borowitzka, 1999), microalgae production for commodity products is still not economically viable as high production costs remain a major bottleneck (Pires *et al.*, 2013).

Numerous growth media have been developed for various microalgae, and physiological manipulation of medium composition is commonly used to promote nutrient-induced shifts in algae biomass (Becker *et al.*, 2013; Procházková *et al.*, 2014). Microalgae require approximately thirty elements for autotrophic growth, but the most important ones are the macronutrients N, P and carbon (C) (Becker *et al.*, 2013). The algal biomass content of N can range from 1 to >10%, and the most used source in algae cultures is nitrate (NO_3^-). Some algae can also utilise other forms of N such as ammonium (NH_4^+) and urea ($(NH_2)_2CO$) (Becker *et al.*, 2013; Procházková *et al.*, 2014). Inorganic phosphate, orthophosphate (PO_4-), is the preferred form of P for algal uptake. Although amounting to only approximately 1% of algal biomass, P is essential for growth and will often be a limiting factor as it is quickly bound to other ions – resulting in precipitation and thus becoming unavailable for algal uptake (Becker *et al.*, 2013; Procházková *et al.*, 2014). Organic phosphates can be made available through hydrolysis (Holtan, Kamp-Nielsen and Stuanes, 1988). Upon limiting P conditions, microalgae can utilise intracellular storages of P that have been saved up during more favourable conditions. This phenomenon is known as "luxury uptake", and it is essential to be aware of when experimenting with new media (Levin and Shapiro, 1965). The N:P ratio can influence the need for other nutrients, i.e. micronutriens (e.g. Fe, Mn, Zn). These nutrients are needed in very small quantities (µ - pg/L), and slightly elevated concentrations could be toxic to the algae (Procházková *et al.*, 2014).

Waste streams from aquaculture could potentially be rich in nutrients required by microalgae, primarily N and P. By utilising aquaculture WSs as a culture medium, one could facilitate wastewater treatment while enhancing the economic viability of microalgae products and contribute to the valorisation of WSs (Pires *et al.*, 2013). Further, co-cultivation in RAS can improve water quality and reduce CO_2 emissions while increasing dissolved oxygen concentrations by photosynthesis (Singh and Ahluwalia, 2013; Ramli *et al.*, 2020). Different algae can be cultivated at different salinities, making microalgae cultivation a solution also for saltwater WSs (Ramli *et al.*, 2020). The utilisation of aquacultural WSs in the cultivation of phototrophic microalgae would ultimately contribute to a zero-waste target which is fundamental when approaching the future circular economy.

This study is a part of the iFishIENCi project under the Waste2Value objective. One of the many goals of Waste2Value is to valorise nutrients in wastewater by using it as a substrate for microalgae growth. This study aimed to test RAS WS samples as growth media for microalgae

and how different medium altercations would affect the algae growth. A total of 22 WS samples (outlet water, sludge, and concentrated sludge) were tested as growth media in the current study. About half of the samples came directly from the aquaculture facilities (mainly outlet water). The rest (mainly sludge and concentrated sludge) had been treated externally with hydrolysis to transform suspended organic nutrients into forms readily available to microalgae. Before preliminary testing (small scale) of the various WSs as growth media for microalgae, the nutrient content (N and P) and other properties (salinity and pH) were mapped. In addition, two WS samples were selected for larger-scale experiments where more detailed analyses (DW, fatty acids, nutrient contents) could be conducted.

3 Materials and Methods

3.1 Waste streams and waste stream analysis

Outlet water, sludge and concentrated sludge from fish farms will further be referred to as waste streams (WS). The identification and selection of WSs was conducted by other parties in the iFishIENCi project. WS samples were supplied by two RAS facilities; Aller Aqua Research (AAR) and AquaBiotech (ABT). Four different batches of WS samples were received as seen in Figure 1. Batches 1 and 2 came directly from the aquaculture facilities. Batch 3 and 4 were sampled at the same time and in mostly the same systems as Batch 1 but had further gone through treatments conducted by LEITAT. A complete overview of the samples received in the different batches is summarised in in Table 1.

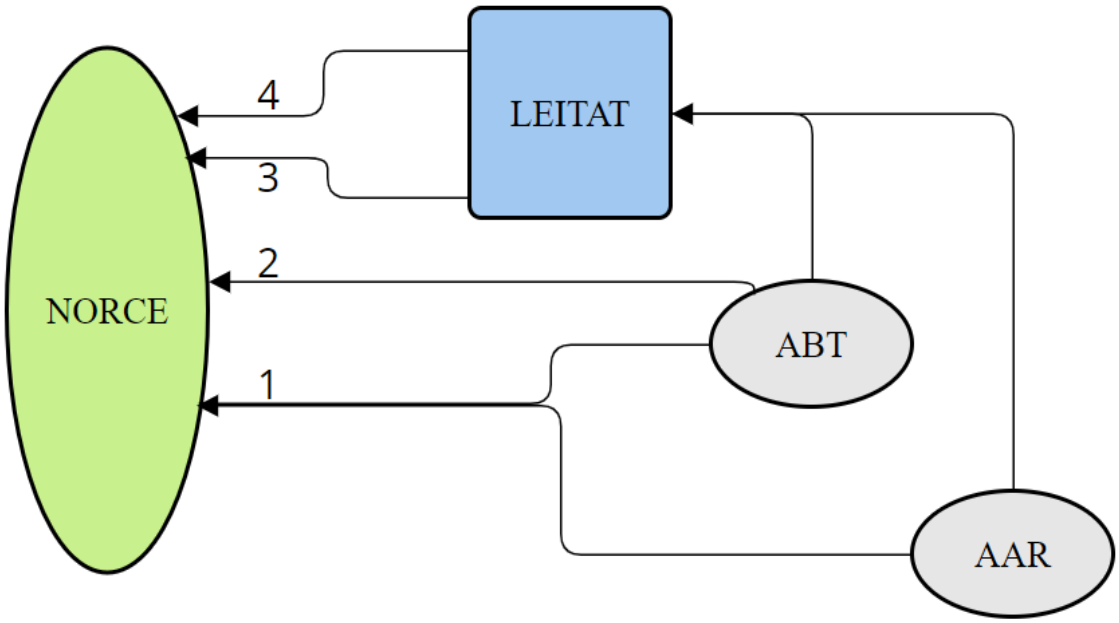

Figure 1 The WS samples came in four batches as displayed in the figure. Baches 1 and 2 came directly from the aquaculture facilities: Aller Aqua Research (AAR) and AquaBioTech (ABT). Batch 3 and 4 also originated from these facilities but had further gone through various treatments at LEITAT.

Table 1 Overview of samples received in Batch 1, 2, 3, and 4. Batches 1 and 2 came directly from the aquaculture facilities, while batch 3 and 4 had gone through further treatments by LEITAT. The column "system ID" is the suppliers (ABT/AAR) name of the system from which a given sample was retrieved. The column "ID" is the name used for the various WS samples in the current research. The first two letters represent the supplier while the last letter represents the WS characteristic. Abbreviations: AA = Aller Aqua Research, AB = AquaBioTech, O =Outlet, S = Sludge, CS = concentrated sludge, FW = freshwater, SW = saltwater.

Batch	Species	System ID	Diet	ID
1	Rainbow trout FW	RAS5_243	Candida	AA1_O
			Control	AA2_O
		RAS1_188	Nanno	AA3_S
				AA4_O
			Control	AA5_S
				AA6_O
		RAS3_ABT3	Conventional	AB1_O
2	Barramundi SW	IFN01_LC	Conventional/Antiox	AB2_O
↓↓ LEITAT ↓↓				
3	Rainbow trout FW	RAS1_188	Control	AA7_S
			Nanno	AA8_S
		RAS2_194	Control	AA9_S
			Antiox	AA10_S
		RAS5_243	Control	AA11_S
			Candida	AA12_S
		RAS3_ABT3	Conventional	AB3_S
				AB4_CS
				AB5_CS
				AB6_CS
				AB7_O
				AB8_O
4	Rainbow trout FW	RAS3_ABT3	Conventional	AB9_CS
				AB10_CS

Aller Aqua Research

The AAR facility in Germany that supplied the samples for this project cultivated rainbow trout in RASs. Three different feed trials in three different RASs were conducted during the sampling time. For every trial with a non-conventional diet, a control group in the same RAS, but different tank, was fed with a conventional diet. The diets tested are named Nanno (RAS1_188), Candida (RAS5_243), and Antiox (RAS2_194). In the Nanno diet, the feed had been formulated with a 30% inclusion rate of *Microchloropsis gaditana* (previously known as *Nannochloropsis gaditana*). The algae had been produced at the National Algaepilot in Mongstad (by NORCE/UiB). In the Candida diet, a 30% inclusion of the *Candida utilis* was formulated in the feed. The yeast was produced by NORCE. The Antiox diet consisted of feed with incorporated liquid antioxidant supplement from *M. gaditana* (3% dose) (supplement produced by LEITAT from *M. gaditana* produced at National Algaepilot Mongstad).

All the cultivation tanks in the AAR facility were equipped with separate sludge collectors as illustrated in Figure 2. Raw sludge was sampled from the mixture with settled particles in these tanks, while Outlet water was sampled from the water above.

Batch 1 contained outlet samples from fish with Candida and Nanno diets, as well as raw sludge samples from fish fed with Nanno and equivalent samples from conventional diets (control). Technical replicas of the raw sludge samples were also shipped to LEITAT. Additionally, LEITAT received raw sludge samples from fish fed with Antiox and Candida diet and their respective controls. This is summarized in Table 1.

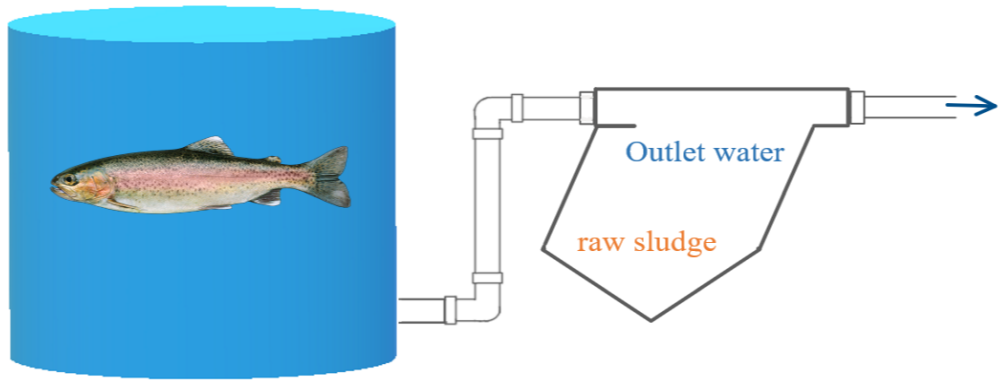

Figure 2 Schematic overview of the AAR RAS with rainbow trout and waste collection points at the sludge collector (right). Raw sludge was sampled from the settled particles in the sludge collector while the top water was sampled as outlet water. All the water samples from AAR originated from these collection points. Picture of trout is borrowed from : https://toppng.com/rainbow-trout-png-rainbow-trout-PNG-free-PNG-Images_184545

AquaBioTech

The ABT facility that supplied the samples for this project is located in Malta. The ABT samples in batch 1, 3, and 4 were retrieved from RAS3_ABT3 cultivating rainbow trout with conventional feed (as seen in Table 1). Each cultivation tank with rainbow trout was equipped with a swirl separator which was the first step in the recirculating process as well as the point for sample collection in this trial. A bottom drain in the fish tank led dirty water and settled solids to the swirl separator. The water circulated in the swirl separator, leaving solid waste to settle before most of the water would exit with the vessels overflow. The settled mixture (>95% water) was periodically evacuated through a bottom drain. This mixture will be referred to as raw sludge. This raw sludge was further treated to create the samples "outlet water" and "concentrated sludge", as described below. A schematic overview of the system is presented in Figure 3.

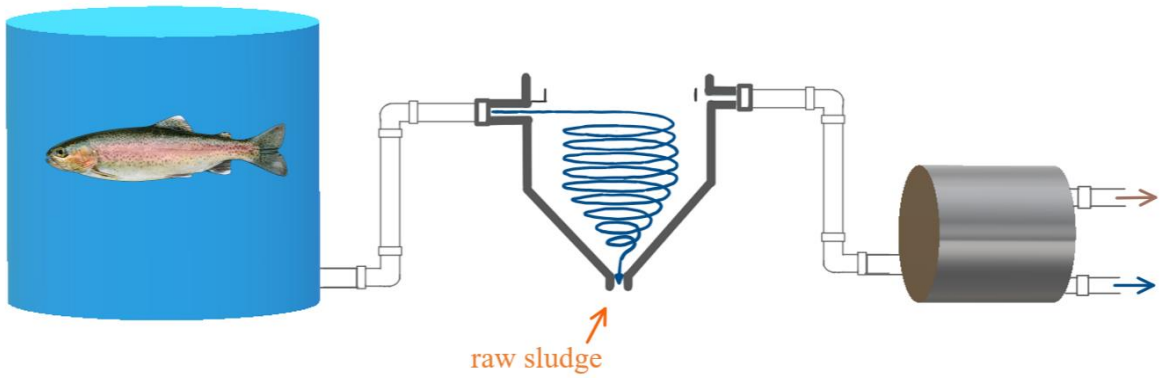

Figure 3 Schematic overview of the ABT RAS with rainbow trout and waste collection point at the swirl separator (middle). To drum filter is illustrated to the right. The WSs AB1 and AB3-10 originated form the collection point at the bottom of the swirl separator marked with an arrow and the text "raw sludge". Picture of trout is borrowed from : https://toppng.com/rainbow-trout-png-rainbow-trout-PNG-free-PNG-Images_184545

The Batch 2 sample was retrieved from a RAS IFN01_LC cultivating Asian sea bass *(Lates calcarifer*) with Antiox diet (same as in AAR Antiox trial) and control groups with conventional diets. The RAS with Asian sea bass did not have swirl separators. The dirty water from both feed groups (Antiox and control diet) were therefore mixed in the pipes headed to the drum

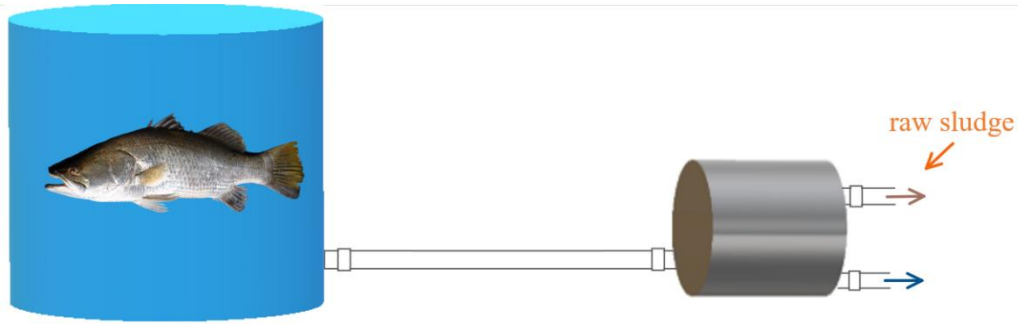

Figure 4 Schematic overview of the ABT RAS with Asian Sea Bass and its collection point. The AB2_O waste stream originated from the collection point marked with an arrow and the text "raw sludge". Picture of barramundi is borrowed from : https://favpng.com/png_view/fish-fish-tilapia-barramundi-png/bP4Qgydm

filter where it passed through a 60µm pore sized filtration screen. The solids that were trapped in the drum filter were removed by backwash with system sump water and collected into a wastewater tank as raw sludge. A schematic overview of the system is presented in Figure 3.

The raw sludge from the Asian sea bass and rainbow trout trial went through several different treatments to simulate WSs from an industrial process. Samples mimicking the effluent water from the system, further referred to as outlet samples, were made by vacuum filtering the raw sludge across a 60µm pore sized mesh and keeping the filtrate. Concentrated sludge samples were made by first allowing the solids in the raw sludge to settle before the supernatant was removed by siphoning. The remaining mixture was passed through a coarse filter with 100µm mesh. Thereafter, the mixture was vacuumed filtered through a 60µm pore sized filters for three hours, leaving a residue of concentrated sludge.

As seen in Table 1, Batch 1 contained one outlet sample (AB1_O) from the Rainbow trout trial. A replica of this sample was shipped to LEITAT (AB7 – 8_O) along with raw sludge (AB3_S) and concentrated sludge (AB4 – 6_CS and AB9 – 10_CS) samples, that were enzymatically treated to liberate more nutrients for microalgae cultivation. These samples were subsequently shipped to NORCE as a part of Batch 3 and 4. Furthermore, Batch 2 contained one large outlet water sample (approximately 30 L divided on 15 flasks) from the Asian sea bass trial (AB2_O).

LEITAT

LEITAT did trials where they looked at insoluble nutrients in sludge and sludge bioconversion into available nutrients for i.e., microalgae. LEITAT received Raw sludge, concentrated sludge, and outlet samples from AAR and ABT, as described earlier. Their methods consisted of different pre-treatments depending on the dry-matter content of the WS sample, and execution of hydrolysis to recover nutrients, mainly N (peptides and amino acids) and P. All sludge samples in Batch 3 were treated enzymatically, except AB7-AB8_O. Only inactivated enzymes were added to these samples to check the effect of the enzyme itself. Replicas of concentrated sludge from ABT were treated with three different enzymes (samples AB4_CS – AB6_CS in Batch 3). In Batch 4 two replicas of concentrated sludge had gone through different treatments. The AB9_CS sample had been treated with enzymatic hydrolysis (different from batch 3) while the AB10_CS sample had been treated with chemical hydrolysis.

Waste stream analysis

The Batch 1 samples were analysed for ammonium, nitrate, total phosphate, and orthophosphate. Salinity and pH were also measured. Measured ammonium and nitrate were used to estimate TN. Most of the samples were filtered with a 0.2 µm sized filter prior to analysis. Some samples had significant turbidity; these were centrifuged at 3500 rpm for 10 min before filtration. The AB1 sample was not filtered prior to analysis as it had been filtered with a 60 µm filter before delivery. One (of 11) batch 2 bottle was sampled and analysed for nitrate, ammonium, and orthophosphate. The salinity and pH were also measured. The sample was not filtrated prior to analysis as it had been filtered with a 60 µm filter before delivery. The batch 3 samples were analysed for TN and TP by LEITAT. Further analyses of salinity and pH were conducted. A nitrate analysis of filtered samples (0.2 µm) was conducted after the samples had been stored thawed for a week. The Batch 4 samples were centrifuged at 5500 rpm for 15 minutes and sterile filtrated (0.2µm) before analysis and experiment. The samples were analysed for ammonium, nitrate, TN, and orthophosphate. The salinity and pH were also measured.

3.2 Standard growth media

A commercial powder fertilizer, YaraVita Rexolin APN, was used as micronutrient source in the standard growth media. The powder, hereafter referred to as APN, contained Boron (B) 0.85%, Copper (Cu) 0.25%, Iron (Fe) 6%, Manganese (Mn) 2.4%, Molybdenum (Mo) 0.25%, Zinc (Zn) 1.3%, and chelating agent (DTPA).

The Bold's Basal and NORCE media were used as controls and in algae stock cultures. Bold's Basal Medium (BBM) was used in FW experiments. The medium has an N:P ratio of 1.71 and was prepared with Reverse Osmosis (RO) water and 2.94 mM $NaNO_3$, 1.29 mM KH_2PO_4, 0.43 mM K_2HPO_4, and 38 mg/L APN. The NORCE medium, developed by the Marine Biotechnology group at NORCE, was used in the SW experiments, and has a N:P ratio of 14.2. The medium was prepared with autoclaved SW, 12.47 mM $NaNO_3$, 0,88 mM KH_2PO_4, and 38 mg/L APN.

3.3 Stock cultures and inoculum preparation

The FW and SW experiments were conducted with either FW *Chlorella vulgaris* (NIVA 108) or SW *Microchloropsis gaditana* (CCMP526, previously known as *Nannochloropsis gaditana* CCMP526), respectively, started from stock cultures. Stock cultures of each microalga were

maintained in 200 ml autoclaved Erlenmeyer flasks. *C. vulgaris and M. gaditana* were cultivated with BBM and NORCE media, respectively. The flasks were capped with aluminium foil and incubated in a growth cabinet at 15 ^{o}C with 14h:10h light/dark cycles. The growth cabinet had shelves with different light intensities, varying from 5– 25 µmol photon $m^{-2}s^{-1}$. The flasks were occasionally swirled and with increased culture density, they were moved to a higher light intensity. Upscaling was done by transferring approximately 5 ml culture to new autoclaved Erlenmeyer flasks and adding approximately 100 ml BBM/NORCE media. This procedure was conducted under a Laminar flow bench (LAF bench). The stock culture OD_{750} was measured before an experiment inoculation and the required culture volume was calculated based on the desired start OD. If needed, the culture volume was collected in falcon tubes, centrifuged at 3000 rpm for 3 min. The supernatant was removed, and the remaining algae biomass was resuspended in Reverse Osmosis (RO) water or SW to achieve the required density for the experiment.

3.4 Experiments

In the following experiments various WSs were tested as growth medium for photosynthetic algae. Ultimately, the testing was done at three different scales: 0.2 mL in well plates, 300 mL in bubble columns, and 20 L in photobioreactors. All WS samples were tested at the smallest scale in the well plate experiments. The outcome was used to develop and conduct further testing of one selected WS in the bubble column experiment and another in the photobioreactor experiment. Positive controls were included in almost all the experiments. The BBM and NORCE media were used as positive controlls in the FW and SW experiments, respectivly.

3.4.1 Well Plate

The 96-well plates without parafilm were used in the six different well plate (WP) experiments (WP1-6). WP1 and WP3-6 were FW experiments (C. *vulgaris*) while WP2 was a SW experiment (M. *gaditana*). The experimental design was based on a trial experiment where it was evident that algae growth was inhibited if the plates were sealed with parafilm, assumingly caused by CO_2 deprivation. A detailed protocol with recipe and plate setup was developed before the start of every experiment. The following procedure was common for the WP experiments. Master mixes (1800µL) with WS sample were prepared in 2 ml Eppendorf tubes. The average N concentration (NH_4+ + NO_3-) in WP1 (~3.6 mM N, AB1_O excluded) was used as a template in order to obtain similar initial growth concentrations. Samples that had noticeably elevated N concentrations were therefore diluted with reverse osmosis (RO) water

(FW) or autoclaved SW so obtain similar conditions. Algae culture (10% = 200 µL) with OD_{750} ~1.0 was added to each master mix. The mixtures were split into two tubes in the experiments where manipulation by supplement of micronutrients was tested. RO or autoclaved SW (10 µl) was added to one tube and APN (0.939 g/L) was added to the other tube. Finally, 3 adjacent wells (biological triplicates) in the well plate were each filled with 200 µL of the mixture. In the experiments without APN the master mix with algae was split directly into 6 adjacent wells (200 µL in each). The outer wells in the plates were filled with RO water to minimize evaporation.

The plates were placed in an empty aquarium with a LAMPEX 14 W (15µmol/m^2/s) light tube mounted horizontally on top. Black plastic was used to cover the aquarium and minimize disturbance from other light sources. The temperature was kept at 22ºC +/- 1 and the plates were rotated regularly to minimize the difference in light availability. The outer wells were refilled with RO water when evaporation was evident. The optical density was measured as described in section 3.5.1.

The different goals and approaches for each well plate experiment (WP1-6) are described below. All quantities in recipes are in µL.

WP1 was conducted to determine the suitability of the Batch 1 WS samples for cultivation of *C. vulgaris.* The samples were tested as growth medium in their original form and after different manipulations. These manipulations include addition of APN, autoclaving, and addition of P to change the N:P ratio. The following N:P ratios were tested: the original ratio as presented in the WS, the altered BBM ratio (N:P ratio = 5.14), and Redfield ratio (N:P ratio = 16) (Redfield, 1934). The altered BBM ratio was used as it is common in commercial production to increase the N concentration (BBM 3xN) to achieve a denser algae biomass (Becker *et al.*, 2013). The estimated TN (est. from nitrate and ammonium, method analyses in section 3.5.7) and measured orthophosphate (see section 3.5.7) was used in Equation 1 to calculate the N:P ratios of the Batch 1 samples. Only orthophosphate was used in this calculation as this is the form preferred for microalgae (Becker *et al.*, 2013).

$$\text{ratio} = \frac{\text{N[mol]}}{\text{P[mol]}}$$

Equation 1 The ratio equation was used to calculate the ratio between nitrogen (N) and orthophosphate (P).

The additional P needed (P_{add}) to reach the BBM 3xN N:P ratio and the Redfield ratio was calculated with Equation 2, a modification of the ratio equation.

$$P_{add}[mol] = \frac{N[mol]}{ratio} - P[mol]$$

Equation 2 A modified version of Equation 1 was used to find additional P needed (P_{add}) to reach a desired ratio. N = measured nitrogen, P = measured orthophosphate, ratio = current N:P ratio.

Stock solutions with 1, 2, 20, and 50 mg/ml KH_2PO_4 were used to alter the N:P ratios. Equation 3, the dilution formula, was used to find the volume of stock solution (v_1) with a given P concentration (c_1) that would add the right P_{add} (c_2) to a given sample volume (v_2).

$$v_1[\mu L] = \frac{v_2[\mu L] * c_2[mol]}{c_1[mol]}$$

Equation 3 The dilution equation was used to calculate the volume of stock solution (v_1) with a given P concentration (c_1) that would add the right concentration of P (c_2) to a given sample volume (v_2).

The N:P data was used to prepare the recipe displayed in Table 2. The wells were prepared in a LAF bench with sterile technique. The OD was measured daily the first eight days, and then every two to three days until the experiment was terminated after 22 days. The AB1_O sample was diluted four times so that the N-concentration would be comparable to the other samples. This was not taken into account when calculating P_{add} for AB1_O. Too much P was therefore added in the Redfield and BBM ratio recipes for AB1_O in WP1.

*Table 2 Recipe for WP1. All the samples were tested autoclaved/not autoclaved and with original/altered N:P-ratios (Redfield and BBM ratio). All quantities are in µL. Abbreviations: AA = Aller Aqua, AB= AquaBioTech, O = outlet, S = sludge .A = autoclaved. *The AB1_O sample was four times diluted but this was not considered when calculating P_{add}. Too much P was therefore added. All quantities are in µL.*

WP1		Redfield ratio				BBM ratio				Original		
		Sample	P_{add}	Water (25-P_{add})	Algae	Sample	P_{add}	Water (25-P_{add})	Algae	Sample	Water	Algae
AA1_O		1775	17.8	7.2	200	1775	8.3	16.7	200	1775	25	200
	.A	1775	11.4	13.6	200	1775	6.9	18.1	200	1775	25	200
AA2_O		1775	16.6	8.4	200	1775	8.1	16.9	200	1775	25	200
	.A	1775	12.6	12.4	200	1775	7.3	17.7	200	1775	25	200
AA3_S		1775	10.7	14.3	200	1775	8.3	16.7	200	1775	25	200
	.A	1775	5.3	19.7	200	1775	6.4	18.6	200	1775	25	200
AA4_O		1775	23.5	1.5	200	1775	9.9	15.1	200	1775	25	200
	.A	1775	12.6	12.4	200	1775	7.1	17.9	200	1775	25	200
AA5_S		1775	10.1	14.9	200	1775	7.6	17.4	200	1775	25	200
	.A	1775	6.2	18.8	200	1775	7.3	17.7	200	1775	25	200
AA6_O		1775	22.9	2.1	200	1775	9.4	15.6	200	1775	25	200
	.A	1775	14.9	10.1	200	1775	7.8	17.2	200	1775	25	200
AB1_O		443,8	8.9	1347.4	200	443.8	13.6	1342.6	200	443,8	25	200
	.A	443,8	7.1	1349.1	200	443.8	12.6	1343.7	200	443,8	1356.02	200

WP2 was conducted to determine the suitability of the WS from Batch 2 for cultivation of *M. gaditana*. The AB2_O sample was used undiluted as it had an N concentration (approximately 4.3 mM N) that was considered to be similar enough to WP1 (average of approximately 3.66 mM N). Manipulations by addition of APN and autoclaving were tested. A negative and a positive control was included with autoclaved SW without additional nutrients and NORCE medium, respectively. The OD was measured, and photographs were taken daily. The experiment was terminated after 7 days.

Table 3 Recipe for W5 where AB2_O was tested original and autoclaved (.A) with and without the addition of APN as growth medium for M. gaditana. A positive (NORCE) and negative (SW) control was included. Abbreviations: AB = AquaBioTech, O = outlet, .A = autoclaved, SW = seawater. All quantities are in µL.

WP2	Sample	SW	Algae
AB2_O	1800	0	200
AB2_O.A	1800	0	200
SW	0	1800	200
NORCE	1800	0	200

WP3 was conducted to determine the suitability of the WS samples from Batch 3 for cultivation of *C. vulgaris*. In WP4, the Batch 3 samples were tested again after being centrifuged (5000 rpm, 15 min) and sterile filtrated (0.2 µm). The plates were kept as sterile as possible during the rest of WP4. Original and centrifuged + sterile filtrated AB1_O was additionally included in WP4. For both experiments manipulation by addition of APN was tested. Analysis of N and P content (by LEITAT) were used to develop the recipe displayed in Table 4, which was used in WP3 and WP4. The dilution factors made N ≈ 3.66 mM N (similar to the average N concentration in WP1) for all media. In both experiments, wells with BBM or RO water were included as positive and negative controls, respectively. For the WP3 experiment the OD was measured daily (14 days), and the wells were photographed on the last day. In WP4 the OD was measured, and photographs were taken daily. The experiment was terminated after 8 days.

*Table 4 Recipe for WP3 and WP4 where the Batch 3 samples were tested as growth medium for C. vulgaris with a positive (BBM) and a negative (RO water) control. Samples with NO_3-N concentrations >3.66 mM N were diluted. All quantities are in µL. Abbreviations: AA = Aller Aqua, AB= AquaBioTech, O = outlet, S = sludge, CS = concentrated sludge, .A = autoclaved.RO = reverse osmosis. *AB1_O was only included in WP4.*

WP3 & 4	x.diluted	Sample	RO water	Algae
AB3_S	4	450	1350	200
AB4_CS	24	75	1725	200
AB5_CS	15	120	1680	200
AB6_CS	19	95	1705	200
AB7_O	5	360	1440	200
AB8_O	5	360	1440	200
AA7_S	3	600	1200	200
AA8_S	4	450	1350	200
AA9_S	3	600	1200	200
AA10_S	4	450	1350	200
AA11_S	1	1800	0	200
AA12_S	2	900	900	200
BBM	1	1800	0	200
RO water	1	0	1800	200
AB1_O*	4	450	1350	200

WP5 was conducted to evaluate the WSs from Batch 4 as a growth media for *C. vulgaris*. The samples were centrifuged at 5500 rpm for 15 min and sterile filtrated before the experiment. Analysis of nitrate, ammonium, and orthophosphate were used to estimate the N:P ratios, and develop the recipe displayed in Table 5. The AB10_CS medium was diluted so that NH_4^+ + NO_3^- ≈ 3.66 mM N (similar to the average N concentration in WP1). The AB9_CS medium was used undiluted as it had a N concentration (4.69 mM N) that was considered to be similar enough to WP1. The WSs were tested with and without the addition of APN. Wells with RO water or BBM were included as positive and negative controls, respectively. The OD was measured and photographs were taken daily. The experiment was terminated after 8 days.

Table 5 Recipe for WP5 where the Batch 4 samples were tested as growth medium for C. vulgaris. Abbreviations: AB = AquaBioTech, CS = concentrated sludge, RO = reverse osmosis. All quantities are in µL.

WP5	**Sample**	**RO water**	**Algae**
AB9_CS	1800	0	200
AB10_CS	1200	600	200
RO water	0	1800	200
BBM	1800	0	200

WP6 was mainly conducted to evaluate the reproducibility of the Well Plate experiments. AB1_O from Batch 1, AB7_O from Batch 3 and BBM were tested with *C. vulgaris*. The recipe from WP1 (original N:P ratio) was used for AB1 and the recipe from WP3/4 was used for AB7_O. The samples were tested without filtration, with 0.2 µm filtration, and with 0.45 µm filtration. The OD was measured, and photographs were taken daily. The experiment was terminated after 8 days.

Well plate data analysis

Excel version 2112 was used for data analysis. Raw data from the Hidex program was filtered, and the optical density measurements (see chapter 3.5.1) of all wells with medium were kept. The average of each biological triplicate/sextuplicate and the standard deviation was calculated. Point diagrams with trendlines were made with the averages of the biological triplicates.

3.4.2 Bubble column

The bubble column experiment was conducted to determine how different manipulations of the AB1_O waste stream from Batch 1 would affect nutrient conversion efficiency and biomass quality in cultivation of *C. vulgaris*. Four different media (1.2 L) were prepared for the bubble column experiment: BBM/control (BBM), AB1_O original (O), AB1 original with micronutrients (Oμ), and AB1 with the same N:P ratio and micronutrient content as BBM (Bμ). All the media were diluted to be prepared with the same N concentration as the original BBM (2.94 mM). Volumes of 261 ml autoclaved AB1 were poured into three sterile 5L flasks to prepare the O, Oμ, and Bμ medium. Micronutrient was added to Bμ and Oμ to give an end concentration of 38 mg APN/L in both media. P stock solution was added to Bμ to give the medium the same P concentration as BBM (1.72 mM P). These procedures were conducted under a LAF bench. Finally, the bottles with media were filled up to 1.2 L with RO water. A positive control with 1.2 L BBM was also prepared. The media were autoclaved, and 1000 ml was transferred to respective 1L flasks. Concentrated *C. vulgaris* inoculum was added to each medium, giving a start OD_{750} ~0.1. The growth media before and after algae addition are pictured in Figure 5. Biological triplicates for each medium were made by transferring 250 ml each to three autoclaved 300 ml bubble column tubes. The tubes were placed in temperature-controlled water tanks at 23 ± 0.5°C. Continuous stirring and CO_2 supply was secured by an aeration tube with flow through of filtered 1% CO_2-enriched air. The bubble columns are

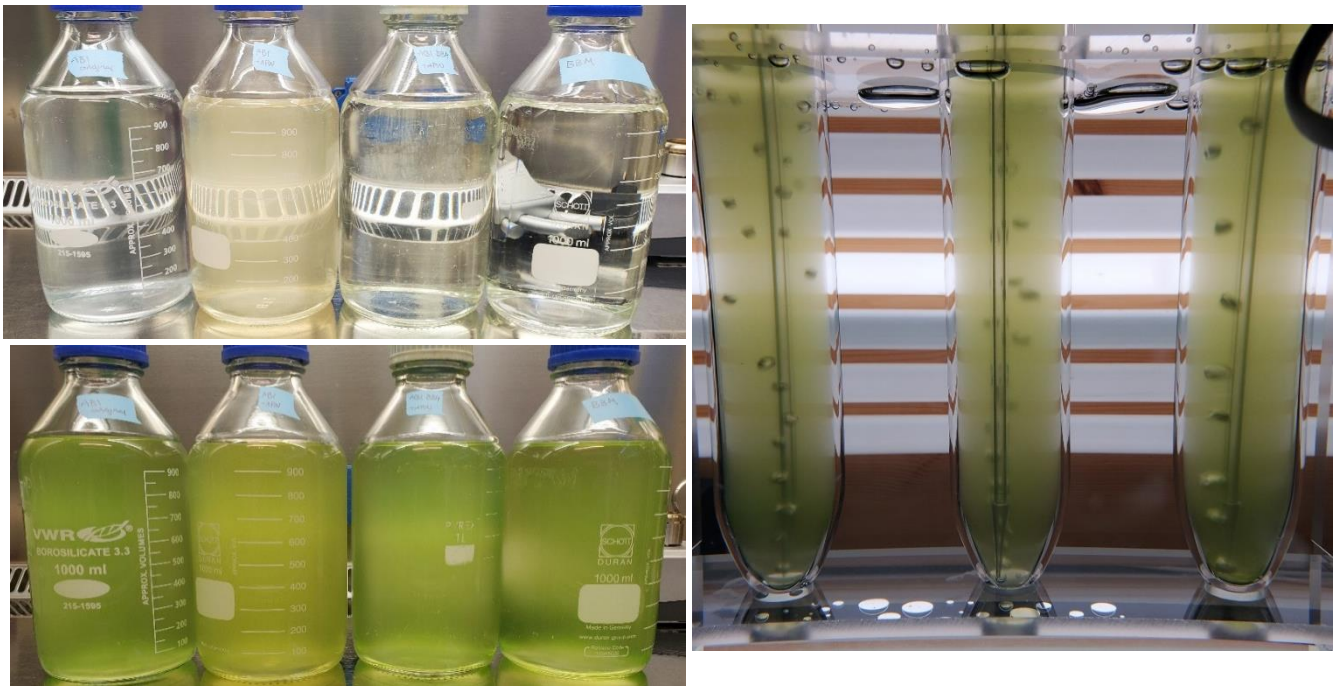

Figure 5 Picture of the autoclaved mediums before (top left) and after algae addition (bottom left). From the left: O, Oμ, Bμ and BBM. Picture on the right is from the first day of the bubble column experiment.

pictured in Figure 5. Manual stirring was also conducted 1-2 times per day. Constant light was attained by an array of six fluorescence tubes (Philips MASTER, TL-D 90 Graphica 58W/95) mounted horizontally behind the water tanks. The light intensity at the start of the experiment was set to 15 µmol photon $m^{-2}s^{-1}$ and was increased daily by one-fold from the mid-growth phase (timed based on OD values). Light measurements were conducted with the LI-250A LI-COR (Bioscience, UK) portable light meter.

Samples were extracted daily from every culture to measure OD (technical duplicates) and Quantum Yield (QY). Additional samples were taken after autoclaving before the algae were added (medium), at the mid-growth phase (S1), and at the stationary stage (S2). These samples were used for analysis of dry weight (S1 and S2), fatty acid analysis (S1), and nitrate (medium, S1 and S2) (see method description in section 3.5). Separate dry weight and fatty acid analyses were done for each biological replica. The samples from the biological replicas were combined for nitrate analysis. Excel version 2112 was used for data analysis. One-way ANOVA tests were used to test for significance differences in culture density with data from the final OD_{750} analysis and dry weight. A p value <0.05 was considered statistically significant. The OD data was also used to calculate specific growth rates as described in 3.5.1.

3.4.3 Photobioreactor

The Photobioreactor (PBR) experiment was conducted as a proof-of-concept at semi-pilot scale by cultivating *M. gaditana* in two highly controlled 25L GemTube RD1-25 tubular PBRs (LGem b.v., Netherlands) seen in Figure 6. The PBRs comprised of a single vertical tubular helix with 12 windings. The tubes were made of borosilicate glass and had an outside and inside diameter of 32 mm and 28 mm, respectively. The PBRs were situated in a temperature-controlled room set to 23^{o}C +/-1. The medium in in one PBR consisted of the undiluted WS sample from Batch 2, AB2_O, with addition of 38 mg APN/L. The other PBR was used as a positive control with NORCE medium. The medium and inoculum were added to the PBRs via the main access point (Figure 6, point 1). The culture circulated through the vertical tubular helix (Figure 6, point 2) by an airflow from the aeration inlet via a humidifier (Figure 6, point 3). Deaeration was allowed by passive flow from the lid of the main access point (Figure 6, point 4). The PBRs were also equipped with thermometers and pH-meters (Figure 6, point 5). The pH-meters were linked to an automatic feedback system that controlled the pH-levels. Once a certain max pH-setpoint was reached (pH=8), a pure CO_2 influx was activated, causing a

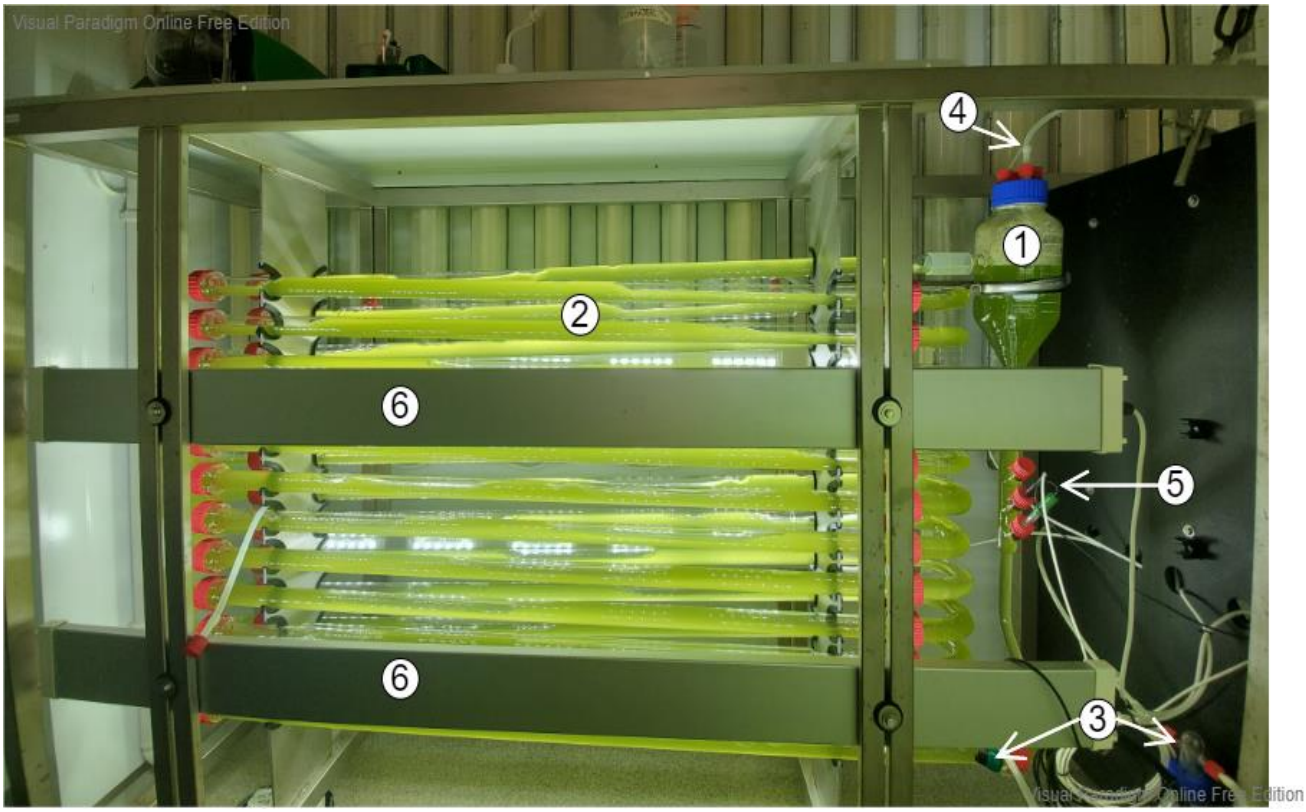

Figure 6 The Photobioreactor used in the experiment containing AB2. Explanation of points: 1. Main access point, 2. Tubular glass helix, 3. Aeration inlet and humidifier, 4. Deaeration, 5. pH-meter and thermometer, 6. Integrated LED-light panels.

decrease in pH. The influx was deactivated when a min pH-setpoint (7,8) was attained. Integrated LED light panels (Figure 6, point 6) allowed precise control of light intensity.

The AB2 PBR was cleaned and washed out with chlorinated water before experiment start. Approximately 19 L (11 bottles) of AB2 sample was added to the reactor. The defrosting time and the turbidity varied among the sample bottles seen in Figure 7. The control reactor was cleaned and filled with chlorinated salt water. Sodium thiosulfate 5% (50 g /L) was added to neutralize chlorine, and the chlorine level was analysed to ensure proper neutralization prior to experiment start. Too much Sodium thiosulfate (approximately 100 mL) was added by mistake by a colleague, which later showed to affect the functionality of the nitrate analyses. This was first discovered after the NORCE stock solution and algae had been added to the reactor.

Figure 7 The bottles containing AB2 sample had visibly different turbidity.

M. gaditana inoculum was added to each reactor, giving a start OD_{750} ~0.3. The nitrate concentration of the algae culture was analysed. The initial light level (day 0) was set to approximately 40 µmol photon $m^{-2}s^{-1}$. On day 1 it was increased to approximately 85 µmol photon $m^{-2}s^{-1}$ (all lights 10%), and further to approximately 150 µmol photon $m^{-2}s^{-1}$ (20%) on day 2. On day 4 the light level was increased to approximately 300 µmol photon $m^{-2}s^{-1}$. Finally, the light level was increased to approximately 360 µmol photon $m^{-2}s^{-1}$ on day 7, where it was maintained until the termination of the experiment (light calibrations by Hanna Böpple).

Samples were extracted daily to measure OD (technical duplicates), QY and nitrate (methods described in chapter 3.5). The specific growth rate was calculated from the OD as described in 3.5.1. These measured values were used to decide the duration of the experiment as well as the mid-growth and stationary phase sampling points where samples would be extracted for fatty acid and dry weight analyses (see methods described in 3.5). Additional samples were extracted for dry weight analyses at the beginning of the experiment approximately 30 min after the last addition to the medium.

3.5 Analyses

3.5.1 Optical density (OD)

In the various experiments optical density (OD) was measured photometrically at 680 and 750 nm to estimate algae density. The OD values were used to construct growth charts and estimate when the cultures entered the mid-growth and stationary phase. These estimations were used to determine the appropriate timepoints for other sampling as well as the termination of the various experiments. In the bubble column and photobioreactor experiments, the OD was also used to calculate specific growth rates. The specific growth rate (μ) was calculated with Equation 4 during the time period (t_1 – t_2) where the OD was considered to have an exponential development (linear graph in logarithmic scale). The OD_{750} on the last day of the exponential growth phase (t_2) is N_2, while the OD_{750} on the first day of the exponential growth phase (t_1) is N_1.

$$\mu\,[d^{-1}] = \frac{\ln(N_2) - \ln(N_1)}{t_2 - t_1}$$

Equation 4 The specific growth rate formula was used to calculate the growth rate (μ) during the exponential growth phase from start(t_1) to finish (t_2). N_2 is the measured OD on t_2 and N1 is the measured OD_{750} on t_1.

The photometric measurements in the well plate experiments were conducted in a Hidex Sense Microplate Reader. Well plates containing the respective growth media were measured and used for blanking. The plates were inserted in the Hidex and shaken for 3 seconds before the OD_{680} and OD_{750} was measured. A dilution series was conducted to determine the range for accurate OD measurements in the Hidex reader. During the second WP experiment (WP3) it was registered that the Hidex reader was sensitive to air bubbles and uneven algae growth as it could result in false readings. After this observation, visual controls were included as a precautionary step to control the data from the Hidex readings. This was done by photographing the plates before every reading. The standard deviation of the biological replicas OD_{750} was calculated.

The photometric measurements for offline samples from the bubble column and PBR experiments were conducted with a spectrometer UV-1201V, Shimadzu Corporation, Kyoto, Japan. Respective media were used for blanking. After ensuring homogeny of the samples, 1 ml was pipetted into a plastic cuvette and placed in the spectrophotometer. To secure correct readings, the samples were diluted with RO water or SW if they had an OD value greater than

the maximum absorbance which was recorded to be 0.2. The data was used for calculation of specific growth rates

3.5.2 QY

The Quantum yield (QY) was measured with the handheld Aquapen AP 110/C fluorometer. The QY is an indicator of the algae stress level and decreasing values are associated with stressful conditions. QY was measured after OD measurement in all experiments expect the well plate experiments. The cuvette was inserted in the AquaPen and kept in the dark for approximately 1 min before measurement.

3.5.3 Fatty acids

Technical duplicated samples of 10 ml were transferred to glass tubes for total fatty acid analysis (TFA). The samples were centrifuged at 3500 rpm for 10 min. The supernatant was removed by siphoning, and the tubes were flushed with nitrogen gas. Finally, the pellets were stored at -20^{o}C until analysis. Total lipid-extraction and derivation to fatty acid methyl esters by direct esterification was conducted externally, according to Steinrücken *et al.* (2017).

3.5.4 Dry weight

Glass microfiber filters (GF/F, 47 mm, Whatman^{TM}) were washed on a filtration unit connected to a vacuum pump with approximately 50 ml MQ water. The filters were contained in numbered glass dishes and placed in a Termaks drying cabinet for about 24 h at 90^{o} C. Thereafter, the dishes were moved to a desiccator to reduce moister absorption while cooling down. The dry filters were weighed (w_1 in Equation 5) with a Mettler Toledo MT5 micro weight (0-5.1 g) before being stored until needed.

Depending on the density of the culture, 1-5 mL sample (x in in Equation 5) was diluted with 5 ml ammonium formate (AF, 31 g/L) for SW samples or RO water for FW samples. A pre-weighed filter was placed on the vacuum pump and 10 mL AF or RO water was poured on the filter before the diluted sample was added. The filter was washed two times with 20 ml AF or RO water before being placed back in the numbered glass dish. This procedure was conducted with technical triplicates. The glass dished were again placed in the Termaks cabined for a minimum of 24 hours at 90^{o}C. Thereafter, the cases were placed in the desiccator to cool down before being weighed again (w_2 in Equation 5) on the Mettler micro weight. Dry weight concentration [mg/mL] was calculated with Equation 5.

$$DW\ [mg/mL] = \frac{w_2\ (mg) - w_1\ (mg)}{x\ (mL)}$$

Equation 5 The dry weight (DW) formula was used to calculate the biomass concentration (DW) in a sample volume (x). A filter would be weighed before (w_1) and after (w_2) filtration of sample.

3.5.5 Salinity

Salinity was measured with a Multi 3510 with a TetraCon® 925 sensor. The sensor was placed in the sample and the ppt-value was noted when the value was stable.

3.5.6 pH

The pH of the samples in Batch 1 and 2 was measured with the PHM210 Standard pH meter from MeterLab®. The volume of the Batch 3 and 4 WS samples were small and muddy. The pH of these samples was therefore measured with pH-paper.

3.5.7 Nutrients

Concentrations of various nutrients were analysed with the Photometer PF-12^{Plus} Photometer PF-12Plus and VISOCOLOR ECO® or NANOCOLOR® tube tests. All the kits have individual specific ranges of detection. Samples with nutrient concentrations that were assumed or confirmed to be outside of a given range were diluted with RO water or SW. Waste sample and test specific reagents were added to a 16 mm glass cuvette as described in the kit instructions. Upon analysis, a three-digit test specific code was entered on the photometer and the chosen test was displayed on the screen. The glass cuvette was wiped with a microfiber cloth and placed in the cuvette slot in the photometer. The various test presented in Table 6 and the instructions can be found at the URL of the listed source.

Table 6 NANOCOLOR and VISOCOLOR kits used to analyse WS samples for various nutrients and chlorine, indicating the range of detection and suitability for seawater (SW) samples. The kit instructions can be found in the listed source.

Name	Parameter	Range [mg/L]	SW	Instructions
NANOCOLOR Ammonium 3	NH_4-N	0.04 – 2.30	1:1 diluted	(MACHERY-NAGEL, no date a)
NANOCOLOR total-Nitrogen TN_b 22	N	0.5-22.0	no	(MACHERY-NAGEL, no date c)
NANOCOLOR ortho- and total-Phosphate 1	P	0.05-1.50	yes	(MACHERY-NAGEL, no date b)
VISOCOLOR ECO Phosphate	PO_4-P	0.2-5.0	yes	(MACHERY-NAGEL, no date f)
VISOCOLOR ECO Nitrate	NO_3-N	1-14	yes	(MACHERY-NAGEL, no date e)
VISOCOLRO ECO Chlorine 2 free and total	Cl_2	0.05 – 2	yes	(MACHERY-NAGEL, no date d)

4 Results

First, the nutrient content of the WS samples was analysed as a basis for medium design. The results are presented in paragraph 4.1. Further, the samples were tested as growth media for microalgae at three different scales: well plate, bubble column and photobioreactor. The aim of the well plates experiments was to determine if the microalgae could grow on the received samples (with/without manipulations such as autoclaving, nutrient addition, sterile filtration, and LEITAT pre-treatment). The aim of the bubble column and photobioreactor experiments was to determine and compare nutrient conversion efficiency, total biomass production and fatty acid content to respective control media. The effects of different nutritional additions (micronutrients and P) were additionally investigated in the bubble column experiment. The results from the various experiments will further be presented in respective chapters.

4.1 Nutritional analysis

The results from the various nutritional analyses, pH and salinity are displayed in Table 7. Samples that came from the same RASs are colour coded to ease comparison, especially of corresponding treated (batch 3 and 4) and direct (batch 1) samples. The samples with the same background colour (grey excluded) came from the same RAS, while different shades of this colour indicate different sample types. Further, the colour coded samples with white font came from fish fed with conventional feed, while black font indicate experimental feed. A table of the average and standard deviation of each "group" can be found in Appendix A:Table 9. There were some minor differences in the samples before/after autoclaving. The salinities of the freshwater (AB9_CS excluded) and saltwater WS samples were 2.2 ± 0.65 and 36.7 ppt, respectively. The FW sample AB9_CS had a salinity of 6.3 ppt. The pH varied from 6-9, with generally higher values for samples in batch 1 (8.59 ± 0.44) and batch 4 (7.50 ± 1.41, analysed with pH-paper) compared to Batch 2 (7.0) and 3 (6.42 ± 0.29, analysed with pH-paper).

Except for AB1_O, the total N (TN) concentrations in the direct sludge and outlet water samples (from batches 1 and 2) were generally similar with 3.71 ± 0.26 mM N and 3.66 ± 0.40 mM N, receptively. The direct outlet water sample, AB1_O, had an TN concentration almost four times higher (~13.77 mM N) than the other direct samples. In batches 1 and 2, the N concentration was estimated based on analysis of ammonium and nitrate. It should be noted that the nitrate kit used in batch 1 had expired, which was only noticed some weeks later. In all the direct samples, ammonium amounted to a small part of the TN concentration compared to nitrate

(batch 1: 0.07 ± 0.05 mM NH_4+, 5.01 ± 3.69 mM NO_3-, batch 2: 0.08 mM NH_4+, 4.19 mM NO_3-).

The total P (TP) concentrations were similar in the direct outlet samples (AB1_O excluded) (0.03 ± 0.01 mM P). TP was not analysed in the direct outlet sample from batch 2 (AB2_O). The orthophosphate concentrations in the direct outlet samples (AB1_O excluded) from batches 1 and 2 were similar (0.11 ± 0.02 mM PO_4-). The direct sludge samples and the outlet sample AB1_O, had TP concentrations of 0.19 ± 0.01 mM P and ~0.31 mM P, respectively. The orthophosphate concentration in the direct sludge samples was 0.18 ± 0.02 mM PO_4-, while the AB2_O sample had a concentration of ~0.27 mM PO_4-. In some cases, the measured orthophosphate concentration exceeded the TP concentration. It should be noted that the orthophosphate kit used in Batch 1 had expired, as was only noticed afterwards.

The average TN concentration in the batches 1 and 2 (AB1_O excluded) samples (3.63 ± 0.32 mM N) should be able to support an algal biomass of approximately 0.817 mg/mL, assuming algal biomass has the chemical formula of $C_{106}H_{263}O_{110}N_{16}P$ (Stumm and Morgan, 1996). The average TP concentration in the batch 1 (AB1_O excluded) outlet samples (0.03 ± 0.01 mM P) should be able to support an algal biomass of approximately 0.106 mg/mL, while the orthophosphate concentration in the AB2_O outlet sample (0.16 mM PO_4-) should be able to support a biomass of approximately 0.569 mg/mL. Further, the average TP concentration in the direct sludge samples (0.19 ± 0.01 mM P) should be able to support an algal biomass of approximately 0.675 mg/mL. The AB1_O sample had TN (~13.77 mM N) and TP (~0.31 mM P) concentrations that should be able to support an algal biomass of approximately 3.058 and 1.101 mg/mL, respectively.

In batch 3 the TN and TP analyses were conducted by LEITAT, while nitrate was analysed in the current research with an expired kit. The various analyses conducted on the batch 4 samples were conducted in the current study. The TN and nitrate concentrations in the treated samples (CS and sludge in batch 3 and 4) varied broadly with 27.44 ± 29.23 mM N and 1.71 ± 2.46 NO_3-. Ammonium was only analysed in the batch 4 samples, and the concentration was low (undetected – 0.02 mM NH_4+). The TP concentrations in batch 3 varied broadly (0.70 ± 0.63 mM P), and so did the orthophosphate concentrations in batch 4 (0.58 ± 0.55 mM PO_4-).

Table 7 Results of the nutritional analyses of the samples in Batch 1-4. Batch 1:direct samples where the total nitrogen concentration was based on ammonium and nitrate. Nitrate and orthophosphate were analysed with expired kits. Batch 2: direct sample where the nitrogen concentration was based on ammonium and nitrate. Nitrate and orthophosphate were analysed with new kits. Batch 3: treated samples were nitrogen and phosphate were analysed by LEITAT. Nitrate was analysed with an expired kit. Batch 4: treated samples where nitrogen was analysed with a total nitrogen kit. Abbreviations: AA = Aller Aqua, AB = AquaBioTech, O = outlet water, S = sludge, CS = concentrated sludge, .A = autoclaved, n.a. = not analysed.
Explanations for colour coded rows: Orange = samples coming from RAS5, Blue = RAS3, Green = RAS1. Different shade on background colour = different sample type, white letter = conventional feed, black letters = experimental feed

Batch	ID	Salinity [ppt]	pH	Tot N [mM]	NH_4^+ [mM]	NO_3^- [mM]	PO_4^- [mM]	tot P [mM]
1	AA1_O	2.9	7.8	3.65	0.03	3.61	0.10	0.02
	AA1_O.A	3.0	8.8	3.20	0.04	3.16	0.12	0.05
	AA2_O	2.9	7.9	3.61	0.03	3.58	0.10	0.04
	AA2_O.A	2.9	8.8	3.34	0.03	3.31	0.12	0.03
	AA3_S	2.8	8.5	4.05	0.16	3.89	0.17	0.19
	AA3_S.A	2.9	9.1	3.41	0.12	3.29	0.19	0.20
	AA4_O	2.7	8.8	4.20	0.03	4.18	0.09	0.03
	AA4_O.A	2.7	9.0	3.24	0.03	3.21	0.11	0.03
	AA5_S	2.8	8.2	3.67	0.17	3.50	0.15	0.17
	AA5_S.A	2.9	9.1	3.73	0.10	3.63	0.19	0.19
	AA6_O	2.7	8.5	3.93	0.03	3.90	0.08	0.04
	AA6_O.A	2.7	9.0	3.50	0.04	3.47	0.11	0.04
	AB1_O	2.2	8.2	14.03	0.09	13.94	0.23	0.26
	AB1_O.A	2.3	8.5	13.52	0.10	13.42	0.32	0.35
2	AB2_O	36.7	7.0	4.27	0.08	4.19	0.16	n.a.

↓↓LEITAT↓↓

Batch	ID	Salinity [ppt]	pH	Tot N [mM]	NH_4^+ [mM]	NO_3^- [mM]	PO_4^- [mM]	tot P [mM]
3	AA7_S	1.8	6	11.00	n.a.	0.93	n.a.	0.27
	AA8_S	2.5	6	12.93	n.a.	0.03	n.a.	0.61
	AA9_S	1.3	6-7	11.21	n.a.	-	n.a.	0.18
	AA10_S	1.4	6-7	13.86	n.a.	0.09	n.a.	0.14
	AA11_S	1.2	6-7	2.14	n.a.	0.98	n.a.	0.11
	AA12_S	2.1	6-7	5.93	n.a.	2.21	n.a.	0.43
	AB3_S	1.6	7	12.86	n.a.	5.77	n.a.	0.27
	AB4_CS	1.6	6-7	84.29	n.a.	-	n.a.	1.39
	AB5_CS	1.6	6-7	53.64	n.a.	-	n.a.	1.04
	AB6_CS	1.1	6	66.50	n.a.	-	n.a.	1.57
	AB7_O	1.4	6-7	n.a.	n.a.	7.06	n.a.	n.a.
	AB8_O	2.6	6-7	n.a.	n.a.	3.39	n.a.	n.a.
4	AB9_CS	6.3	8-9	4.69	0.02	0.62	0.97	n.a.
	AB10_CS	1.6	6-7	96.58	-	5.90	0.19	n.a.

4.2 Well Plate

The Well Plate experiments were conducted to evaluate whether the different WSs could be suitable as growth media for algae.

4.2.1 Method accuracy and reproducibility

A dilution series was performed to test the linear OD range in the HIDEX (Picture 1, Appendix B:Figure 20). The results showed that cultures with an OD_{750} lower than 0.2 or higher than 0.6 did not give an accurate correlation with biomass concentration. Therefore, the Well Plates experiments were terminated when $OD_{750} > 0.6$ for most samples.

Picture 1 Dilution series conducted to determine the range for accurate OD measurements in the Hidex

Throughout the experiments it was observed occasionally incoherence between the visual observations and recorded OD values. As an example, the growth curves (based on OD_{750}) of *Chlorella vulgaris* in AB7_O and BBM in WP3 are displayed in Figure 8 along with the day 14 visual control depicted on the left. By visual control, the algae density in the AB7_O wells (top picture) seemed lower than the BBM wells (bottom picture). At the same time, the average OD_{750} values recorded were 0.615 and 0.425 for AB7_O and BBM, respectively. This illustrates a disagreement between the visual controls and the recorded OD values. Uneven algae growth within a well was observed regularly throughout the WP experiments (to the side, concentrated in the middle, absent in the middle), and one or more air bubbles would also often appear in the wells. This could have contributed to inaccurate OD values.

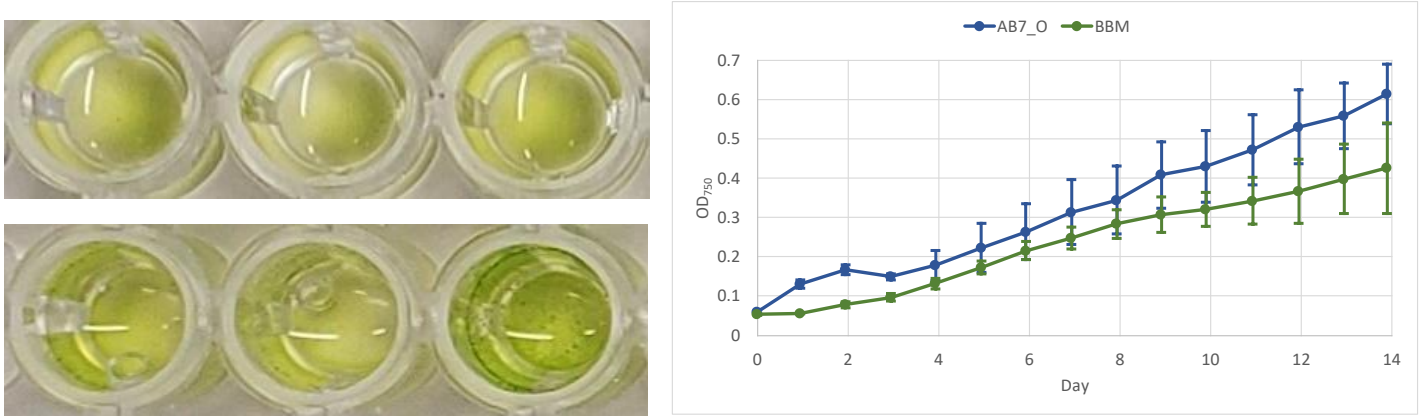

Figure 8 Left: The wells are pictured on day 14 of WP3. The top and bottom wells contain AB7_O and BBM, respectively. The graph shows the OD_{750} values with standard deviation recorded n throughout the experiment. The AB7_O and BBM wells had a final average OD_{750}= 0.615 and OD_{750}= 0.425, respectively, based on the three wells.

The reproducibility of the WP experiments was tested in WP6. This was conducted to get a better understanding of how sterile filtration could affect the samples suitability as growth media, and to investigate whether the same results would be attained when testing at a different time and with different degrees of filtration. The sterile filtration had no effect on the AB7_O media as all wells showed algae growth. The three-month-old BBM however only had algae growth when sterile filtrated (both 0.2 and 0.45µm). The 0.45µm sterile filtrated AB1_O medium had no algae growth, while the 0.2µm filtrated and original AB1_O did. As the results demonstrated uncertainty concerning the OD analysis and the WP method, it was decided that only binary results (growth (G) or no growth/failure (F)) could be acceptable as noted outcomes.

4.2.2 WP1-WP6 results

The binary results, growth/failure (G/F), of the WP experiments are presented in Table 8. These were based on the OD data and the visual controls (if recorded). Various OD_{750} growth curves, OD_{680} to OD_{750} ratios, standard deviations (WP2-6), and final visual controls (WP2-6) can be found in Appendix B:. All the unmanipulated samples from Batches 1 and 2 had positive binary outcomes (G), except AA5_S (F). Growth was only registered in AA5_S after manipulation by autoclaving. Manipulation by N:P ratio adjustments (increasing P concentration) did not change any failure results (F) to growth results (G) (possibly negative effect of P addition in some media, but not consistent for all). Only 5 of the 12 batch 3 samples had algae growth. Sterile filtration did not endorse algae growth in any media. Algae only grew in AB3_S and AB7_O with and without the addition of APN, respectively. The binary outcomes for the WSs in batch 4 were based on the OD measurements alone because the pigmentation of the samples made algae growth undetectable by visual control (Appendix B: Picture 5). The final OD analysis indicated algae growth in both samples. It should be noted that the negative control in this WP experiment had a positive binary outcome (see Appendix B: Figure 43 and Picture 5). The binary results for all the samples are summarised in Table 8.

Table 8 Overview of the binary results from the Well Plate experiments based on visual control and OD. All WSs were tested with and without the addition of micronutrients. Moreover, some samples were autoclaved, others were sterile-filtrated (white cells in the three columns "original, autoclaved, and filtered" indicate "not analysed". The Batch 1 and 2 samples are direct samples from the RAS, while Batch 3 and 4 were first treated by enzymatic/chemical hydrolysis at LEITAT. The binary outcome G means that algae growth was registered in the WS with and without the addition of micronutrients (green cells). The binary outcome F means that no algae growth was registered neither with nor without the addition of micronutrients (red cells). Cases where manipulation by addition of micronutrients affected the binary outcome or where other issues should be noted are marked with a footnote: [1]only algae growth with APN, [2] only clear growth without APN, [3]uneven growth, no growth registered in OD, [4]AB1_O 0.45 µm filtrated did not have growth but 0.2 µm did.

Abbreviations: AA = Aller Aqua, AB= AquaBioTech, O = outlet water, S = sludge, CS = concentrated sludge

Batch	ID	original	autoclaved	filtered
1	**AA1_O**	G	G	
	AA2_O	G	G	
	AA3_S	G	G	
	AA4_O	G	G	
	AA5_S	F	G	
	AA6_O	G	G	
	AB1_O	G	G	F[4]
2	**AB2_O**	G		
3	**AA7_S**	F		F
	AA8_S	F		F
	AA9_S	F		F
	AA10_S	F		F
	AA11_S	G		G
	AA12_S	G		G[3]
	AB3_S	G[1]		F
	AB4_CS	F		F
	AB5_CS	F		F
	AB6_CS	F		F
	AB7_O	G[2]		G
	AB8_O	G		G
4	**AB9_CS**			G
	AB10_CS			G

4.3 Bubble column

The bubble column experiment was conducted to determine how different manipulations of nutrient levels in an AB1_O (further referred to as AB1) based medium would affect the growth of *C. vulgaris* in terms of biomass concentration reached, total nutrient conversion efficiency, and fatty acid content. AB1 was selected for the bubble column experiment because it was suitable as a growth medium for *C. vulgaris* in WP1. It was also selected because it was the largest sample in volume, and it could be used four times diluted because of its high nitrogen concentration. Three different versions of the AB1 WS were tested: the original (O), the original with the addition of micronutrients (Oμ), and with the addition of micronutrients and equal N:P-ratio to BBM by adding P (Bμ). BBM was used as a positive control.

The growth curves based on OD_{750} are presented with standard deviation in Figure 9, and the sampling points for dry weight and fatty acids, S1 and S2, are highlighted. All the media had a start OD_{750} of approximately 0,1. The specific growth rates (days 6-9) for O, Oμ, Bμ, and BBM were 0.56, 0.60, 0.67, and 0.73 d^{-1}, respectively. All media had the same initial N-concentration of (2.94 mM N) which should be able to support an algal biomass of approximately 0.653 mg/mL, assuming algal biomass has the chemical formula of $C_{106}H_{263}O_{110}N_{16}P$ (Stumm and Morgan, 1996).

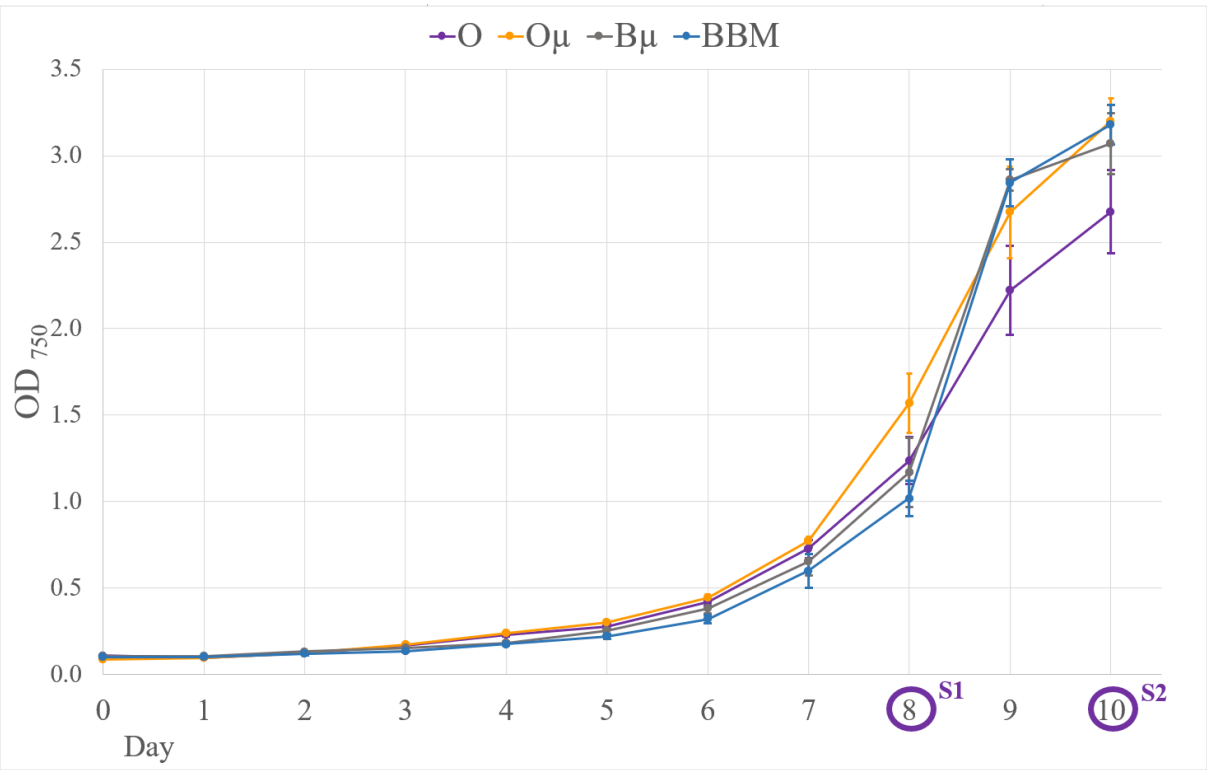

Figure 9 OD_{750} growth curves with standard deviation based on biological triplicates in the Bubble column experiment. Media : O = original AB1, Oμ = original AB1 with APN, Bμ = AB1 with N:P ratio equal BBM and APN, BBM = Bold's Basal medium/Control. S1 was the sampling point for the mid growth phase, S2 was the sampling point for the stationary phase

The O and Oμ P-concentration of 0.07 mM should be able to support an algal biomass of 0.09 mg/mL, while the Bμ and BBM P-concentration (1.72 mM) should be able to support an algal biomass concentration of 6.11 mg/mL. The dry weight at mid-growth (S1) and stationary phase (S2) is presented in Figure 10. The final biomass concentration (day 10) was similar for all the media with averages of 0.75, 0.84, 0.84, and 0.87 mg/mL for O, Oμ, Bμ, and BBM, respectively. The only a significant difference in OD_{750} was between O and Oμ on day 10 with a p-value of 0.030, while for dry weight the only statistical significant difference was between O and BBM at the stationary stage, with a p-value of 0.043. There were no other statistically significant differences ($p > 0.05$).

The Quantum Yield (QY) was measured daily and is presented in Figure 11. The QY was relatively stable and above 7 for day 1-8. It was a bit lower on day 0 (0.6 – 0.65). The QY started to drop for all the media on day 9, however generally more for both the media with original AB1 N:P ratio, O and Oμ. At day 10 the QY varied from approximately 0.5 to a 0.62.

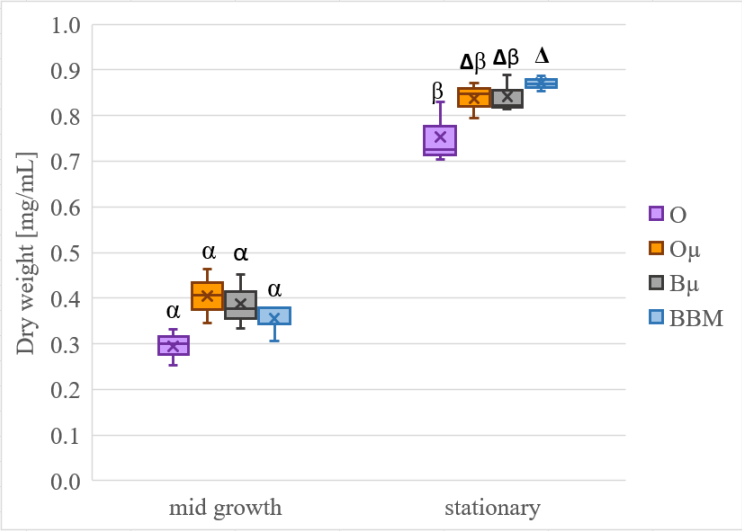

Figure 10 The dry weight was analysed at the mid growth and stationary phase for the O, Oμ, Bμ, and BBM medium in the Bubble column experiment. The data is presented in a box plot with mean value (X), average (horizontal line), interquartile (box), and standard deviation of the averages of biological triplicates. The only statistically significant difference was between O and BBM at the stationary phase (p=0,043), indicated by letters β and Δ. Media : O = original AB1, Oμ = original AB1 with APN, Bμ = AB1 with N:P ratio equal BBM and APN, BBM = Bold's Basal medium/Control.

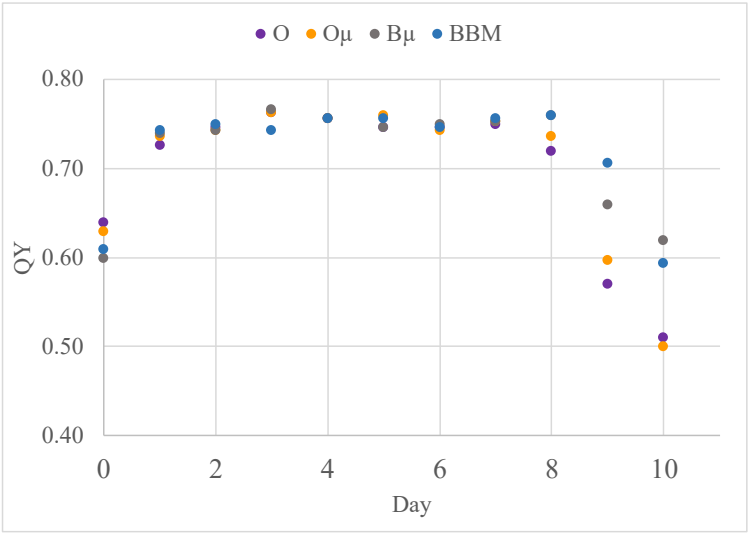

Figure 11 The QY was registered daily in all media in the Bubble column experiment. Media: O = original AB1, Oμ = original AB1 with APN, Bμ = AB1 with N:P ratio equal BBM and APN, BBM = Bold's Basal medium/Control

Samples for fatty acid analysis were collected at the mid-growth phase (S1 in Figure 9) and the results are presented in Figure 12. Compared to Bμ and BBM, the WS media with original N:P ratio (and lower P content), O and Oμ, generally had a higher total fatty acid (TFA) content per biomass (Figure 12). Specifically, they had more of the saturated FAs (SFA) 18:0 and 16:0, and more of the monounsaturated FA (MUFA) 18:1 n-9. O and Oμ also had a bit less of the polyunsaturated FAs (PUFA) 18:3 n-3, and 16:3 n-3, while other PUFAs as 16:2 n-6 and 18:2 n-6 were approximately equal to Bμ and BBM. The relative fatty acid content is presented in Appendix C:Figure 48.

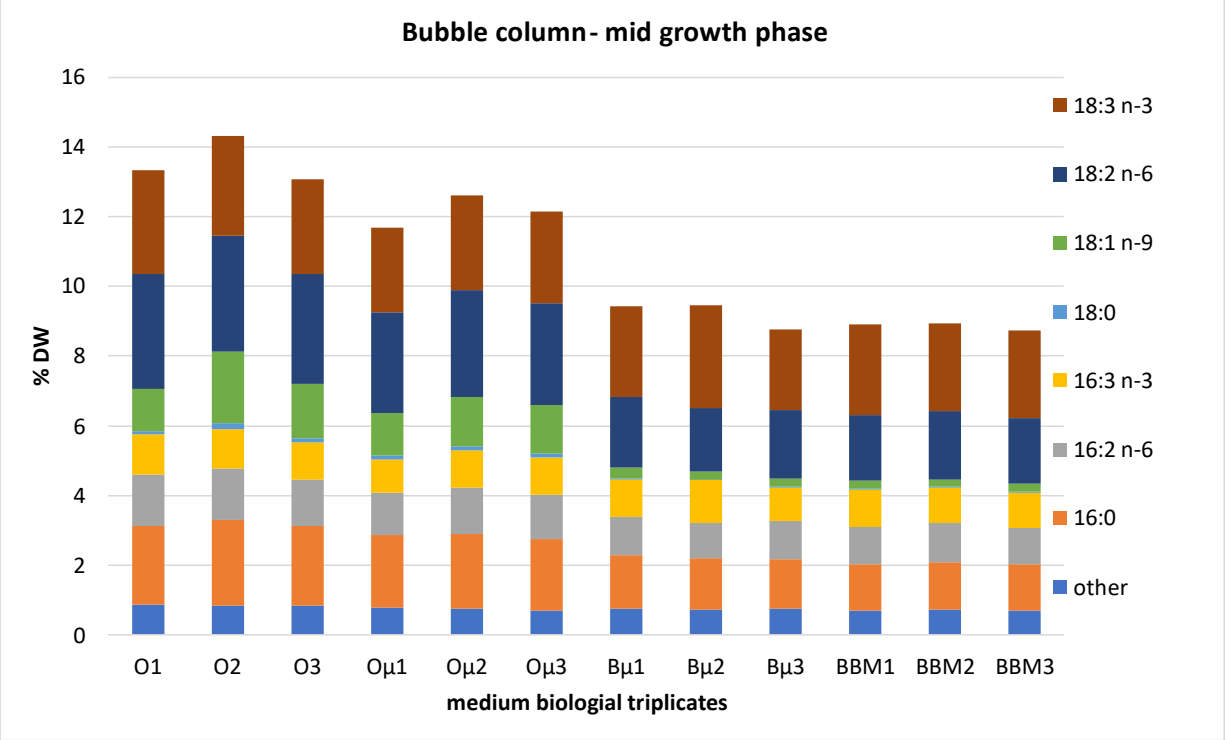

Figure 12 The fatty acid content of the various media in the bubble column experiment was analysed at mid growth stage. Samples from each biological triplicate was analysed (1-3). The quantities are presented as % of dry weight (DW). O = original waste stream, μ = addition of micronutrients, B = waste stream with N:P ratio equal BBM, BBM = Bold's Basal Medium/control. The different colours indicate the different fatty acids as shown in the figure legend. Branched alcohol and unknown fatty acids have been combined under "other".

Samples for nutrient analyses of the various media were collected before addition of algae, at the mid-growth (S1 Figure 9), and the stationary phase (S2 Figure 9). The samples from the biological triplicates were mixed before the analyses. The results from the TN and nitrate analysis are presented in Figure 14. Generally, the results indicate a slightly higher concentration of nitrate than nitrogen. It should again be noted that the nitrate analyses were conducted with an expired kit, which was only noticed some weeks later. As intended, all the

media had approx. equal N concentrations at the medium sampling point (3.23 +/- 0.10 mM N and 3.48 +/- 0.21 mM NO_3-). At the mid-growth sampling point, all the media had used much of the TN and nitrate available. The average concentrations in O, Oμ, and BBM were 0.68 ±0.08 mM N and 0.57 +/- 0.26 mM NO_3-. The concentrations were noticeably lower in the Bμ medium with 0.36 mM N and 0.06 mM NO_3-. Further, no nitrate could be detected at the stationary stage in any media (<0.07 mM NO_3-) and only very low amounts of TN were still found. The results from the orthophosphate analysis are presented in Figure 13. The initial P concentration in O, Oμ, Bμ and BBM was 0.04, 0.02, 1.61 and 1.61 mM PO_4-, respectively. Again, it should also be noted that the orthophosphate kit had expired. Throughout the various sampling points, the PO_4- concentration was substantially higher in the Bμ and BBM medium compared to O and Oμ. Although present at relatively low concentrations in O and Oμ (0.01 – 0.04 mM PO_4-) orthophosphate was never undetectable in any of the phases. However, the concentration at the mid-growth phase (0.02 mM PO_4- for O and Oμ) was almost identical to that of the stationary phase (0.02 and 0.01 mM PO_4- for O and Oμ, respectively). The P concentrations in Bμ and BBM were descending throughout the various sampling points and was almost identical between the media.

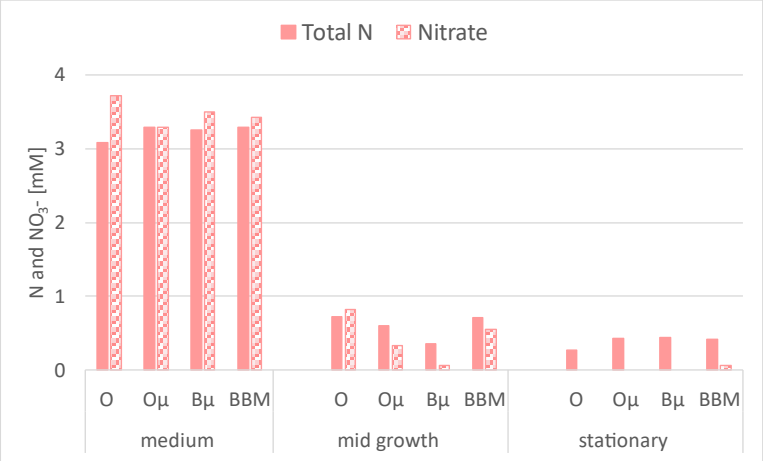

Figure 14 In the bubble column experiment the nitrate and nitrogen concentration in the various media (O, Oμ, Bμ, BBM) were analysed before the algae were added (medium), at the mid growth and stationary phase. The samples from the biological triplicates were mixed before the analyses.

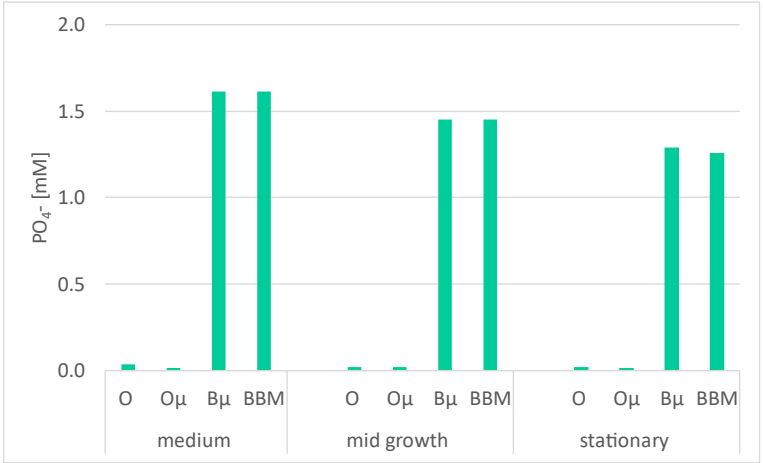

Figure 13 In the bubble column experiment the orthophosphate concentration in the various media (O, Oμ, Bμ, BBM) was analysed before the algae were added (medium), at the mid growth and stationary phase. The samples from the biological triplicates were mixed before the analyses.

4.4 Photobioreactor

The photobioreactor (PBR) experiment was conducted to test aquaculture wastewater as nutrient source for *M. gaditana* in a proof-of-concept at semi pilot-scale. The AB2_O outlet water (further referred to as AB2) from Batch 2 was intended for this experiment as it was the only WS with a big enough sample size. Prior to the PBR experiment, growth of *M. gaditana* in AB2 was tested in WP2 were it got a positive (G) binary result. In the PBR experiment, AB2 was used undiluted with the addition of micronutrients. NORCE medium was used as a positive control in a second PBR run in parallel.

The growth curves based on OD_{750} and the measured nitrate concentration in AB2 are presented in Figure 15. The initial nitrate concentration measured in the medium was 2.63 mM NO_3-. The dry weight of the culture at the beginning (Day 0), at mid-growth (S1), and at the stationary phase is presented in Figure 17. The stationary samples for the two cultures were taken at different days as seen in Figure 15 (S2a and S2b). The data from the QY analysis are presented in Figure 16. Both cultures started with an OD_{750} of about 0.3 and approximately equal dry weights (~ 0.19 and 0.14 mg/mL for AB2 and NORCE, respectively, beginning, Figure 17). The cultures had near identical growth curves until day 5. In this period (day 0-5), the specific growth rate for AB2 and NORCE was 0.59 and 0.55 d^{-1}, respectively. The biomass

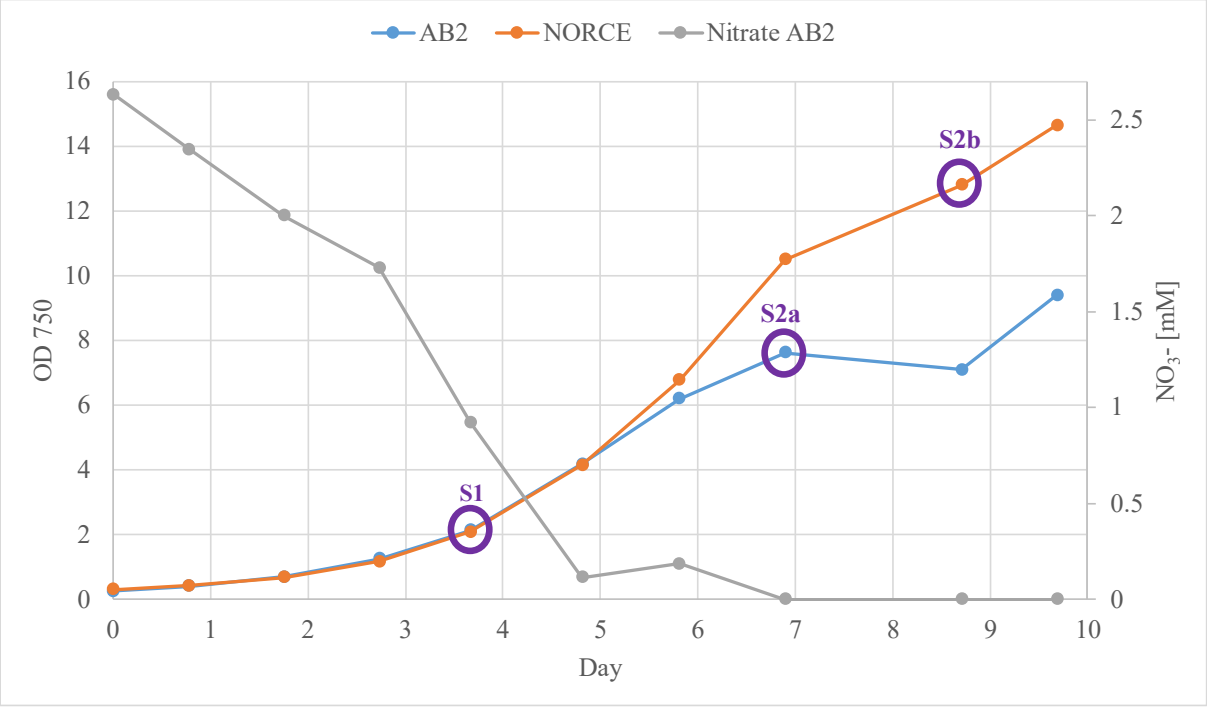

Figure 15 Growth curves for AB2 and NORCE (based on OD_{750}) during the photobioreactor experiment. S1 is the mid growth phase sampling point for both mediums. S2a and S2b is the stationary sampling point for AB2 and NORCE, respectively. The nitrate concentration in the AB2 medium is also included in the figure. The nitrate concentration for NORCE was undetectable throughout the experiment because too much thiosulphate was added (explained in methods).

concentration at day 4 (Figure 17, mid-growth phase) was 0.67 and 0.60 mg/mL for AB2 and NORCE, respectively. Only approximately 1/3 of the nitrate content remained in AB2 at day 4, and the culture had entered the stationary phase by day 7, at which point nitrate was undetectable by analysis. Thus, day 7 was the stationary sampling point for the AB2 medium. Although nitrate never was detected in the NORCE medium because of thiosulfate, it was known that it had an initial nitrate concentration over 6 times the concentration of AB2. The timing of the stationary sampling point for the NORCE media was therefore based on QY and OD_{750}. On day 9, the QY had decreased slightly from the previous measurement (see Figure 16) and the OD_{750} growth curve was no longer exponential (Figure 15). Further, the colour of the medium had started to change from green to a yellowish brown. These observations were all interpreted as indications of growth limitations due to nutrient deficiency. Thus, day 9 was the stationary sampling point for the NORCE medium. The initial nitrate concentration measured in the AB2 medium (~2.63 mM NO_3-) should be able to support an algal biomass of 0.58 mg/mL, assuming algal biomass has the chemical formula of $C_{106}H_{263}O_{110}N_{16}P$ (Stumm and Morgan, 1996). At the stationary sampling point, the average biomass concentration of the AB2 and NORCE medium was 2.36 and 4.81 mg/mL (stationary, Figure 17). The final algae biomass obtained was 2.17 mg/mL in AB2 and 4.67 mg/mL in NORCE. The AB2 culture had a drop in QY on day 2 (QY = 0.62), apart from this it was quite stable around 7 from day 0-5. The QY dropped from day 6 and was at its lowest point on day 9 at 0.52. It increased slightly at day 10 to 0.55. The NORCE culture had a stable QY around 7 from day 0-7 before decreasing slightly to 0.63 on day 10.

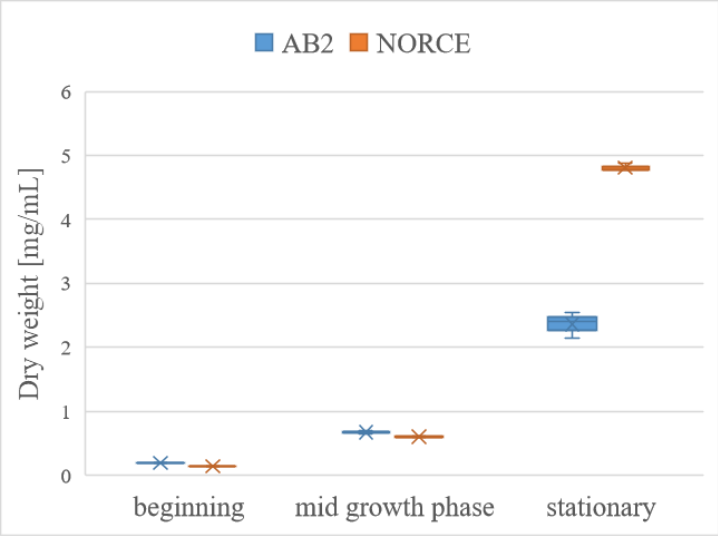

Figure 17 The dry weight was analysed at the beginning, mid-growth, and stationary phase for the AB2 and NORCE medium in the PBR experiment. The data is presented in a box plot with mean value (X), average (horizontal line), interquartile (box), and standard deviation of technical triplicates.

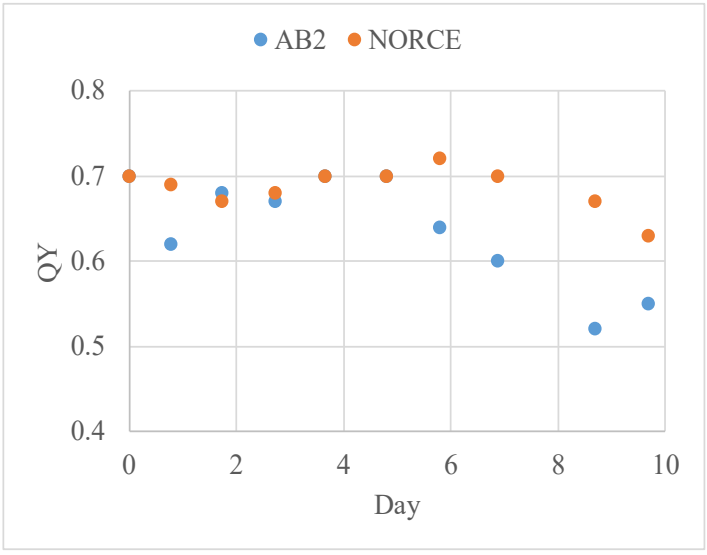

Figure 16 The Quantum Yield (QY) for the AB2 and the NORCE medium was measured throughout the experiment.

Samples for FAs analysis were collected at the mid-growth and stationary phase (S1, S2a, and S2b in Figure 15, respectively). The average results of technical duplicates are presented in Figure 19. There was an increase in TFA content per biomass in AB2 at stationary phase compared to mid-growth stage. The TFA per biomass decreased in the NORCE medium from the mid-growth to the stationary phase. The relative fatty acid contents are presented in Figure 18. From mid-growth to the stationary phase, both media had a slight increase in 18:2 n-6 (PUFA) and a more prominent increase in 18:1 n-9 (MUFA). Both media also had a decrease in the omega-2 FA 20:5 n-3. Further, AB2 had an increase in 20:3 n-6 (PUFA), 18:0 and 16:0 (SFA), and 18:1 n-7 (MUFA).

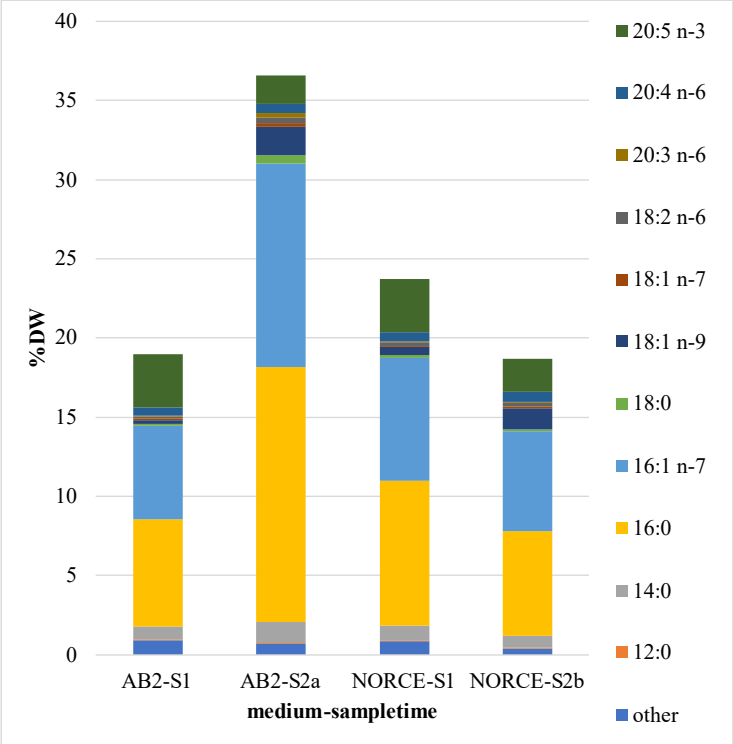

Figure 19 The total fatty acid (FA) content of M. gaditana on the various media in the PBR experiment was analysed at mid growth phase (S1) and at the stationary phase (S2a or S2b). The quantities are presented as % of dry weight (DW). Branched alcohol, unknown FA, and FA that were <0.2 % of DW have been combined under "other".

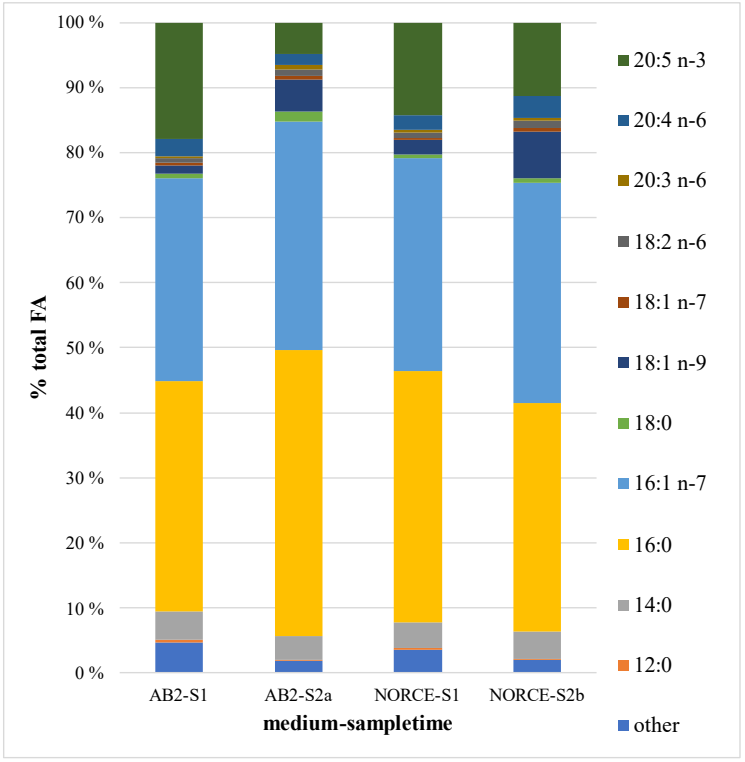

Figure 18 The relative fatty acid content in M. gaditana grown on the various media in the PBR experiment was analysed at mid growth phase (S1) and at the stationary phase (S2a and S2b). The quantities are presented as % of total fatty acid content. Branched alcohol, unknown FA, and FA that were <0.2 % of DW have been combined under "other".

5 Discussion

Fish pollute their surrounding waters with faeces, carbon dioxide and TAN. In RASs, soluble particles are removed from the circulating waters and TAN is converted to nitrate in biofilters. These processes result in different waste streams, mainly outlet water and sludge. This study aimed to investigate whether these waste streams could function as growth media for microalgae. This was accomplished by answering a series of questions:

1. What is the nutritional content of the direct WSs, and how do they compare to standard growth media?
2. How did the nutritional content in the LEITAT-treated WSs compare to the direct WSs?
3. Can microalgae grow on these various WSs on a small scale, and can manipulations like autoclaving, sterile filtrating, micronutrient and/or P addition change this?
4. How does the addition of micronutrients and P affect the algae growth in the bubble column experiment?
5. How does the algae growth rate in WS medium compare to a control on a larger scale?

5.1 Nutritional content of direct WS samples

According to literature, about 30 elements are required in a growth medium for microalgae (Procházková *et al.*, 2014). The micro nutritional requirements for algae growth can be complex and influenced by the availability of macronutrients (Procházková *et al.*, 2014). The analyses conducted in the current paper only allow evaluation of the N and P macronutrient concentrations. Next to carbon dioxide, these two elements have the most impact on the growth medium costs (Ruiz *et al.*, 2016). The nutritional contents of the WS samples are presented in Table 7, and the averages and standard deviation of the various "groups" can be found in Appendix A:Table 9.

All the direct outlet water WS samples (AB1_O excluded) had elevated but similar TN concentrations (3.66 ± 0.40 mM N) to standard BBM (2.94 mM N). Nitrate, a form of nitrogen readily available for microalgae (Becker *et al.*, 2013), comprised most of the TN concertation (3.62 ± 0.39 mM NO_3-). The direct outlet samples had approximately 2% (0.03 ± 0.01 mM P) of the BBM TP concentration (1.72 mM P). The AB1_O outlet water WS sample had almost four times the nitrate concentration (~13.77 mM NO_3-) and approximately ten times the TP concertation (~0.31 mM PO_4-) of the other direct outlet water samples (Table 7). This nitrate concentration would expose the cultivated rainbow trout to a high risk of health impacts as it is

over two times the advised upper limit (approximately 5 mM NO_3-) (Davidson *et al.*, 2014). No reports of reduced fish health were received; therefore, it is assumed that the concentration was much lower in the fish tanks. AB1_O was the only sample collected from the ABT system with swirl separators. As described in the introduction, the nutritional content can vary depending on many factors, especially related to the operations of the RAS. Therefore, it could be assumed that the separator or the following filtration steps resulted in elevated NO_3- and PO_4- concentrations relative to the rest of the production water and relative to the other outlet water samples. The nutritional content of reject water will generally be noticeably higher than outlet water (Bregnballe, 2015). Therefore, it seems like the sampling method and filtration steps of the AB1_O sample made its qualities more similar to reject water than outlet water.

As mentioned in the introduction, because P is mainly excreted from fish in particulate form and N is mainly dissolved, sludge commonly contains a larger fraction of the TP and a smaller fraction of TN than outlet water (Bregnballe, 2015). As expected, the direct sludge samples, AA3_S and AA5_S, had higher TP concentrations (0.19 ± 0.01 mM P) than the corresponding (from the same RAS with the same feed) direct outlet water samples AA4_O and AA6_O, respectively (0.03 ± 0.01 mM P). However, the TN concentrations in the direct sludge samples (3.71 ± 0.26 mM N) were approximately equal to the concentration in the corresponding direct outlet water samples (3.72 ± 0.43 mM N). Further, the orthophosphate and nitrate concentrations of the direct sludge samples (0.18 ± 0.02 mM PO_4-, 3.58 ± 0.25 mM NO_3-) were also similar to the direct corresponding outlet water samples (0.10 ± 0.02 mM PO_4-, 3.69 ± 0.43 mM NO_3-), while the ammonium concentrations (0.14 ± 0.03 mM NH_4+) were higher in sludge than outlet samples (0.03 ± 0.00 mM NH_4+). This implies that the main difference between the sludge and outlet water samples was particulate P and the dissolved nutrient composition was roughly the same, except for ammonium. Compared to BBM, the direct sludge samples had approximately 11% of the BBM P concentration (1.71 mM P) and, as the direct outlet samples, a bit elevated but similar nitrate concentrations to BBM.

The broad variation of RAS setups makes it challenging to compare the nutrient concentrations in the various WS samples to samples analysed in other studies. The direct outlet samples (AB1_O excluded) had similar TN, TP, and orthophosphate concentrations to outlet water from another RAS (2.64 mM TN, 0.36 mM TP, 0.12 mM PO_4-), but higher nitrate (>5 times) and lower ammonium (<13%) concentrations (0.71 mM NO_3-, 0.31 mM NH_4+) (Cabell *et al.*, 2019). The difference in nitrate to ammonium ratio could be explained by the biofilters in the

respective RASs having different nitrification efficiencies. Moreover, the nitrate concentrations in the outlet samples (AB1_O excluded) from the current study (3.62 ± 0.39 mM NO_3-) compare better to the upper nitrate limit advised for RAS with rainbow trout (approximately 5 mM NO_3-) (Davidson *et al.*, 2014). Sludge from the mentioned RAS was also analysed; however, all the nutrient concentrations, except nitrate, were over a hundred times higher (402,29 mM TN, 23.5 mM NH_4, 177.55 mM P, orthophosphate was not analysed) than in the direct sludge samples from the current study. The nitrate concentrations in the sludge samples from the current study were almost 40 times higher than in the sludge sample from the mentioned study (0.08 mM NO_3-) (Cabell *et al.*, 2019). The extensive variations between the sludge samples illustrate that comparing the nutrient concentrations of WSs from different RASs is challenging, especially after various water treatment steps.

Some TP values in batch 1 were slightly lower than the orthophosphate values, which was unexpected as orthophosphate is a part of the TP. It should be noted that the orthophosphate and nitrate kit used in the analyses of this batch (and nitrate in batch 3) had expired, which may have led to inaccurate analyses. Nevertheless, the orthophosphate and TP values, and the TN and nitrate values were similar, and it is assumed that the occasional minor inaccuracies had an overall insignificant impact. It should also be noted that there were some small differences in the nutritional contents of the samples before and after autoclaving (batch 1). As there were no consistencies in these differences, it is thought that they were mainly caused by inaccurate analysis with the expired kits. Therefore, the average (~) of these values (before and after autoclaving) has been used in the discussion. Further, there were no consistent categorical differences in nutritional contents regarding feed type (control vs experimental).

Whereas all samples (both outlet water and sludge) showed sufficiently high N concentrations, most of them were lacking severely in P compared to the standard growth medium BBM. While comparing the nutrient concentrations of WSs to that of BBM can be useful, it is important to note that many standard growth media with different nutrient concentrations and N:P ratios have been developed for different purposes (Becker *et al.*, 2013). BBM, or a modified BBM enriched with three times more nitrate (BBM 3xN), are standard media used in to cultivate many algal species (Becker *et al.*, 2013). The standard BBM has an N:P ratio of 1.72. Generally, this is considered to be a medium where N is limiting for algae growth, especially at high growth rates when more N is needed compared to P per biomass produced (Stumm and Morgan, 1996; Becker *et al.*, 2013). Modified BBM (N:P ratio of 3.4) and the NORCE medium (N:P ratio of

14.2), used as a control in the PBR experiment, were specifically designed to grow algae at high densities. Therefore, they have relatively high nitrate concentrations (NORCE has approximately 4.24 times more N than standard BBM) compared to standard BBM.

5.2 LEITAT nutrient recovery

There is generally a broader range in the nutritional content of the 14 LEITAT-treated samples (batches 3 and 4 in Table 7) compared to the direct samples (batches 1 and 2). The treated outlet water samples, AB7_O and AB8_O, and the direct AB1_O sample were subsamples of the same sample, with only inactivated enzymes added to AB7_O and AB8_O (blanks). Surprisingly, AB7_O and AB8_O had lower nitrate concentrations (7.06 and 3.39 mM NO_3-, respectively) than the AB1_O sample (~13.77 mM NO_3-). However, other nutrient concentrations were not analysed. It should be noted that, in contrast to the direct samples, all the LEITAT samples were sterile filtrated before analyses. However, it is more probable that the nutritional difference originated from the sampling, processing, and storage/shipment procedures. Especially the latter seems plausible since the AB1_O sample was sent directly to Norway, whereas AB7_O and AB8_O originated from an AB1_O replica that was sent to Spain (LEITAT) first.

Further, the nitrate concentrations in the LEITAT-treated sludge samples, AA11_S (0.98 mM NO_3-), AA12_S (2.21 mM NO_3-), and AB3_S (5.77 mM NO_3-), were lower than in the corresponding direct outlet water samples AA2_O (~3.44 mM NO_3-), AA1_O (~3.39 mM NO_3-), and AB1_O (~13.77 mM NO_3-), respectively. As described in the introduction, it is expected that a sludge sample has a lower nitrate concentration than the outlet water. However, the nitrate concentration was also lower in the treated sludge samples, AA7_S (0.93 mM NO_3-) and AA8_S (0.03 mM NO_3-), compared to the corresponding direct sludge samples, AA5_S (~3.56 mM NO_3-) and AA3_S (~3.59 mM NO_3-), respectively. The concentrated sludge (CS) samples from batch 4, AB9_CS and AB10_CS, were treated with enzymatic and chemical hydrolysis, respectively. The enzymatic treatment was dissimilar to the treatments administered on the batch 3 CS samples. Of the CS samples, nitrate was only detectable in AB9_CS (0.62 mM NO_3-) and AB10_CS (5.90 mM NO_3-) from batch 4. Again, the nitrate concentrations were lower than in the corresponding direct outlet sample, AB1_O (~13.77 mM NO_3-). These results could indicate that the nitrate concentration in all the samples had been negatively affected, particularly in the CS samples from batch 3, where no nitrate could be detected. This could be

an effect of the LEITAT treatments; however, as similar nitrate deficiencies were observed in the blanks (AB7_O and AB8_O) also, losses during storage and shipment should be considered.

In contrast to the nitrate concentration, the TN concentration was higher in most of the treated sludge samples compared to the corresponding direct samples (except for AA11_S and AB3_S). As mentioned, all the treated samples were sterile-filtered before analyses. The measured TN (and TP, as discussed below) can thus be assumed to be mainly soluble as the particulate matter had been removed. The batch 3 CS samples and AB10_CS from batch 4 had very high concentrations of TN (75.25 ± 18.97 mM N) relative to all other samples, including the other batch 4 sample, AB9_CS (4.69 mM N). This indicates that some LEITAT treatments, especially the treatment administered on the CS samples in batch 3 and the chemical hydrolysis administered on the AB10_CS sample, successfully liberated N, but in other forms than nitrate. The dominant form of N in the treated samples is unknown as only nitrate N was analysed in the majority of the samples (AB9_CS and AB10_CS had 0.02 and 0 mM NH_4+, respectively). However, based on the use of enzymes, LEITAT mainly expected to liberate peptides and amino acids. More extensive analyses would have been preferred to reveal if this indeed was achieved. However, the availability of the liberated N compounds would be tested in the well plate experiments (discussed in the next chapter).

All the sludge and concentrated sludge samples (except AB3_S) from batch 3 had a higher TP concentration than the corresponding direct outlet water and sludge samples. The AB3_S sample had a TP concentration (0.27 mM P) approximately equal to the corresponding direct original outlet water sample AB1_O (~0.26 mM P) but lower than the autoclaved AB1_O sample (0.35 mM P). The TP concentrations were not analysed in the batch 4 samples. However, based on orthophosphate, the chemically hydrolysed AB10_CS sample was the only CS sample that did not have an elevated P concentration (0.19 mM PO_4-) compared to the corresponding direct outlet water sample, AB1_O (~0.27 mM PO_4-). As described in the introduction and demonstrated in section 5.1, it is indeed expected that sludge contains a higher concentration of P than outlet water. The presented results can therefore imply that some treatments (particularly the treatment administered on AB3_S and perhaps AB10_CS) had a rather negative effect on the TP concentration. At the same time, the treated sludge samples, AA7_S (0.27 mM P), and AA8_S (0.61 mM P) had higher TP concentrations than their corresponding direct sludge samples, AA5_S (~0.18 mM P) and AA3_S (~0.20 mM P), respectively. These results indicate that the enzymatic LEITAT treatment could have had a P

liberating effect; however, it is uncertain as only two treated sludge samples could be compared to direct sludge samples. Unfortunately, orthophosphate was not analysed in batch 3 as it had just been discovered that the kit was expired. It is therefore also unknown what potential P compounds were liberated with the treatment and if it was a form readily available for microalgae.

As mentioned in the introduction, different sample types (outlet water, sludge, concentrated sludge) will generally have different nutrient concentrations. Therefore, the value of comparing a direct outlet water sample with a treated sludge sample is limited. Aside from the blanks, only two LEITAT-treated samples (AA7_S and AA8_S) could be compared to corresponding direct samples of the same sampling type (AA5_S and AA3_S). Even though the basis for comparison is better for these samples, there are still other factors than the treatment itself that could have had unknown effects (ex., sampling and storage/shipment procedures).

The analyses indicate that some nutrients potentially were liberated through the LEITAT treatment. However, presence is one aspect, while bioavailability, which was tested in the following experiments, is another.

5.3 Well plate

As described in the well plate chapter, section 4.2.1, multiple sources of error limit the admissible results to binary: i) the deviations between visual controls and OD, ii) the relatively high standard deviation in OD between biological replicates (max. SD recorded was for the AB9_CS sample in WP5, SD = 0.34, see Appendix B:Table 13), and iii) the very limited range of measurable OD (0.2 - 0.6 OD_{750}) that correlated accurately to biomass increase, making it difficult to set-up the experiment in such a way that different nutrient concentrations would be sufficient to measure growth but limiting before light would become limiting or the cell density too high to determine. Still, the experiments gave a first impression of whether microalgae could grow on the various WS media (see binary results in Table 8). Almost all the direct unmanipulated samples had positive binary results (G). In one case, the presence of APN was necessary for a positive binary result; the same was observed for autoclaving in another case. Half of the unmanipulated batch 3 LEITAT-treated samples had positive binary results. One of these samples had a negative binary result after being sterile filtrated. The batch 4 LEITAT-treated samples only had positive binary results, indicating that the treatments had improved from batch 3. These samples were only tested after being sterile filtrated.

The predictions of algae biomass production based on N and P concentrations (see section 4.1) indicated that P would be the limiting macronutrient in batches 1 and 2 WS media. In a study by Daneshvar *et al.* (2018), *C. vulgaris* was cultivated in BBM 3xN, and the effects of different nutrient levels were investigated. The study found that a low P concentration was a severely limiting factor for C. *vulgaris*, as no growth was registered in BBM with ≤25% of the original P concentration (≤0.43 mM P) (Daneshvar *et al.*, 2018). In the current research however, all the outlet water sample media in WP had algae growth (Table 8) even when tested without additional P, though most of these had orthophosphate concentrations at approximately 6% BBM (0.11 ± 0.02 mM PO_4-) (Table 7). Although this contradicts the findings by Daneshvar *et al.* (2018), it should be noted that it is typical for microalgae to have intracellular storage of phosphate obtained by *luxury uptake* during comfortable growth conditions, i.e. from inoculum production (Levin and Shapiro, 1965). The intracellularly stored P can be sufficient to support algae growth for several generations (Solovchenko *et al.*, 2019). Therefore, it is reasonable to assume that the algae cultures were or were going to be limited by the low P concentrations but that the observed growth was attained by the utilisation of intracellular P stored during the inoculum production phase. Since the results were binary, it was not distinguished between more and less growth. Consequently, the additional P might have led to better growth in the microalgal cultures than those without, but this was imperceptible.

One of the two direct sludge samples had a positive binary result when tested untreated in WP, while the other only had growth when autoclaved (section 4.2.2). This could imply that some direct WSs can contain inhibitors or harmful contaminants that are neutralized/killed by autoclaving. In addition to OD_{750} measurements to determine total biomass increase, OD_{680} measurements were conducted to determine the increase in chlorophyll (Griffiths *et al.*, 2011). The rather constant OD_{680} to OD_{750}ratios (as seen in Appendix B:), indicate that chlorophyll was increasing and thus most probably microalgal biomass, and not just total biomass, which also could have been contaminants such as bacteria.

Only half of the LEITAT samples (batches 3 and 4) had positive binary results in WP (Table 8). However, both samples in batch 4 had positive binary results, indicating that the treatments administered in this batch were more successful than the batch 3 treatments. At the same time, it should be noted that the negative control also had a positive binary outcome in the batch 4 WP experiment, WP5 (see Appendix B:Figure 43 and Picture 5). Further, the binary results of the batch 4 samples were based on OD_{750} alone, as the pigmentation of the samples made algae

growth undetectable by visual control. These factors add uncertainty to the binary outcomes of the batch 4 WP experiment.

All the LEITAT-treated samples with growth had available nitrate, but there were also samples that had available nitrate that had no growth. Furthermore, the LEITAT samples generally had more solubilized P than the direct samples (see results from nutritional analysis in Table 7). This implies that nitrogen in the form of nitrate was a prerequisite for growth, and, based on the results from batches 1 and 2, sufficient P would be available for a positive result, but that other inhibiting factors were present. An inhibiting factor was perhaps introduced or produced by the enzymes in LEITATs treatment or already present in the sludge samples. As sterile filtration did not endorse algae growth in any samples (section 4.2.2), it is unlikely that the inhibition came from bacteria in these samples. The OD_{680} to OD_{750} ratio also showed no indication of biomass increase by contaminants (as seen in Appendix B:). It would have been interesting to test if autoclaving could promote growth in these samples as it did in the direct sludge sample. This was not tested because of the small sample volume. Moreover, filtration was prioritized over autoclaving as high temperatures might have influenced the sludge composition and nutrient availability, and the main interest was to understand the suitability of the enzyme treatments to liberate nutrients for microalgae growth.

Manipulation by APN addition gave some contradictory results. The AB7_O sample did not have algae growth when micronutrients were added in contrast to observed growth without additional micronutrients, while AB3_S only had algae growth when they were present. However, the original micronutrient concentrations of the various WSs were unknown. As slightly elevated micronutrient concentrations can be toxic for microalgae (Procházková *et al.*, 2014), the addition of APN could have led to unfavourable or even toxic micronutrient levels in some samples, as perhaps seen in AB7_O. While other samples, like AB3_S, might have had too low original micronutrient concentrations and would therefore depend on APN addition to function as a growth medium.

However, determining the binary outcome could sometimes be challenging, and occasionally the visual observations had to be the determining factor. For example, AB3_S with additional micronutrients showed very little and delayed (started at day 8) growth in OD readings Appendix B:Figure 38). However, the binary result was considered positive since some growth was observed in the visual control (see Appendix B: Picture 3). The difference between no growth (without APN) and little growth (with APN) for the AB3_S samples might have been

smaller than the binary outcome suggests. Another inconsistency was seen in the WP experiments with AB1_O. The sample did not have algae growth when 0,45 µm sterile filtrated but did when it was 0,2 µm sterile filtrated. Here, a clear difference was seen both in the OD readings and the visual controls (Appendix B: Figure 45 and Picture 6). However, the reason for the inconsistencies in the manipulation effect is unclear. One explanation could be that perhaps the small sample volume (≤1800 µL) weakened the WP method and made the reproducibility low. It would be interesting to see if more reliable results could be obtained using the 48 well-plate with larger well volumes than the 98 well-plates.

Although being limited to binary results is a disadvantage, as it would be interesting to compare the microalgae growth rates on the various WSs and the biomass concentrations achieved per unit WS, the WP method also has some clear advantages. Throughout the WP experiments, the 22 different WSs were tested with different manipulations amounting to over 150 different triplicates/sextuplicates. With the time available, it would have been nearly impossible to test all these replicas on a larger scale. Although it is important to be aware of its limitations, the WP method offered a quick and efficient large-scale screening of WSs as growth media for microalgae. Manipulation by adding an initially possibly limiting nutrient could be interesting in WP, but only if the nutrient source was truly lacking (e.g., one of the micronutrients as perhaps seen in AB3_S). Manipulation by P-addition was tested in WP1 as it was expected that the low P concentrations in the majority of the direct samples would be a growth-limiting, or even inhibiting, factor, as described by Daneshvar *et al.* (2018). However, P-addition was essentially not interesting in this experiment, as even the media with the lowest P-values had algae growth in WP. Manipulation by autoclaving/filtration could be interesting in WP as possible bacterial contaminations could have a negative/inhibiting effect on algae growth (as indicated in the AA5_S sample, which only had a positive binary outcome when autoclaved). Ultimately, the WP method served its purpose as a tool for preliminary testing.

5.4 Bubble column

Three AB1_O-based media were tested on a larger scale (300 ml) in the bubble column experiment as growth media for C. *vulgaris* (section 4.3). This experiment aimed to determine how the addition of micronutrients and P would affect the growth of *C. vulgaris* and the final biomass concentration in AB1_O based media. Therefore, it was crucial that the algae growth became limited by nutrient deprivation before the culture density could lead to limitations due to light or CO_2 availability. The AB1_O medium had an N concentration of ~13.78 mM (Table

7). This concentration could have supported an algae biomass of >3 mg/mL, assuming algal biomass has the chemical formula of $C_{106}H_{263}O_{110}N_{16}P$ (Stumm and Morgan, 1996). The AB1_O WS was therefore diluted so that algae growth eventually would become limited by N-deprivation. After dilution, the initial N concentration would approximately equal BBM (2,94 mM N), which was also used as a control medium. The initial actual N concentrations of the three AB1_O-based media were slightly elevated compared to the estimation, averaging at 3.23 ± 0.10 mM N. Two AB1_O media were prepared without changing the original N:P ratio of 41.91. One of these was kept as the original AB1_O medium (O), and the other had the addition of APN (Oμ). The P concentrations in these media were calculated to be about 0.07 mM P (4% of BBM) based on the measured concentration of the original AB1_O sample. However, the actual initial concentrations of P in the final media were measured at 0.04 and 0.02 mM PO_4- for O and Oμ, respectively. The third AB1_O-based medium (Bμ) was designed with a P concentration equal to BBM (1.72 mM P) and the addition of APN. The actual initial P concentration in Bμ was a bit lower (1.61 mM PO_4-) but equal to the BBM medium.

Generally, the OD_{750} and DW showed little variations between the media during the entire experiment period, and there were no categorically equal significant differences. The only significant differences were between O and Oμ in OD_{750} on day 10 (p-value of 0.3) and between O and BBM in the stationary DW samples (p-value of 0.043). The specific growth rates for *C. vulgaris* in all the media (0,56, 0,6, 0,67, and 0,73 d^{-1} for O, Oμ, Bμ, and BBM, respectively) were substantially lower than the maximum specific growth rate recorded for *C. vulgaris* (SAG 211-11b) (2.0 d^{-1}) (Lakaniemi *et al.*, 2012). However, the conditions of the current experiment were not designed to be optimal. The media had intentional low initial N and P concentrations, and algae growth was eventually nutrient limited in all the media. The specific growth rate found by Daneshvar *et al.* (2018) was 0.321 d^{-1} for *C. vulgaris* (CCAP 211/11B) grown under more similar conditions to the current experiment (250 mL flask vs 300 mL in bubble column, 80 -100 μmol photon $m^{-2}s^{-1}$ vs 15 - 120 μmol photon $m^{-2}s^{-1}$, 25 ± 2°C vs 23 ± 0.5°C, and BBM 3xN vs standard BBM). The results from the current experiment indicate that the growth of *C. vulgaris* was virtually unaffected by the low P concentration (0.07 mM P). This was unexpected as, according to Daneshvar *et al.* (2018), the initial P concentration in O and Oμ of ≤0.04 mM PO_4- (≤0.02 % of BBM) should have been a limiting or even an inhibiting factor for the growth of *C. vulgaris*. However, further results in the current experiment indicate that the O and Oμ

media might have been P-limiting but that the observed growth was obtained by utilisation of P from inoculum (discussed later).

Total nitrogen and nitrate were only measurable at low quantities (<1.00 mM) in the mid-growth phase (Figure 14). However, it is evident that this nitrate was still available for algae utilization as it was completely depleted in all media at the stationary phase. The Bμ medium had a considerably lower nitrate concentration at the mid-growth phase than the other media. This could be explained by inaccuracies caused by the nitrate kit being expired. However, the TN was also lower in this culture, giving reason to believe that other unknown factors, like method uncertainty, influenced the N level. Samples from the respective biological triplicates were pooled because of the total sample quantity required throughout the experiment compared to medium size (samples were collected for additional analyses apart from the aforementioned in the section 3.5, these were not conducted due to further mentioned reasons in section 5.6). Consequently, as information regarding the variation between the replicas was not attained, it was not possible to consider the reproducibility and method uncertainty in the experiment.

The P concentrations in the O and Oμ media at the mid-growth phase were low (0.02 mM PO_4- for both media) and roughly the same as at the stationary phase (0.02 and 0.01 mM PO_4-, respectively) (Figure 13). Although lower P concentrations have been used in P-limiting media (0.0045 mM P) in other research (Passarge *et al.*, 2006), the stability of the concentrations from mid-growth to the stationary phase makes it apparent that the O and Oμ media might have been P-limiting at the mid-growth phase, if not earlier. Further, several analyses indicated stress reactions exclusively in the O and Oμ media, which may imply P-deprivation. Decreasing QY is an indicator of stress, and the QY decline (starting at the mid-growth phase, day 8) was indeed more prominent in the media without P addition (Figure 11). Moreover, slight shifts in the lipid composition of the O and Oμ cultures compared to Bμ and BBM (more SFA and MUFA and less of some PUFAs) were observed. Although the variations were minor, this could also be interpreted as an indicator of stress by P-deprivation (Morales, Aflalo and Bernard, 2021). Increased TFA concentration is also an indicator of stress, and the TFA analysis revealed that the O and Oμ cultures had slightly increased concentrations (approximately 12.5% of DW) at the mid-growth phase compared to Bμ and BBM (approximately 9% of DW). Generally, nutrient limitation would result in a much higher lipid concentration in *C. vulgaris* (>20% for P-limitation, ≤40% for N-limitation) (Illman, Scragg and Shales, 2000; Liang *et al.*, 2013). This could indicate that *C. vulgaris* at the mid-growth phase only recently had run out of P in O and

Oμ. These results suggest that the O and Oμ cultures were stressed prior to the Bμ and BBM cultures, and it is reasonable to assume that P-deprivation caused the stress.

The final biomass concentrations (0.75, 0.84, 0.84, and 0.87 mg/mL for O, Oμ, Bμ, and BBM, respectively), exceeded the amount predicted by both the P (0.09 mg/mL in O and Oμ, 6.11 mg/mL in Bμ and BBM) and the N (0.653 mg/mL) concentrations in all the media (section 4.3). It should however be noted that the initial biomass concentration has not been withdrawn from these numbers. This was because the large samples that would have been needed at the start of the experiment to get accurate DW results (because of the low biomass concentrations) were impossible to attain with the available medium in the bubble columns. A theoretical estimation of initial biomass concentration could have been calculated from the start OD. However, this would require a calibration curve of *C. vulgaris* cultivated under normal conditions in the bubble column, which was not attained. Further, unexpectedly high biomass concentrations could be (at least partially) explained by the algae having a different chemical composition than suggested by the general formula.

The initial P concentration in O and Oμ (≤0.04 mM PO_4-) should have been inadequate for the growth of *C. vulgaris*, according to Daneshvar *et al.* (2018). However, the initial nutrient concentrations in the media were only analysed before algae inoculum addition. The possibility of unintentional nutrient manipulation by inoculum addition can therefore not be excluded. Moreover, as suggested in the WP experiment, the O and Oμ cultures may also have maintained their growth partly by utilising intracellular P storages obtained through previous luxury uptake in the inoculum phase (Levin and Shapiro, 1965). However, this effect would be much less pronounced in the current experiment compared to WP as larger volumes with higher final biomass densities were reached. A repeated batch bubble column experiment could have been conducted to investigate this matter further.

The bubble column experiment supports previous findings that *C. vulgaris* can utilise nutrients in wastewater from RAS (Milhazes-Cunha and Otero, 2017). However, low P concentrations (≤0.04 mM PO_4-) could be a limiting factor, as indicated in a study by Halfhide *et al.* (2014), where the initial P concentration of 0.06-0.08 mM P in RAS outlet water was considered to be P-limiting for growth of *Chlorella sp.* (NIVA CHL-137).

5.5 PBR

The SW waste stream sample, AB2_O, was tested as a growth medium for *M. gaditana* in a photobioreactor as a proof-of concept semi-pilot-scale experiment (section 4.4). The 19 L AB2_O sample (further referred to as AB2) was used undiluted with the addition of APN in a 25L GemTube RD1-25. A positive control with *M. gaditana* in NORCE medium was cultivated in a parallel PBR. The final algae biomass concentrations obtained in AB2 and NORCE were 2.17 mg/mL and 4.81 mg/mL, respectively (Figure 17). As the NORCE medium was designed to achieve high densities of microalgae, it was not surprising that the culture ended up with a biomass concentration that was more than double of the AB2 medium. The final biomass concentration obtained in the AB2 medium was almost four times the amount predicted (0.58 mg/mL) given the initial nitrate concentration (2.63 mM N) and the general chemical formula for microalgae. This could be explained by *M. gaditana* having a different chemical composition than suggested by the general formula.

In contrast to the bubble column experiment, the nitrate concentration was measured continuously throughout the PBR experiment (Figure 15). Therefore, unintentional nitrate addition through inoculum addition can be excluded as a source of error. The P concentration in the AB2 media was unknown (discussed below). Further, the specific growth rate for M. *gaditana* (/day 0-5) were 0.59 and 0.55 d^{-1} in the AB2 and NORCE medium, respectively. Indicating that although the nutritional concentration in AB2 only facilitated for a low final biomass concentration relative to NORCE medium, the composition was suitable for cultivation of *M. gaditana* as the cultures had similar growth rates.

The growth rates of *M. gaditana* found in the current research are higher than reported for the same strain with modified f/2 medium in flat-panel PBRs (0.29 d^{-1}), with a similar light path (30 mm vs 32 mm in our tubular PBRs) and less illumination (100 µmol photon m^{-2} s^{-1} vs 40 – 360 µmol m^{-2} s^{-1}) (Sung *et al.*, 2017). Another study by Karthikaichamy *et al.* (2018) found a similar growth rate (0.52 d^{-1}) for the same *M. gaditana* strain with f/2 medium in 500 mL Erlenmeyer flasks under 12/12h light/dark cycle (150 µmol photon m^{-2} s^{-1}).

The 19 L of AB2_O WS sample used in the PBR experiment was distributed over 11 bottles during sampling. Nutritional analyses were conducted on a sample from one of the bottles prior to the preliminary testing in WP2. This sample had nutritional concentrations of 4.27 mM N, 0.08 mM NH_4+, 4.19 mM NO_3-, and 0.16 mM PO_4- (results presented in Table 7). However, the nitrate concentration in the final AB2 medium on the first day of the PBR experiment was

2.63 mM NO_3^- (section 4.4). Further, the various sample bottles varied in turbidity and defrosting time (Figure 7). This implies that the content of the 11 bottles containing AB2_O sample was not homogenous. Additional analyses of the AB2 medium were not conducted as its deviation from the previously analysed AB2_O sample was only discovered after the PBR experiment was terminated. Therefore, apart from nitrate, the nutrient concentrations and consequently the N:P ratio in the PBR is unknown.

The AB2 culture had an evident increase in TFA % per biomass from the mid-growth to the stationary phase sampling point (Figure 19). The medium was depleted of nitrate at the stationary sampling point (Figure 15). It can therefore be assumed that further growth was limited by nitrate deprivation. It is however unknown if this was the only nutrient limitation or if the culture was limited by P, as we saw for some media in the bubble column experiment. The NORCE medium, however, had a slight decrease in TFA % per biomass from the mid-growth to the stationary phase sampling point. As the nitrate concentration in the NORCE medium could not be detected due to thiosulfate (explained in section 3.4.3), the timing of the stationary sampling point was solely based on the OD and QY. In hindsight, too much of the timing was based on the OD, and more attention should have been paid to the QY. After all, the OD samples were eventually 100x diluted to fit the photometer's measuring rage, which could easily result in significant uncertainties. For example, the development at the end of the AB2 curve (Figure 15) was likely affected by uncertainties connected to high dilution factors. The growth curve descended after the stationary sampling point before a quite rapid growth was registered at the end of the experiment. It is more likely that the biomass concentration was slowly increasing but levelling off between day 7 and day 10. The QY on the day of the stationary sampling point of the NORCE culture was at 0.67 (Figure 16), which indeed was a slight decrease from the previous day but not as low as one would expect in a stationary culture. Based on the QY, the following day (day 10, QY = 0.63) or even the day after would be a more accurate timing of the stationary sampling point, in which case the biomass concentration of the culture would most likely be higher.

Even though the N:P ratio of the WS medium was unknown and the stationary sampling point of the control medium was poorly timed, the experiment still demonstrated that untreated SW waste stream medium could be used to cultivate M. *gaditana* under non-axenic conditions and that the microalgae were able to utilise and remove roughly all available nitrogen. The phosphate concentration was unknown, and, as in the other experiments, it should be assumed

that the algae had intermediate storage of P, which would limit/delay the registration of stress by external P deprivation (Levin and Shapiro, 1965).

The final biomass concentration obtained with WS medium in the PBR experiment (2,17 mg/mL) was lower than in the bubble column experiment with WS medium with APN (Oμ, 0,85 mg/mL). However, the initial nitrate concentration was lower in the PBR WS medium (2.63 mM NO_3-) compared to the bubble column WS medium (3.23 ± 0.01 mM NO_3-). Based on the nitrate concentration, and the fact that it was depleted in both experiments, it would be expected that the final biomass concentration would be higher in the bubble column compared to the PBR experiment. At the same time, two different algae were used in these experiments, and they may have very different N concentrations in their biomass.

A rough estimate of possible production quantities will further be made to better understand the valorisation potential of microalgae cultivation on RAS wastewater. As mentioned in the introduction, a RAS facility generally has a water exchange rate of 300 – 400 L/kg feed per day. Given the biomass obtained in the PBR experiment (2.17 mg/mL), roughly 0.8 kg of algae could be produced per kg feed per day with the exchange water (given the same qualities as AB2). The daily amount of feed will however vary depending on factors such as cultivated species, fish size and count, feeding regime, pellet quality and feed efficiency, and facility size. The feed-to-fish conversion rate varies with fish size but is generally up to 1 in RAS (Bregnballe, 2015). For simplicity, a rough estimate could therefore be made, and one could say that, given the results in this experiment, 0.8 kg *M. gaditana* could be produced per kg fish produced in RAS. Even with the much lower predicted microalgae yield on RAS water (0.58 mg microalgae / mL), around 0.2 kg *M. gaditana* could still be produced per kg fish produced in RAS. These numbers show the potential relevance of valorising these WSs with microalgae. These estimations are however merely circumstantial, and various details, especially concerning the practicability of large-scale algae production on wastewater from RAS, should be included in more elaborate estimations.

5.6 Methodological considerations

Although perhaps not significant for the overall results of the various experiments, the inconsistencies in the execution of sample analysis weakened the comparability between the batches. All the nutrient analysis kits (listed in Table 6), except NANOCOLOR total-Nitrogen, could have been used for all the samples, and it would have been preferred to ease comparison between the batches. This was however not possible because of kit delivery delays and

occasional small sample volumes. It would obviously also be preferred to use unexpired kits in all analyses. Further, more comprehensive analyses should be conducted in bubble column and PBR experiments in further research. It was intended to analyse total organic carbon, bacterial count, elements, and microbiome in the bubble column and PBR experiment. However, this was not conducted due to equipment availability and delivery delay of aquaculture samples.

Moreover, steps to limit the intracellular storage of P should have been conducted. For example, in the final preparation of algal inoculum, it would have been preferred to use a low nutrient medium to limit unintended nutrient addition to experiment media. The possibility intracellular P storage being a rather big source of error is recognized in the entirety of the thesis, but especially in the WP experiments, as the binary results offer little nuance and room for evaluating the intracellular P storage effect.

It was intended to conduct a repeated batch in the PBR experiment. This could have exposed P-limiting factors in the WS medium. The experiment was however limited to a single batch due to the sample size and nutrient levels, leaving no room for dilution.

5.7 Future possibilities

One must integrate or facilitate to create opportunities for wastewater valorisation through microalgae production. RAS facilities can integrate microalgae phycoremediation as a water treatment step. Several studies have suggested that a microalgal unit could replace the denitrification step and increase the recirculating degree in RASs (Halfhide *et al.*, 2014; Milhazes-Cunha and Otero, 2017). Increasing the recirculating degree would be an advantage, not only with respect to production costs but also because it would lessen the pollution pressure in the recipient waters. In a study by Gao *et al.* (2016), a membrane photobioreactor (MBPR) that allowed continuous cultivation of *C. vulgaris* on aquacultural wastewater was developed. The writer found that the MBPR achieved a nutrient reduction comparable to traditional biofilters with similar hydraulic retention time. However, this wastewater had up to 10 times lower nutrient concentrations (0.49 mM N, 0.01 mM P) than the average of the various direct outlet samples in batches 1 and 2 in this research (AB1_O excluded). Although much further research is required, this indicates that an MBPR could potentially replace the traditional biofilter. To ensure that higher nutrient concentrations could be treated as well, the size of the photobioreactor, type of microalgae and possibly optimised growth conditions would be essential parameters for improvement. *C. vulgaris* is already considered a fast-growing algae species with a reported growth rate of 2.2 d^{-1}. However, for example, *Chlorella sorokiniana* has

an even higher reported growth rate (5.9 d^{-1}) (Morales, Aflalo and Bernard, 2021). High nutrient removal rates would also be expected with such high growth rates. Further, the additional water treatment steps required in RASs to prevent the waters from becoming anoxic due to the aerobic bacteria in traditional biofilters (oxygenation, aeration) could be eliminated if replaced with MBPR (Gao *et al.*, 2016; Milhazes-Cunha and Otero, 2017). Alternatively, another participant can use the wastewater of the RAS facility to produce microalgae externally (though in the vicinity, as transport of large amounts of water will be costly). However, as available information on WS quantities is limited, and most outlet water from RAS is currently released to coastal water, potential users would benefit from a platform where available WSs are quantified and categorized, and which facilitates for involvement of external parties such as microalgae producers. This could be challenging as there are no standardised RAS setups, and the nutritional content of WSs will vary. External microalgae production on RAS wastewater medium could also be interesting on site (parallel production to the aquaculture facility). Both facilitation and integration will depend on further research, technological developments, and a knowledge-sharing platform. Further research should also be conducted regarding nutrient recovery through hydrolysis treatments, as conducted by LEITAT for this thesis. It would especially be interesting to see if a treated sample with more liberated nutrients could be combined with outlet samples to give a more balanced nutrient profile.

6 Concluding remarks

This thesis demonstrated that WSs from SW and FW RAS could be utilised as growth media for SW M. *gaditana* and FW C. *vulgaris*, but that relatively low nutrient concentrations, especially in P, can be a limiting factor. Enzymatic and chemical hydrolysis conducted on sludge samples by LEITAT did often liberate P. However, the WP experiments with these samples had low success rates, which may indicate inhibiting factors introduced by the treatment. Moreover, the results demonstrated that the nutritional content of WSs can vary, especially depending on the sampling method. Further research should be conducted on optimal WS collection and subsequent microalgae valorisation.

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Appendix A: Nutrient concentrations

Table 9 Averages and the standard deviation (SD) of different groups have been calculated. Abbreviations: O =outlet water, CS = concentrated sludge. Notes: a – corresponding outlet samples to the sludge samples in batch 1, b – AB2_O was not analysed, c – the batch 3 outlet samples (AB7_O and AB8_O) were not analysed

	Salinity [ppt]		pH		TN [mM]		NH4+ [mM]		NO3- [mM]		PO4- [mM]		TP [mM]	
Groups	average	SD	average	SD	average	SD	average	SD	average	SD	average	SD	average	SD
Batch 1	2.74	0.23	8.59	0.44	5.08	3.70	0.07	0.05	5.01	3.69	0.15	0.07	0.12	0.11
Batch 1 (not AB1_O)	2.83	0.11	8.62	0.46	3.63	0.32	0.07	0.05	3.56	0.31	0.13	0.04	0.08	0.08
Outlet water batch 1 and 2 (not AB1_O)	6.58	11.30	8.39	0.68	3.66	0.40	0.04	0.02	3.62	0.39	0.11	0.02	0.03[b]	0.01[b]
Sludge batch 1	2.85	0.06	8.74	0.47	3.71	0.26	0.14	0.03	3.58	0.25	0.18	0.02	0.19	0.01
Corresponding outlet samples[a]	2.70	0.00	8.82	0.22	3.72	0.43	0.03	0.00	3.69	0.43	0.10	0.02	0.03	0.01
Batch 3	1.68	0.49	6.42	0.29	27.44[c]	29.23[c]	n.d.	n.d.	1.71	2.46	n.d.	n.d.	0.70[c]	0.63[c]
Sludge batch 3	1.70	0.47	6.43	0.35	9.99	4.33	n.d.	n.d.	1.43	2.07	n.d.	n.d.	0.43	0.53
CS batch 3	1.43	0.29	6.33	0.29	68.14	15.39	n.d.	n.d.	0.00	0.00	n.d.	n.d.	1.34	0.27
outlet water batch 3	2.00	0.85	6.50	0.00	n.d.	n.d.	n.d.	n.d.	5.23	2.60	n.d.	n.d.	n.d.	n.d.
Batch 4 (CS)	3.95	3.32	7.50	1.41	50.63	64.97	0.01	0.01	3.26	3.74	0.58	0.55	n.d.	n.d.

Appendix B: WP

Dilution series Hidex

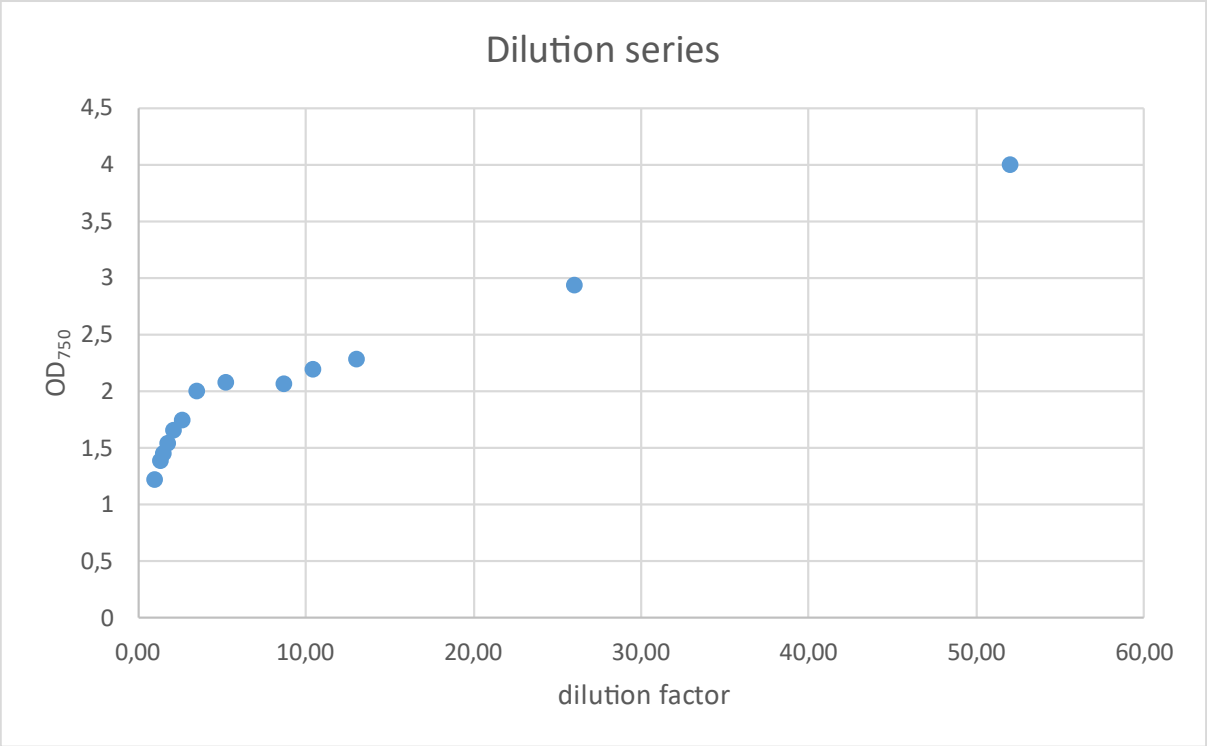


Figure 20 A dilution series was conducted to determine the range for accurate OD measurements in the HIDEX. The rage for accurate detection wat determined to be OD_{750} 0.2 – 0.6.

WP1

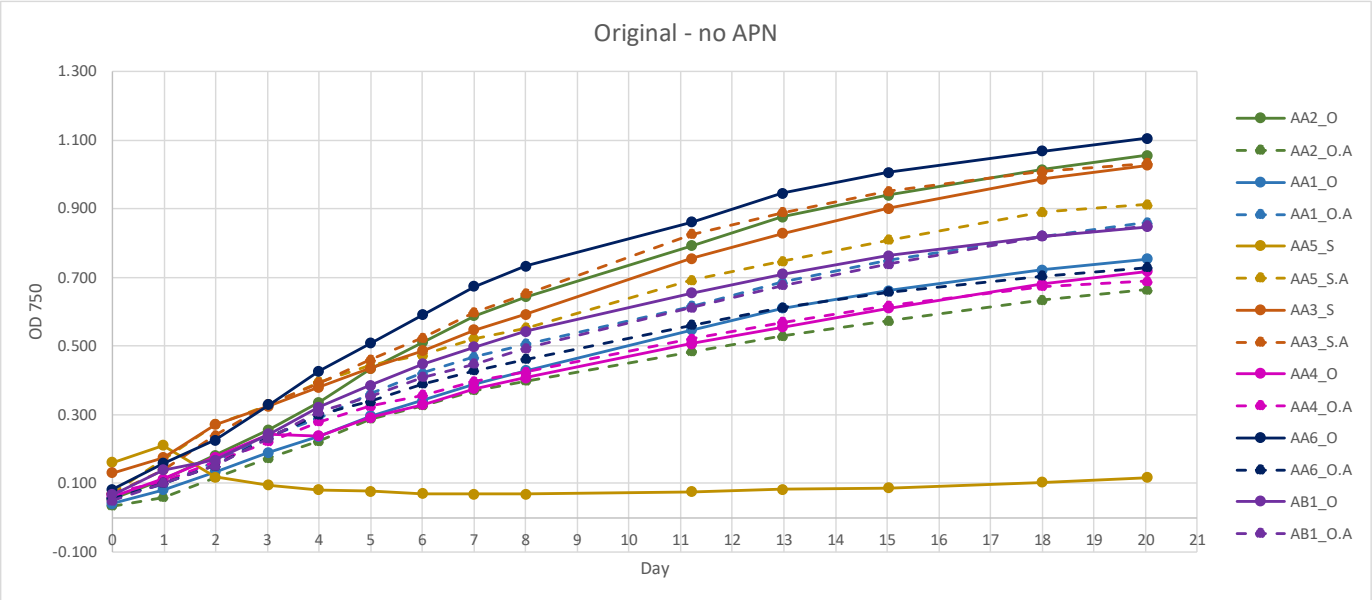


Figure 21 OD_{750} chart from WP1 with original N:P ratio and without the addition of APN. Abbreviations: AA = Aller Aqua, AB= AquaBioTech, O = outlet, S = sludge .A = autoclaved.

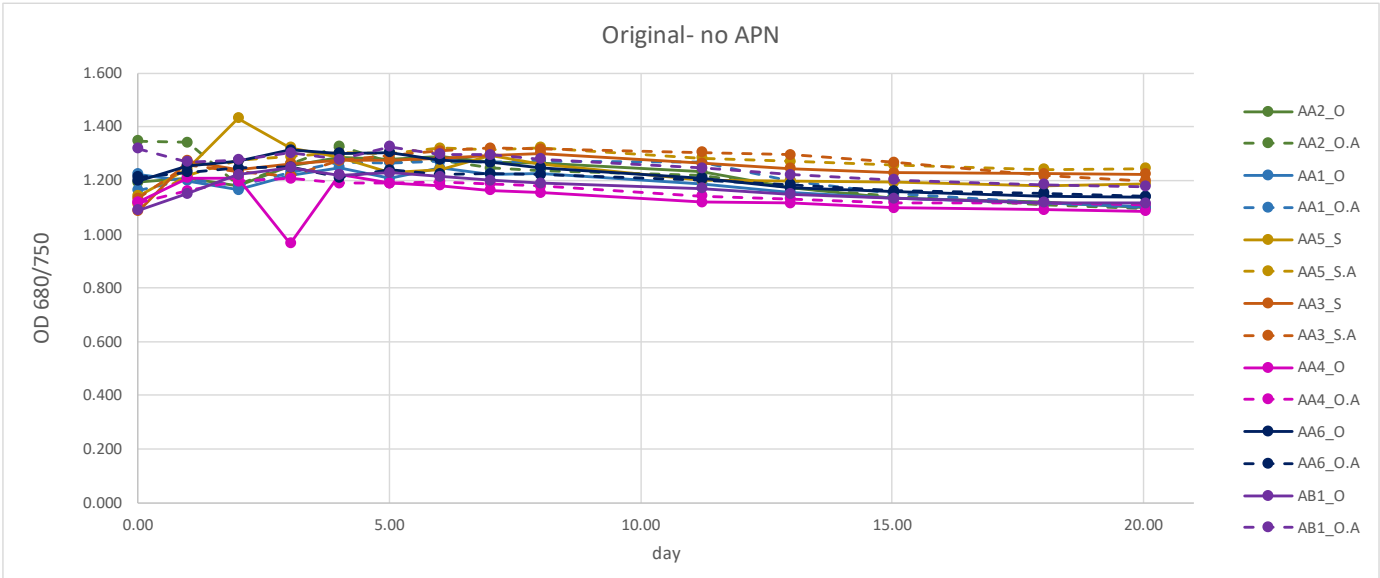


Figure 22 OD_{750}/OD_{680} chart from WP1 with original N:P ratio and without the addition of APN. Abbreviations: AA = Aller Aqua, AB= AquaBioTech, O = outlet, S = sludge .A = autoclaved.

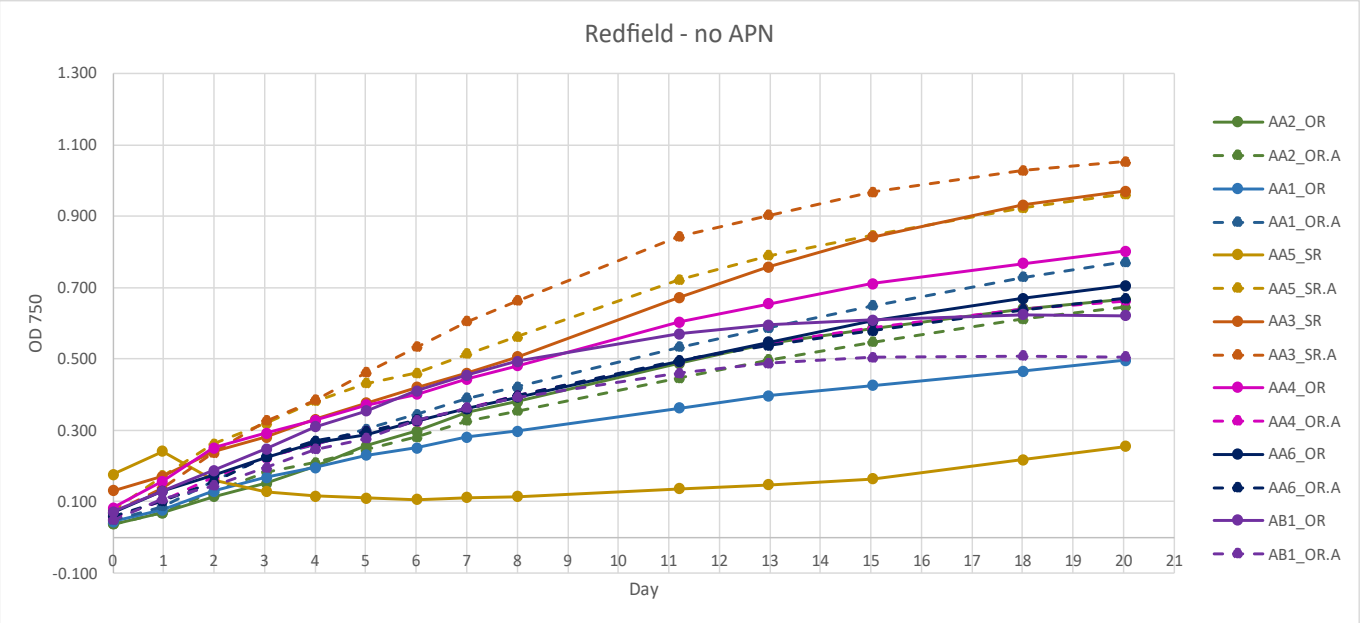


Figure 23 OD_{750} chart from WP1 with Redfield N:P ratio and without the addition of APN. Abbreviations: AA = Aller Aqua, AB= AquaBioTech, O = outlet, S = sludge .A = autoclaved.

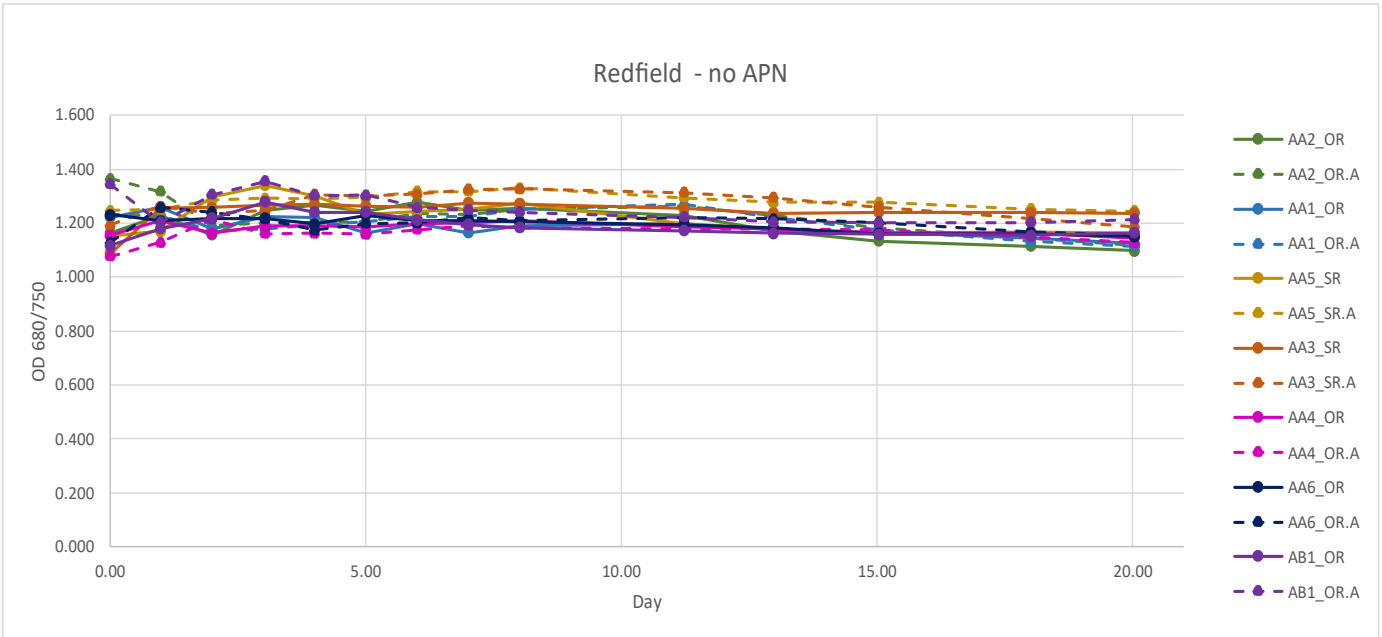


Figure 24 OD_{750}/OD_{680} chart from WP1 with Redfield N:P ratio and without the addition of APN. Abbreviations: AA = Aller Aqua, AB= AquaBioTech, O = outlet, S = sludge .A = autoclaved.

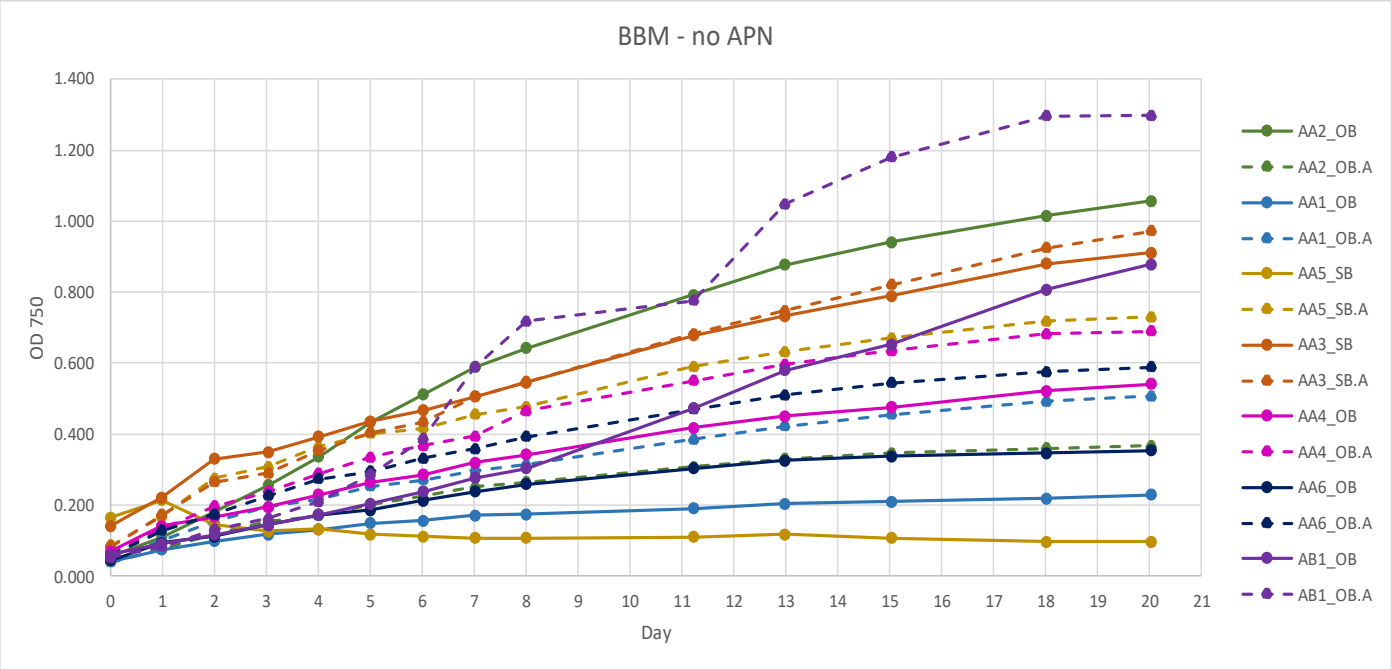


Figure 25 OD_{750} chart from WP1 with BBM N:P ratio and without the addition of APN. Abbreviations: AA = Aller Aqua, AB= AquaBioTech, O = outlet, S = sludge .A = autoclaved.

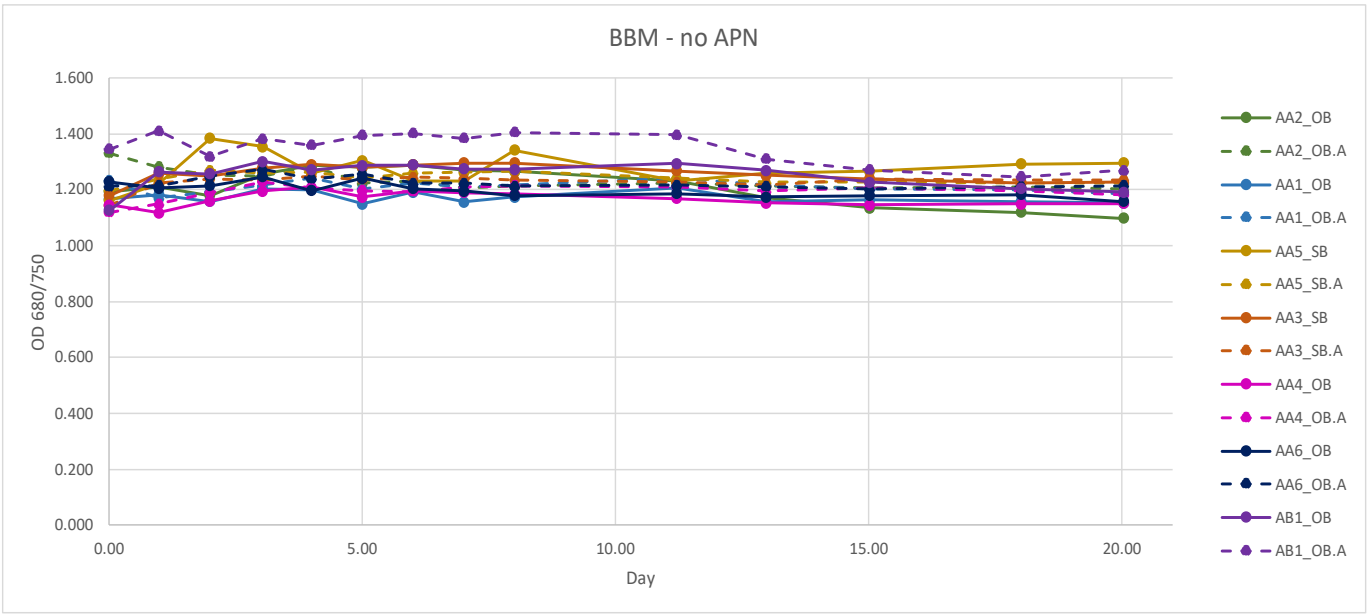


Figure 26 OD_{750}/OD_{680} chart from WP1 with BBM N:P ratio and without the addition of APN. Abbreviations: AA = Aller Aqua, AB= AquaBioTech, O = outlet, S = sludge .A = autoclaved.

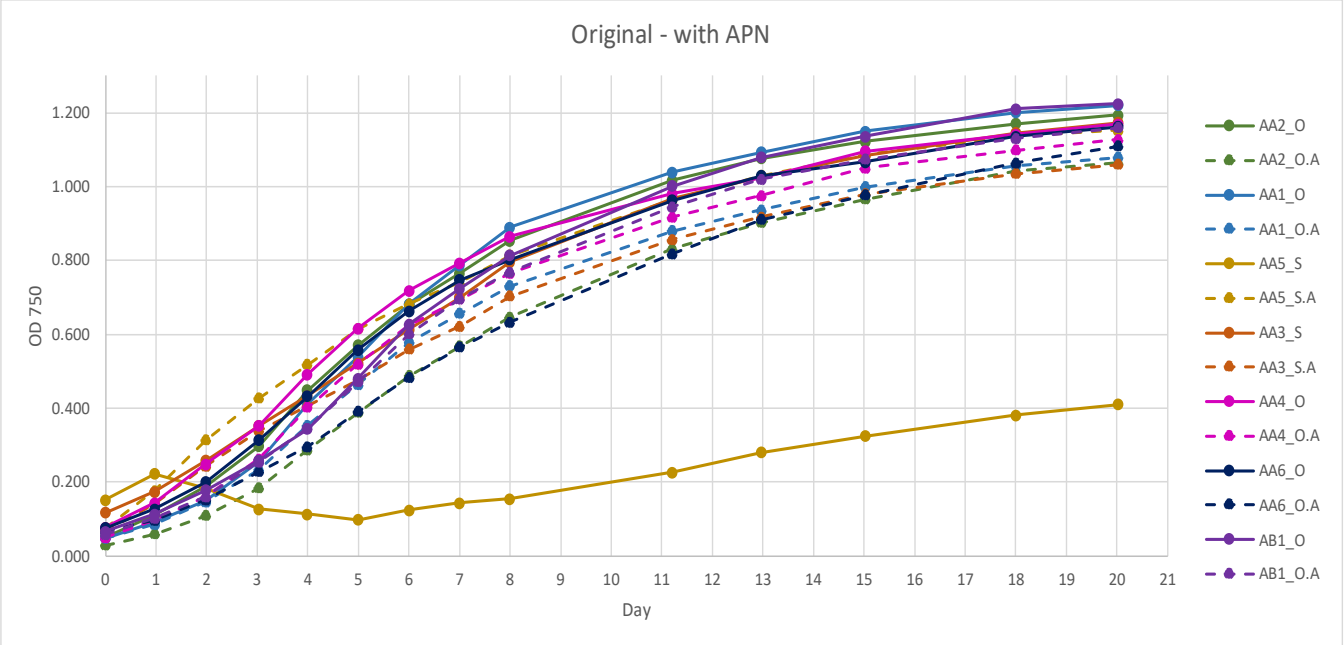


Figure 28 OD_{750} chart from WP1 with original N:P ratio and with the addition of APN. Abbreviations: AA = Aller Aqua, AB= AquaBioTech, O = outlet, S = sludge .A = autoclaved.

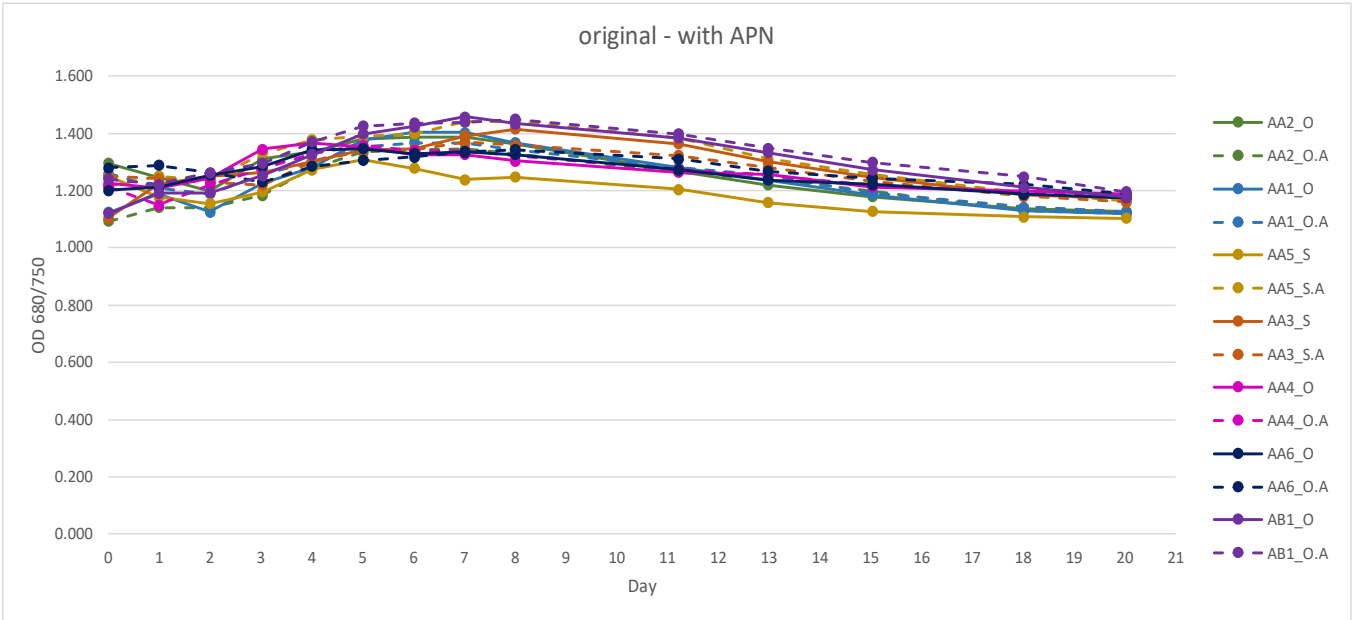


Figure 27 OD_{750}/OD_{680} chart from WP1 with original N:P ratio and with the addition of APN. Abbreviations: AA = Aller Aqua, AB= AquaBioTech, O = outlet, S = sludge .A = autoclaved.

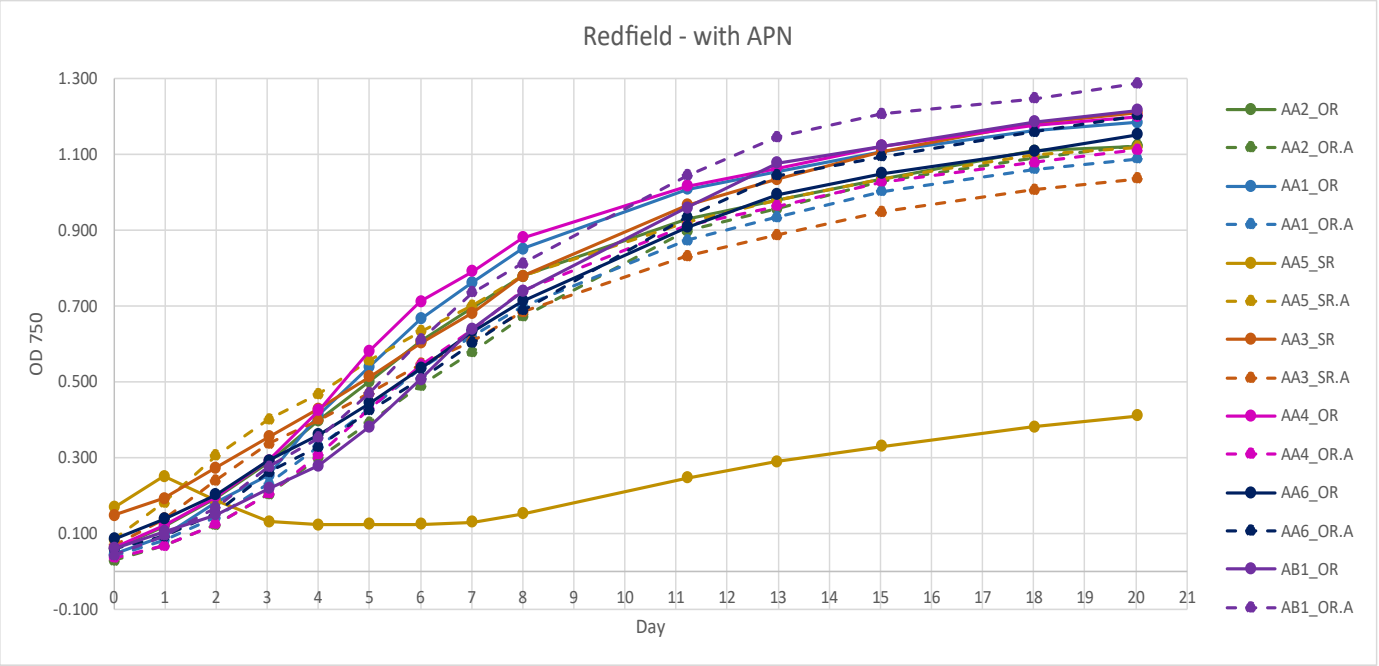


Figure 29 OD_{750} chart from WP1 with Redfield N:P ratio and with the addition of APN. Abbreviations: AA = Aller Aqua, AB= AquaBioTech, O = outlet, S = sludge .A = autoclaved.

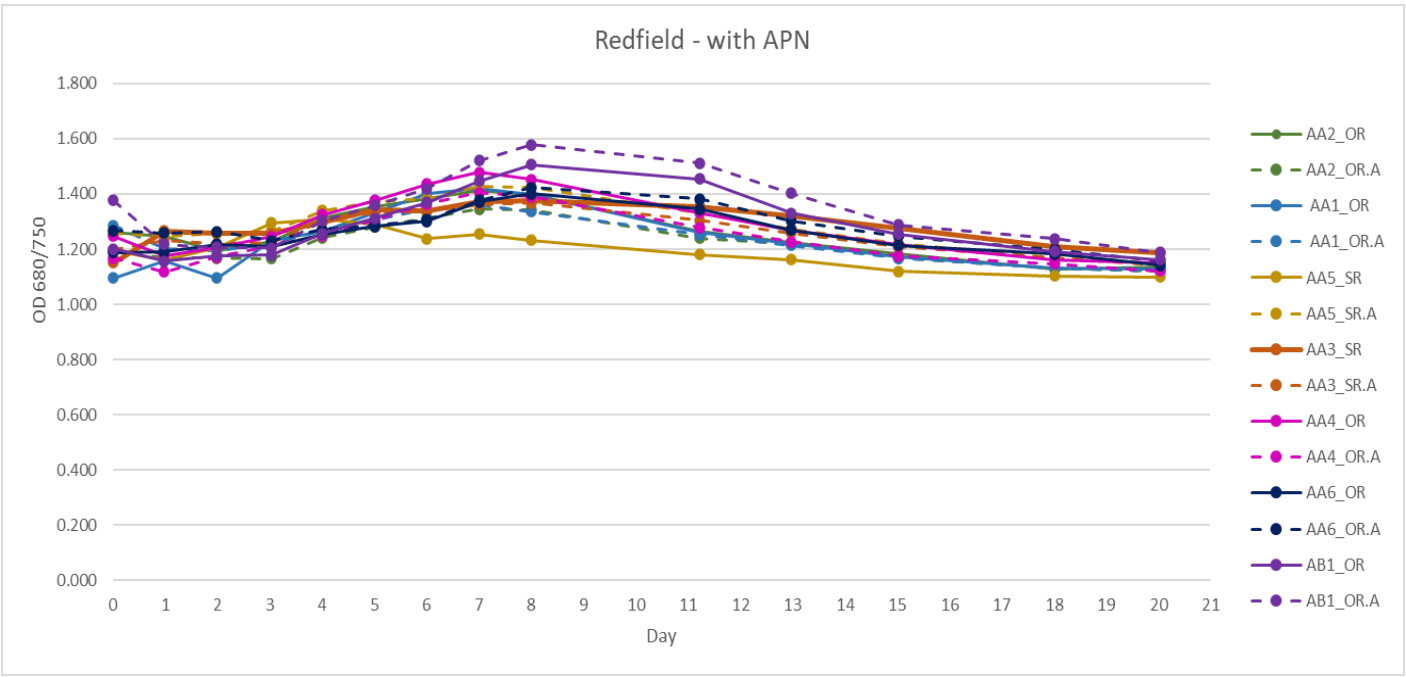


Figure 30 OD_{750} /OD_{680} chart from WP1 with Redfield N:P ratio and with the addition of APN. Abbreviations: AA = Aller Aqua, AB= AquaBioTech, O = outlet, S = sludge .A = autoclaved.

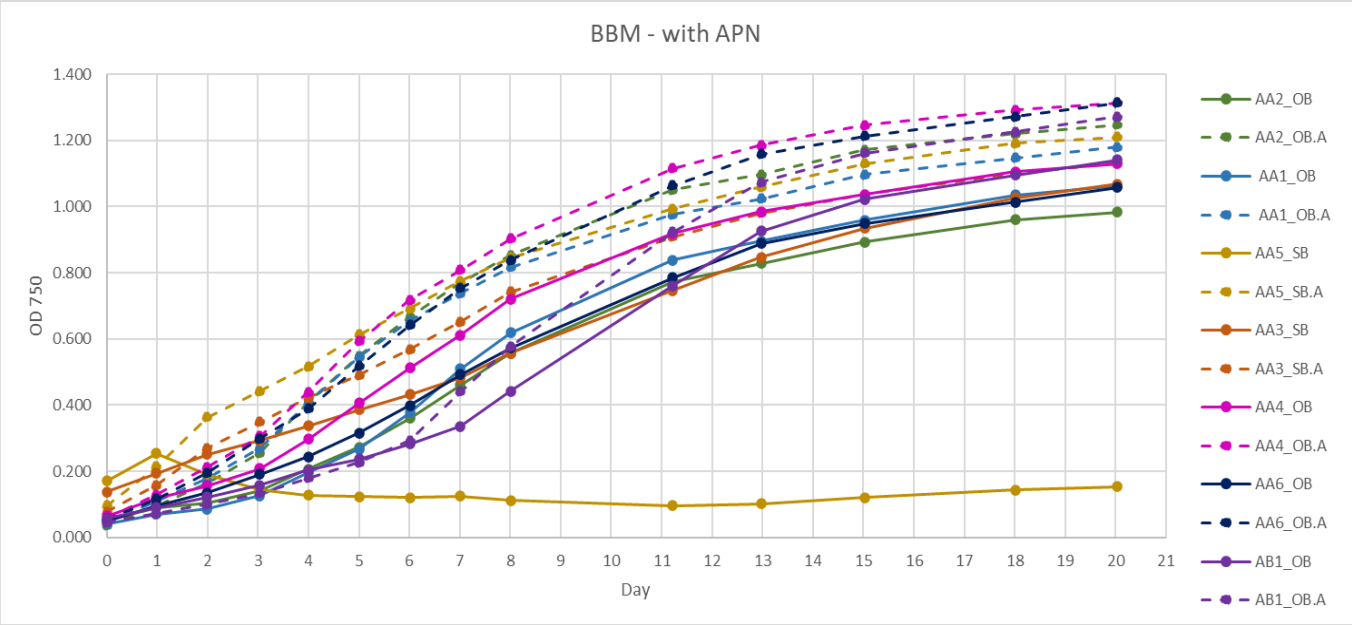


Figure 32 OD_{750} chart from WP1 with BBM N:P ratio and with the addition of APN. Abbreviations: AA = Aller Aqua, AB= AquaBioTech, O = outlet, S = sludge .A = autoclaved.

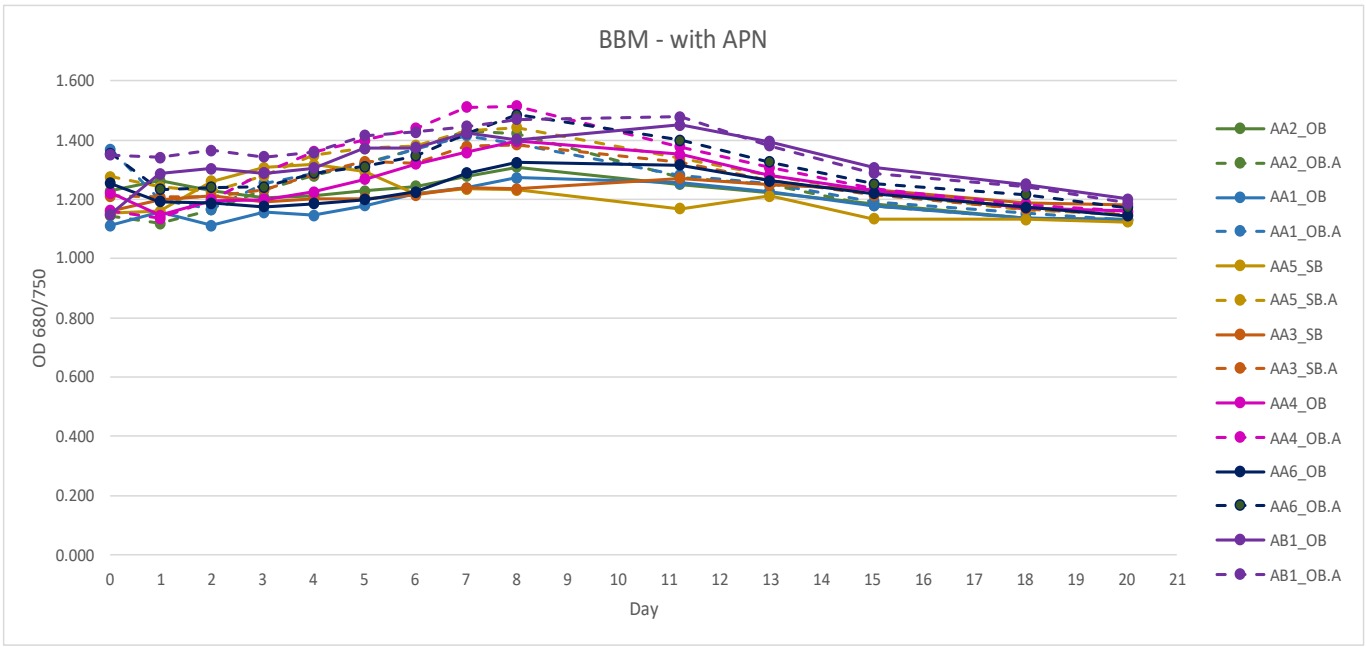


Figure 31 OD_{750}/OD_{680} chart from WP1 with BBM N:P ratio and with the addition of APN. Abbreviations: AA = Aller Aqua, AB= AquaBioTech, O = outlet, S = sludge .A = autoclaved.

WP2

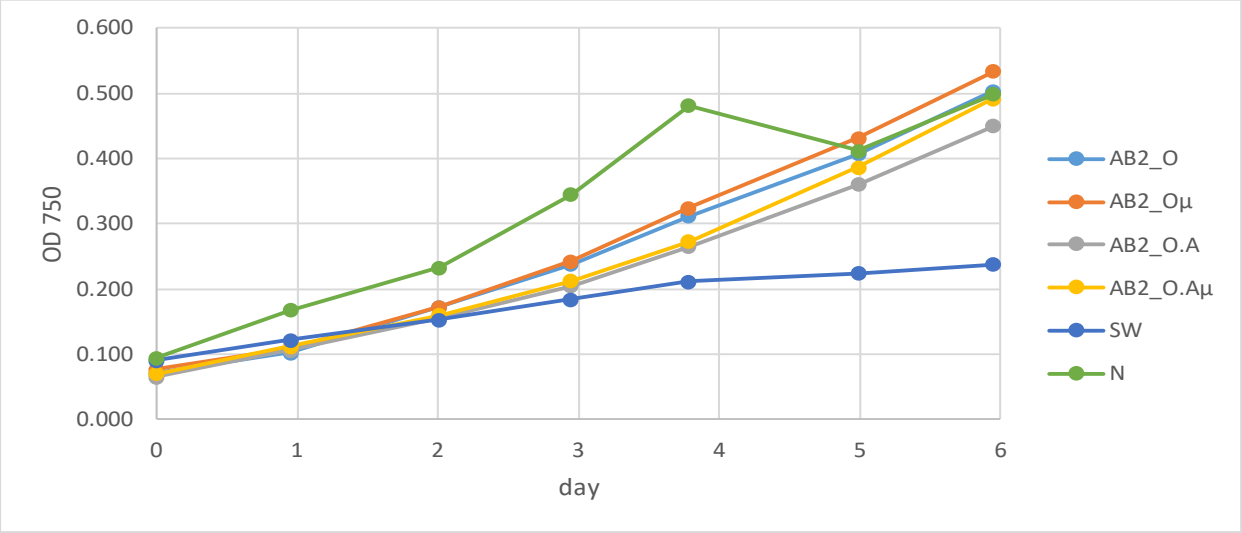


Figure 33 OD_{750} chart from WP2 where the batch 2 WSs were tested with different manipulations (autoclaving (.A), micronutrient addition (μ)) as growth media for M. gaditana. Abbreviations: AB= AquaBioTech, O = outlet, μ = APN, .A = autoclaved, SW = salt water (negative control), N = NORCE medium (positive control).

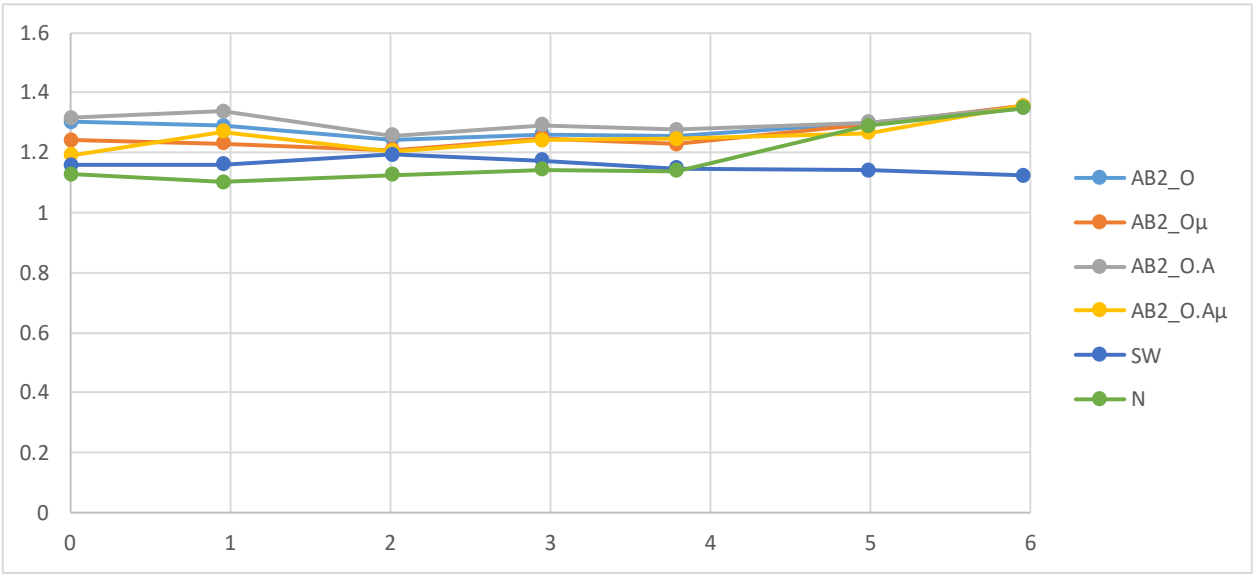
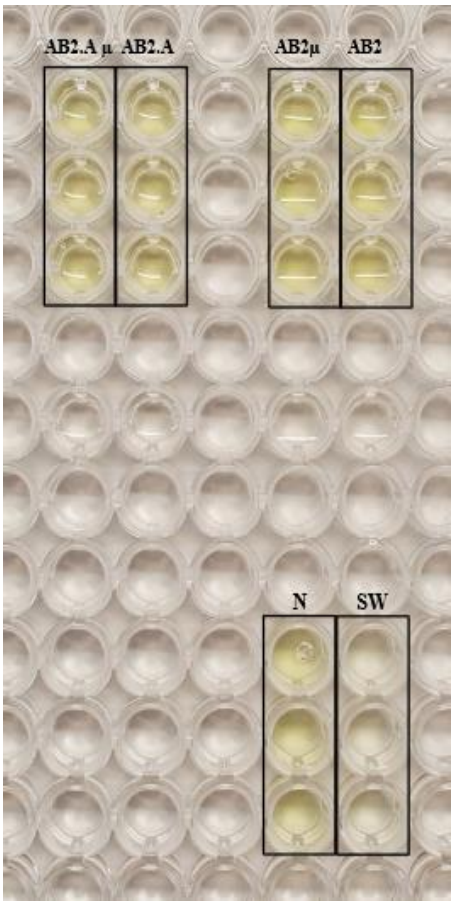


Figure 34 OD_{680} / OD_{750} chart from WP2s where the batch 2 WS were tested with different manipulations (autoclaving (.A), micronutrient addition (μ)) as growth media for M. gaditana. Abbreviations: AB= AquaBioTech, O = outlet, μ = APN, .A = autoclaved, SW = salt water (negative control), N = NORCE medium (positive control).



Picture 2 Visual control on the final day of WP3 where the batch 2 WSs were tested with different manipulations (autoclaving (.A), micronutrient addition (µ)) as growth media for M. gaditana. Abbreviations: AB= AquaBioTech, O = outlet, µ = APN, SW = salt water (negative control), N = NORCE medium (positive control).

Table 10 SD of the OD_{750} in WP2 where the batch 2 WSs were tested with different manipulations (autoclaving (.A), micronutrient addition (µ)) as growth media for M. gaditana. Abbreviations: AB= AquaBioTech, O = outlet, µ = APN, SW = salt water (negative control), N = NORCE medium (positive control).

Medium	day						
	0.00	0.95	2.01	2.95	3.79	4.99	5.95
	standard deviation - biological triplicates						
AB2_O	0.00	0.01	0.01	0.02	0.02	0.03	0.03
SW	0.01	0.01	0.01	0.01	0.01	0.02	0.03
AB2_Oµ	0.00	0.00	0.01	0.01	0.01	0.01	0.01
N	0.00	0.01	0.04	0.13	0.23	0.07	0.11
AB2_O.A	0.00	0.00	0.00	0.00	0.00	0.00	0.00
AB2_O.Aµ	0.01	0.01	0.01	0.01	0.01	0.02	0.02

WP3

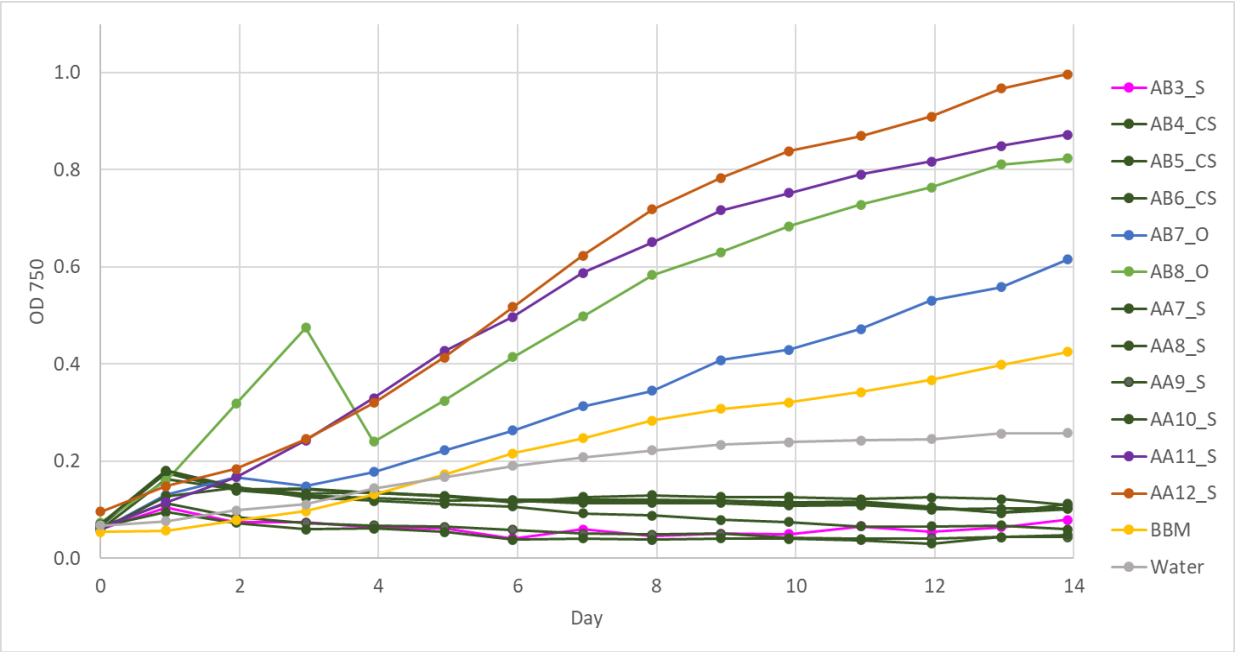


Figure 35 OD_{750} chart from WP3 where the unmanipulated batch 3 WSs were tested as growth media for C. vulgaris. Abbreviations: AA = Aller Aqua, AB= AquaBioTech, O = outlet, S = sludge CS = concentrated sludge, BBM = Bolds basal medium (positive control).

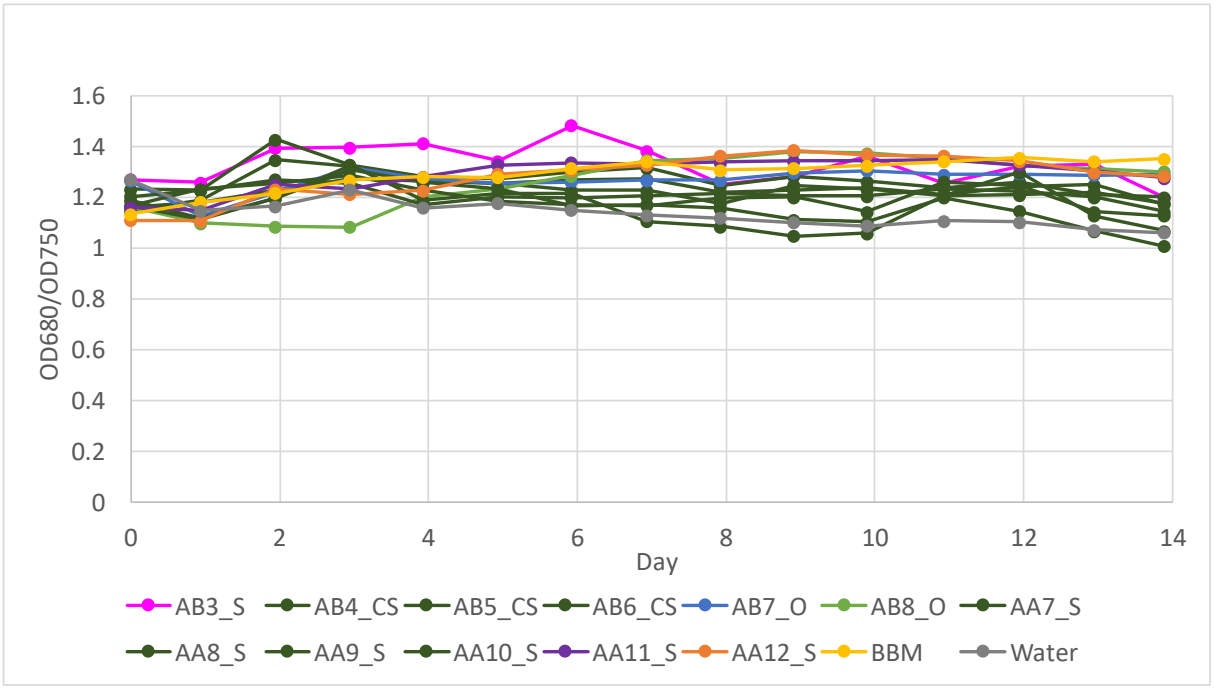


Figure 36 OD_{680} / OD_{750} chart from WP3 where the unmanipulated batch 3 WSs were tested as growth media for C. vulgaris. Abbreviations: AA = Aller Aqua, AB= AquaBioTech, O = outlet, S = sludge CS = concentrated sludge, BBM = Bolds basal medium (positive control).

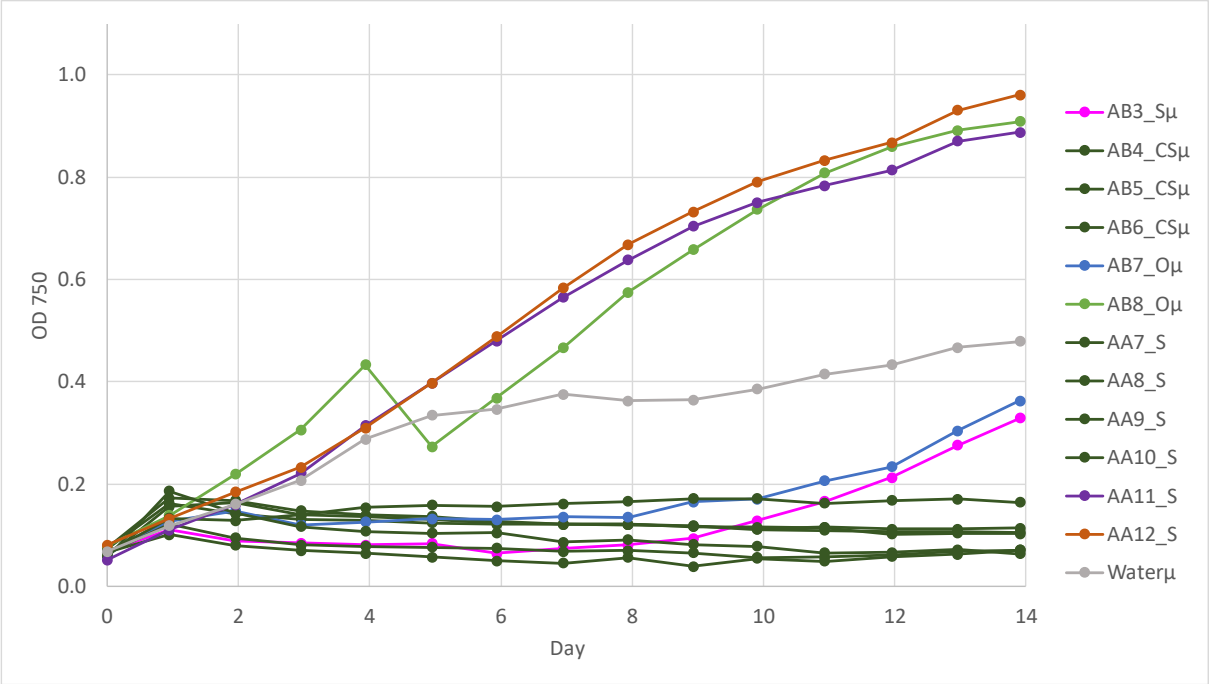


Figure 38 OD_{750} chart from WP3 where the batch 3 WSs with micronutrients were tested as growth media for C. vulgaris. Abbreviations: AA = Aller Aqua, AB= AquaBioTech, O = outlet, S = sludge CS = concentrated sludge, μ = APN, BBM = Bolds basal medium (positive control)

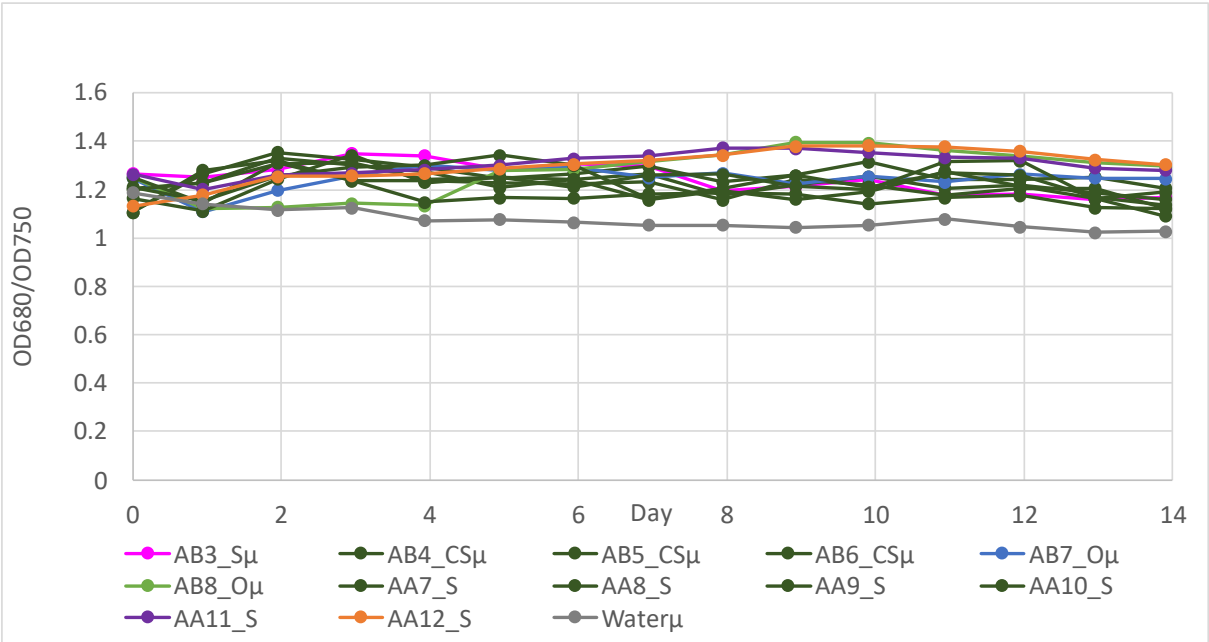


Figure 38 OD_{680} / OD_{750} chart from WP3 where the batch 3 WSs were tested with the addition of micronutrients as growth media for C. vulgaris. Abbreviations: AA = Aller Aqua, AB= AquaBioTech, O = outlet, S = sludge CS = concentrated sludge, μ = APN, BBM = Bolds basal medium (positive control)

Picture 3 Visual control on the final day of WP3 where the batch 3 WSs were tested with the addition of micronutrients as growth media for C. vulgaris. Abbreviations: AA = Aller Aqua Research, AB= AquaBioTech, µ = APN, W = water (negative control) , BBM = Bolds basal medium (positive control)

Table 11 SD of the OD_{750} of biological triplicates in WP3 where the batch 3 WSs were tested with and without the addition of micronutrients as growth media for C. vulgaris. Abbreviations: AA = Aller Aqua, AB= AquaBioTech, O = outlet, S = sludge CS = concentrated sludge, µ = APN, BBM = Bolds basal medium (positive control

Medium	day														
	0.00	0.94	1.95	2.95	3.93	4.94	5.93	6.94	7.94	8.92	9.90	10.93	11.95	12.95	13.90
	standard deviation - biologicl triplicates														
AB3_S	0.01	0.00	0.01	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02
AB3_Sµ	0.01	0.01	0.02	0.01	0.01	0.01	0.01	0.02	0.02	0.03	0.02	0.03	0.04	0.06	0.06
AB4_CS	0.00	0.01	0.01	0.01	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.03	0.01
AB4_CSµ	0.01	0.01	0.00	0.01	0.00	0.00	0.01	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01
AB5_CS	0.00	0.01	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
AB5_CSµ	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
AB6_CS	0.00	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.01	0.02	0.01	0.01	0.01	0.01	0.01
AB6_CSµ	0.01	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.00
AB7_O	0.01	0.01	0.01	0.01	0.04	0.06	0.07	0.08	0.09	0.08	0.09	0.09	0.09	0.08	0.08
AB7_Oµ	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.02	0.03	0.05	0.06	0.08	0.10	0.12	0.14
AB8_O	0.01	0.10	0.32	0.50	0.02	0.07	0.10	0.11	0.11	0.12	0.12	0.12	0.13	0.13	0.13
AB8_Oµ	0.02	0.08	0.18	0.25	0.38	0.04	0.05	0.07	0.08	0.08	0.11	0.09	0.09	0.09	0.09
BBM	0.00	0.00	0.01	0.01	0.01	0.02	0.02	0.03	0.04	0.04	0.04	0.06	0.08	0.09	0.12
BBMµ	0.00	0.00	0.01	0.01	0.01	0.02	0.03	0.03	0.04	0.04	0.04	0.04	0.04	0.04	0.04
AA7_S	0.00	0.00	0.01	0.01	0.01	0.01	0.02	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.01
AA7_Sµ	0.01	0.01	0.00	0.01	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.03
AA8_S	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.01	0.01	0.01
AA8_Sµ	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
AA9_S	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.01	0.01
AA9_Sµ	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01
AA10_S	0.00	0.01	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
AA10_Sµ	0.00	0.01	0.00	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.00	0.00	0.00	0.01	0.00
AA11_S	0.00	0.01	0.01	0.01	0.02	0.03	0.04	0.05	0.06	0.06	0.05	0.04	0.04	0.04	0.04
AA11_Sµ	0.01	0.00	0.01	0.03	0.05	0.07	0.11	0.13	0.15	0.16	0.18	0.17	0.17	0.16	0.17
AA12_S	0.01	0.01	0.02	0.02	0.03	0.04	0.06	0.06	0.05	0.06	0.06	0.05	0.06	0.05	0.06
AA12_Sµ	0.01	0.00	0.00	0.01	0.02	0.04	0.06	0.07	0.08	0.09	0.09	0.10	0.09	0.09	0.09
water	0.01	0.02	0.02	0.02	0.02	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03
waterµ	0.01	0.07	0.10	0.15	0.23	0.25	0.22	0.23	0.18	0.16	0.19	0.23	0.25	0.28	0.30

WP4

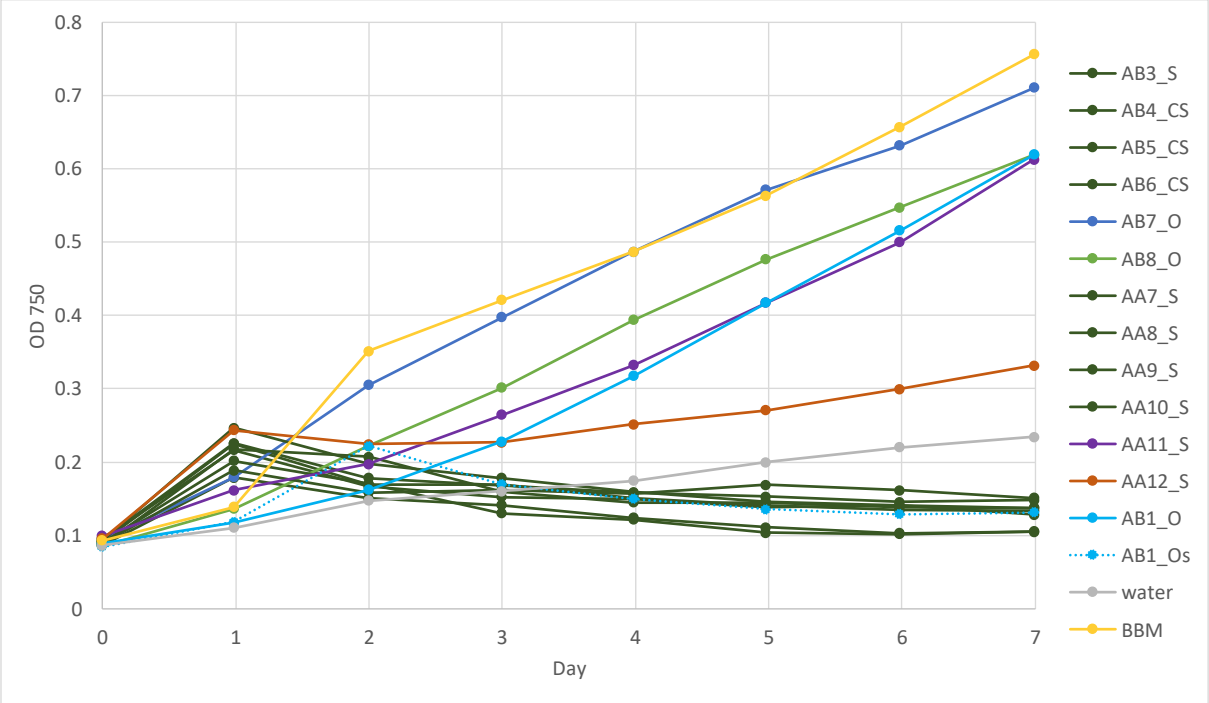


Figure 39 OD_{750} chart from WP4 where the batch 3 WSs were tested sterile-filtered without the addition of micronutrients as growth media for C. vulgaris. Abbreviations: AA = Aller Aqua, AB= AquaBioTech, O = outlet, S = sludge CS = concentrated sludge, s = sterile filtrated, BBM = Bolds basal medium (positive control)

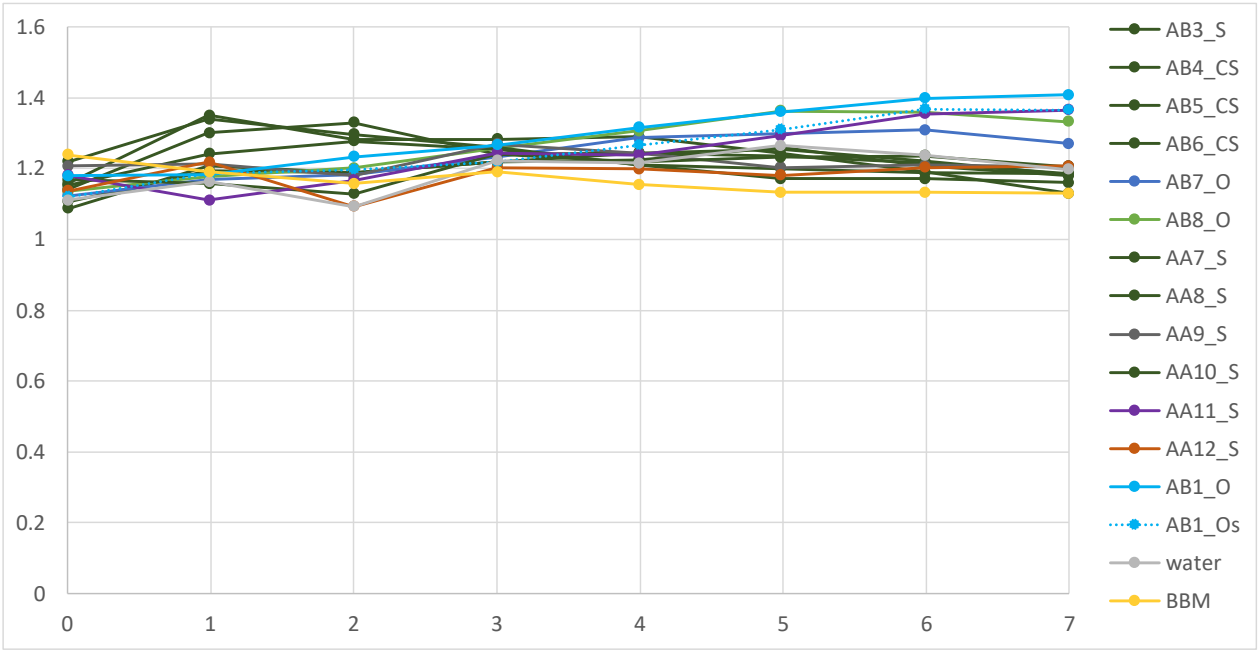


Figure 40 OD_{680} / OD_{750} chart from WP4 where the batch 3 WSs were tested sterile-filtered without the addition of micronutrients as growth media for C. vulgaris. Abbreviations: AA = Aller Aqua, AB= AquaBioTech, O = outlet, S = sludge CS = concentrated sludge, s = sterile filtrated, BBM = Bolds basal medium (positive control)

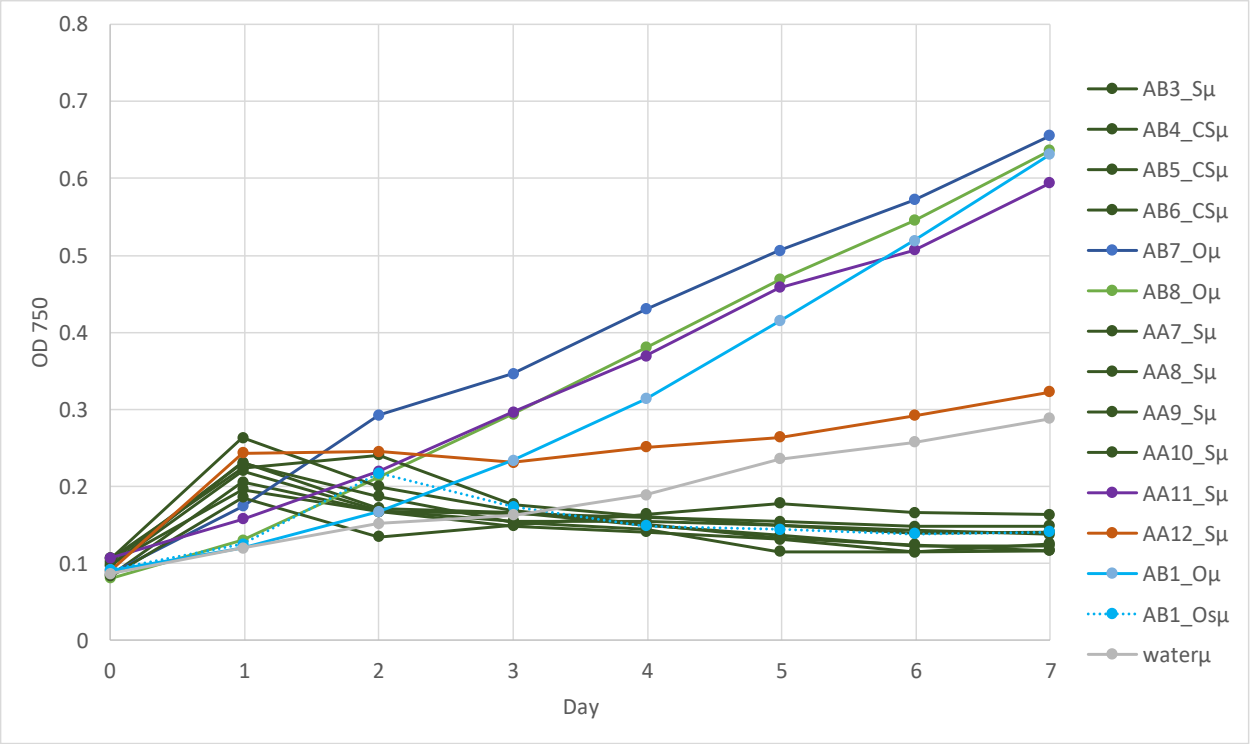


Figure 42 OD_{750} chart from WP4 where the batch 3 WSs were tested sterile-filtered with the addition of micronutrients as growth media for C. vulgaris. Abbreviations: AA = Aller Aqua, AB= AquaBioTech, O = outlet, S = sludge CS = concentrated sludge, μ = APN, s = sterile filtrated

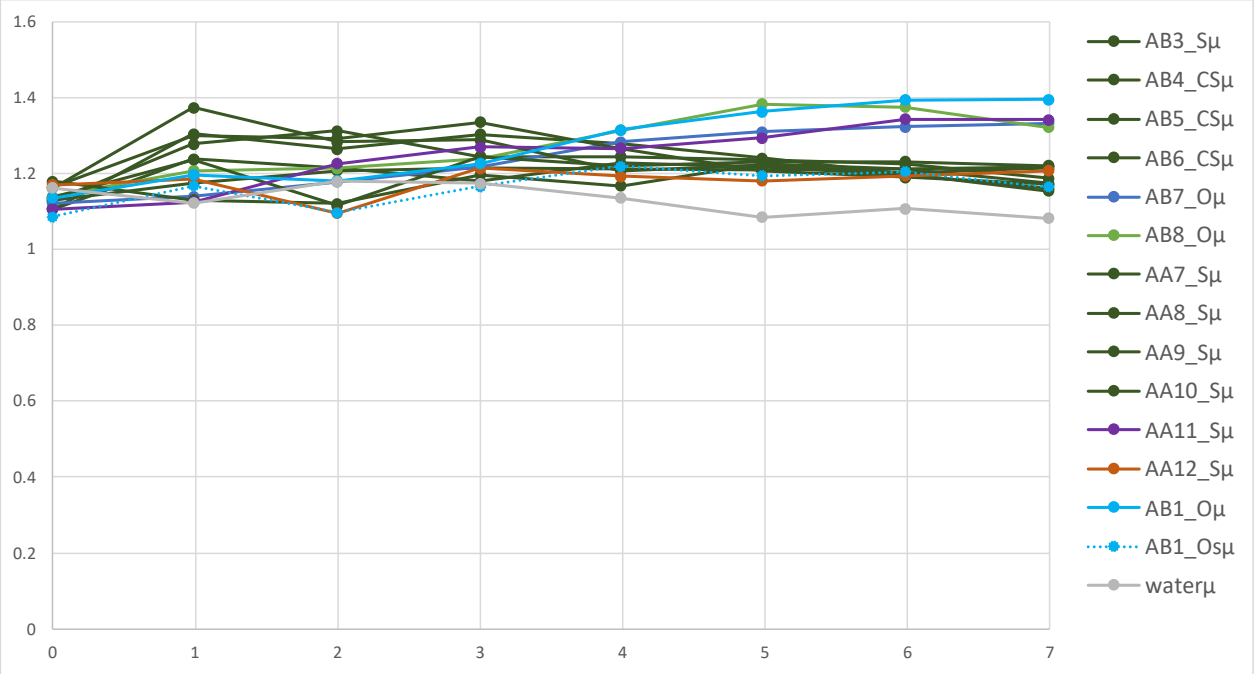
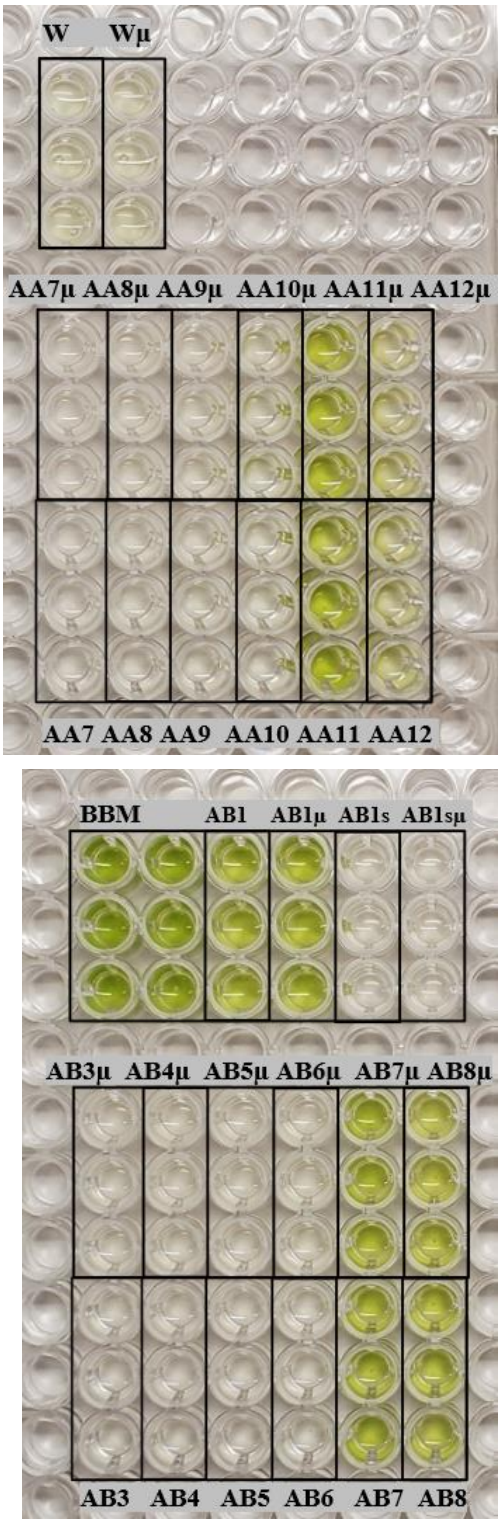


Figure 41 OD_{680} / OD_{750} chart from WP4 where the batch 3 WSs were tested sterile-filtered with the addition of micronutrients as growth media for C. vulgaris. Abbreviations: AA = Aller Aqua, AB= AquaBioTech, O = outlet, S = sludge CS = concentrated sludge, s = sterile filtrated

Table 12 SD of the OD_{750} of biological triplicates in WP4 where the batch 3 WSs were tested sterile-filtered with and without the addition of micronutrients as growth media for C. vulgaris. Abbreviations: AA = Aller Aqua, AB= AquaBioTech, O = outlet, S = sludge CS = concentrated sludge, s = sterile filtrated, BBM = Bolds basal medium (positive control)

Medium	day							
	0.00	0.99	2.00	3.00	3.99	4.98	5.99	6.99
	standard deviation - biological triplicates							
AB3_S	0.02	0.01	0.01	0.00	0.00	0.00	0.00	0.01
AB3_Sµ	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.01
AB4_CS	0.01	0.01	0.01	0.00	0.01	0.00	0.01	0.01
AB4_CSµ	0.00	0.01	0.01	0.00	0.01	0.01	0.01	0.01
AB5_CS	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.01
AB5_CSµ	0.00	0.01	0.00	0.01	0.01	0.01	0.02	0.01
AB6_CS	0.00	0.01	0.01	0.01	0.01	0.02	0.01	0.01
AB6_CSµ	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.01
AB7_O	0.01	0.01	0.01	0.02	0.02	0.03	0.03	0.05
AB7_Oµ	0.00	0.01	0.01	0.02	0.03	0.03	0.03	0.03
AB8_O	0.00	0.01	0.01	0.01	0.01	0.02	0.02	0.01
AB8_Oµ	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.01
BBM	0.01	0.02	0.02	0.02	0.02	0.01	0.02	0.03
AB1_O	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.00
AB1_Oµ	0.01	0.01	0.01	0.01	0.01	0.00	0.01	0.01
AB1_Os	0.00	0.01	0.01	0.01	0.01	0.00	0.00	0.00
AB1_Osµ	0.00	0.01	0.01	0.00	0.00	0.00	0.01	0.01
AA7_S	0.01	0.00	0.00	0.00	0.01	0.00	0.01	0.01
AA7_Sµ	0.01	0.01	0.01	0.01	0.02	0.01	0.01	0.02
AA8_S	0.01	0.00	0.00	0.01	0.01	0.01	0.02	0.01
AA8_Sµ	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01
AA9_S	0.01	0.01	0.01	0.01	0.00	0.00	0.01	0.01
AA9_Sµ	0.01	0.00	0.01	0.01	0.01	0.01	0.01	0.01
AA10_S	0.01	0.02	0.02	0.01	0.01	0.01	0.01	0.01
AA10_Sµ	0.00	0.00	0.00	0.01	0.01	0.01	0.01	0.01
AA11_S	0.02	0.01	0.02	0.05	0.07	0.08	0.10	0.09
AA11_Sµ	0.02	0.01	0.01	0.02	0.02	0.02	0.02	0.02
AA12_S	0.01	0.02	0.01	0.02	0.03	0.04	0.06	0.08
AA12_Sµ	0.00	0.01	0.01	0.01	0.02	0.03	0.04	0.05
water	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.02
waterµ	0.00	0.00	0.00	0.01	0.03	0.08	0.10	0.13



Picture 4 Visual control on the final day of WP4 where the sterile-filtered batch 3 WSs were tested as growth media for C. vulgaris with and without micronutrients. Abbreviations: AA = Aller Aqua Research, AB= AquaBioTech, µ = APN, W = water (negative control), BBM = Bolds basal medium (positive control)

WP5

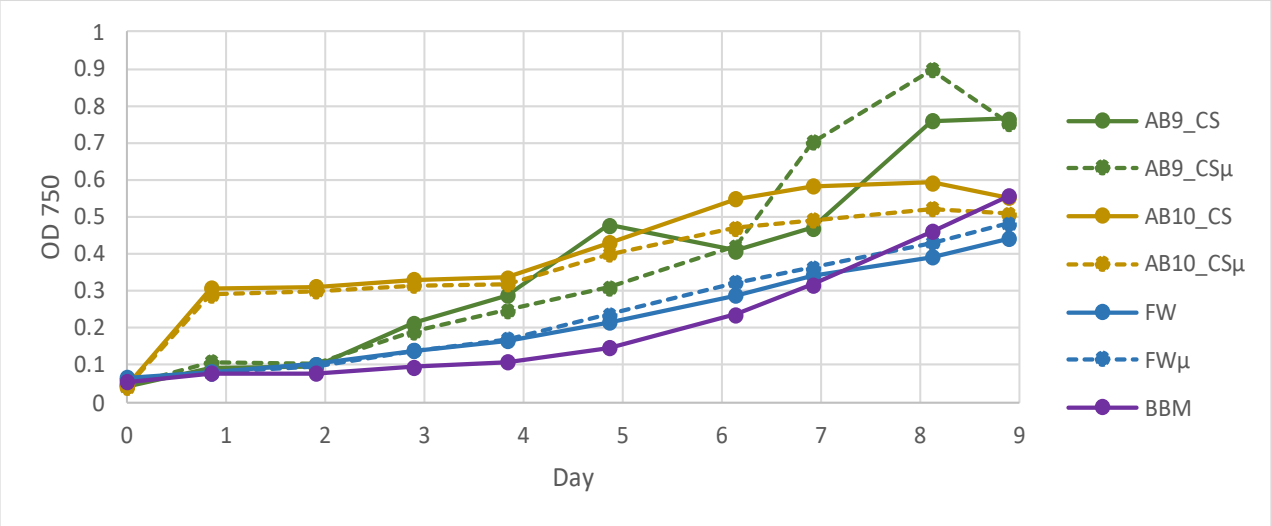


Figure 43 OD_{750} chart from WP5. Abbreviations: AB = AquaBioTech, CS = concentrated sludge, μ = APN, FW = fresh water (negative control), BBM = Bold's basal medium (positive control)

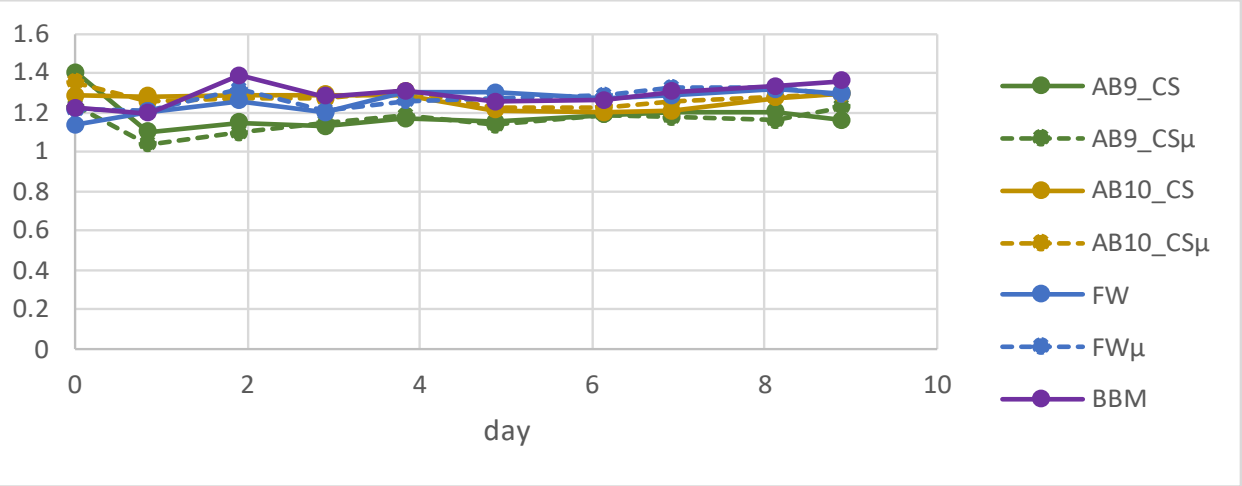
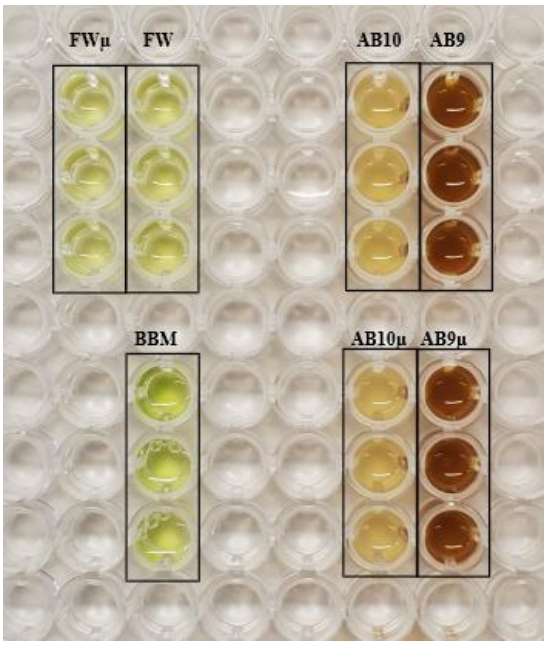


Figure 44 OD_{680} / OD_{750} chart from WP5. Abbreviations: AB = AquaBioTech, CS = concentrated sludge, μ = APN, FW = fresh water (negative control), BBM = Bold's basal medium (positive control)



Picture 5 Visual control on the final day of WP5 where the batch 4 WSs were tested sterile-filtered as growth medium for C. vulgaris with and without the addition of micronutrients. Abbreviations: AB = AquaBioTech, CS = concentrated sludge, µ = APN, FW = fresh water (negative control), BBM = Bold's basal medium (positive control)

Table 13 SD of the OD_{750} of biological triplicates in WP5 where the batch 4 WSs were tested sterile-filtered with and without the addition of micronutrients as growth media for C. vulgaris. Abbreviations: AA = Aller Aqua, AB= AquaBioTech, CS = concentrated sludge, BBM = Bolds basal medium (positive control), FW = fresh water (negative control)

Medium	day									
	0.00	0.84	1.90	2.90	3.83	4.87	6.14	6.92	8.12	8.89
	standard deviation - biological triplicates									
AB9_CS	0.00	0.00	0.01	0.07	0.12	0.34	0.18	0.12	0.26	0.31
AB9_CSµ	0.01	0.02	0.01	0.01	0.02	0.01	0.14	0.34	0.13	0.16
AB10_CS	0.00	0.01	0.02	0.03	0.04	0.02	0.04	0.04	0.03	0.04
AB10_CSµ	0.00	0.01	0.02	0.01	0.02	0.01	0.03	0.03	0.04	0.05
FW	0.01	0.00	0.01	0.00	0.01	0.01	0.01	0.02	0.02	0.02
FWµ	0.01	0.00	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.01
BBM	0.00	0.00	0.00	0.00	0.00	0.02	0.05	0.06	0.09	0.10

WP6

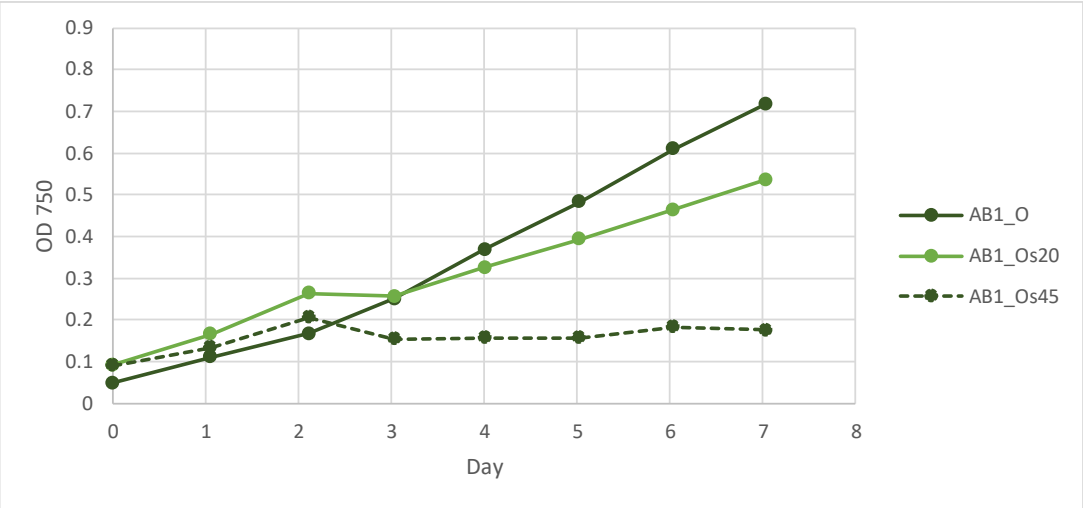


Figure 45 OD_{750} chart from WP6 where the AB1_O WS from batch 1 was retested as growth medium for C. vulgaris 20µm and 40µm sterile filtrated. Abbreviations: AB= AquaBioTech, O = outlet, s=sterile-filtered, 20 = 20µm filter, 45 = 45µm filter

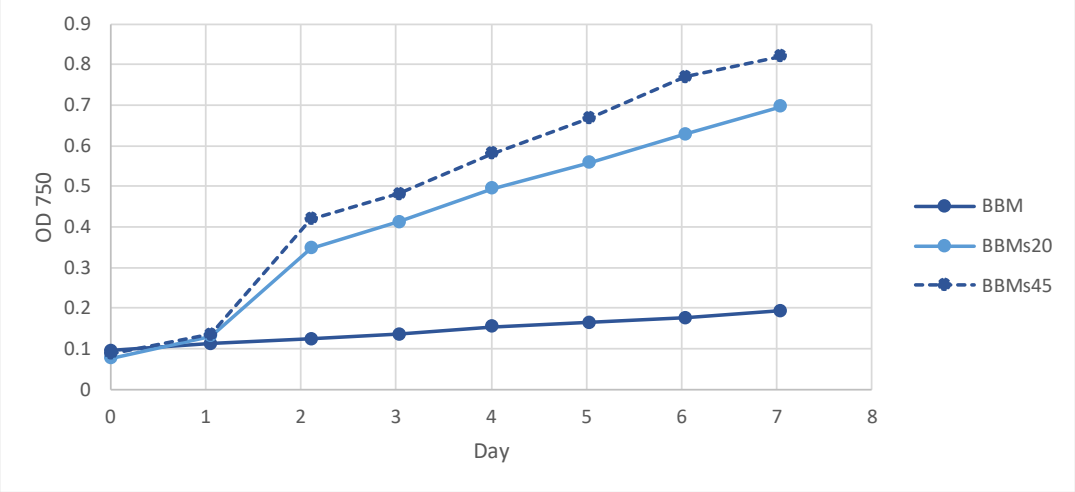


Figure 46 OD_{750} chart from WP6 where three-month-old BBM was retested as growth medium for C. vulgaris 20µm and 40µm sterile filtrated. Abbreviations: s=sterile-filtered, 20 = 20µm filter, 45 = 45µm filter, BBM = Bolds basal medium

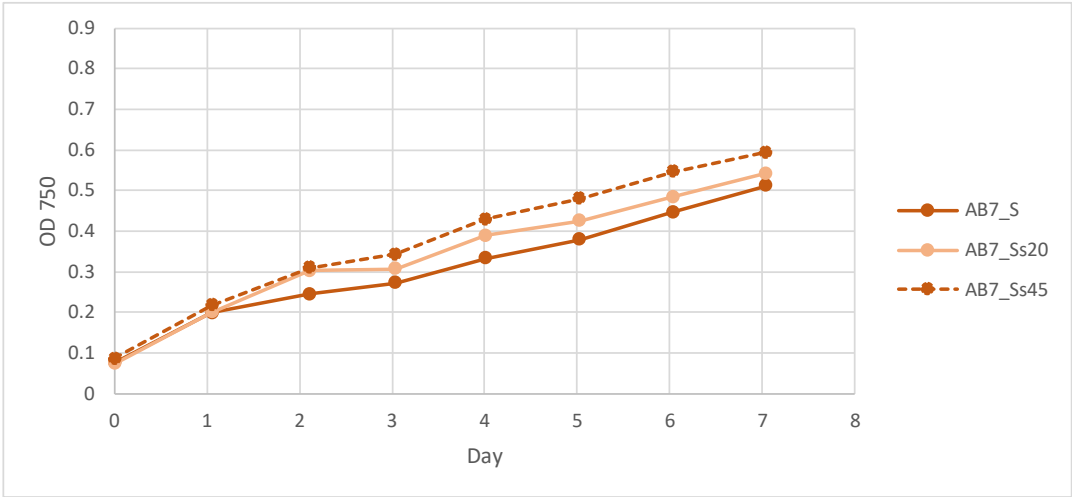
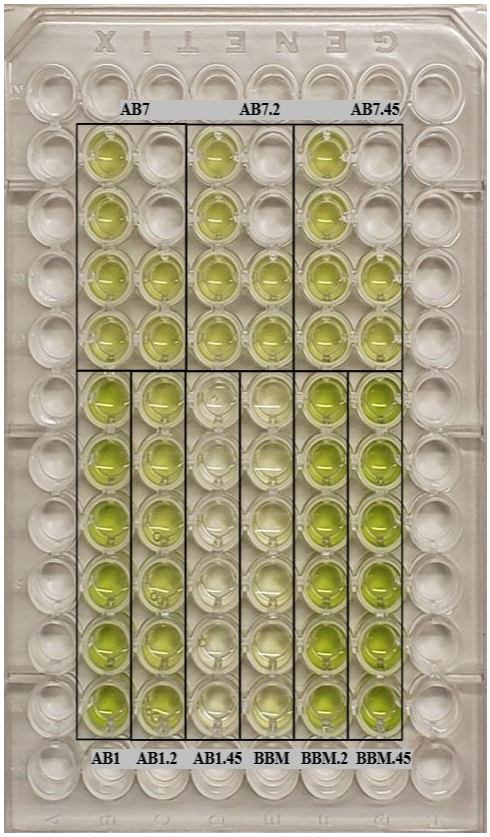


Figure 47 OD_{750} chart from WP6 where the AB7_S WS from batch 3 was retested as growth medium for C. vulgaris 20µm and 40µm sterile filtrated. Abbreviations: AB= AquaBioTech, S = sludge, s=sterile-filtered, 20 = 20µm filter, 45 = 45 µm filter



Picture 6 Visual control on the final day of WP6 where AB1_O, AB7_S and BBM were retested as growth media for C. vulgaris 20µm and 40µm sterile filtrated. Abbreviations: AB= AquaBioTech, BBM = Bolds basal medium, s = sterile filtrated, 2 = 20µm filter, .45 = 40µm filter

Table 14 SD of the OD_{750} of biological sextuplicate in WP where AB1_O, AB7_S and BBM were retested as growth media for C. vulgaris 20µm and 40µm sterile filtrated. Abbreviations: AB= AquaBioTech, BBM = Bolds basal medium, s = sterile filtrated, 20 = 20µm filter, .45 = 40µm filter

Medium	day							
	0.000	1.050	2.106	3.031	4.007	5.024	6.039	7.037
	standard deviation - biological sexduplicates							
AB1_O	0.01	0.01	0.00	0.01	0.01	0.02	0.02	0.02
AB1_Os20	0.01	0.06	0.15	0.01	0.02	0.04	0.06	0.08
AB1_Os45	0.00	0.01	0.03	0.02	0.03	0.03	0.07	0.03
BBM	0.01	0.00	0.01	0.01	0.02	0.03	0.03	0.04
BBMs20	0.01	0.02	0.04	0.04	0.03	0.03	0.03	0.02
BBMs45	0.00	0.00	0.01	0.01	0.01	0.02	0.02	0.01
AB7_S	0.01	0.01	0.01	0.02	0.02	0.02	0.02	0.02
AB7_Ss20	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.03
AB7_Ss45	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.03

Appendix C: Bubble column

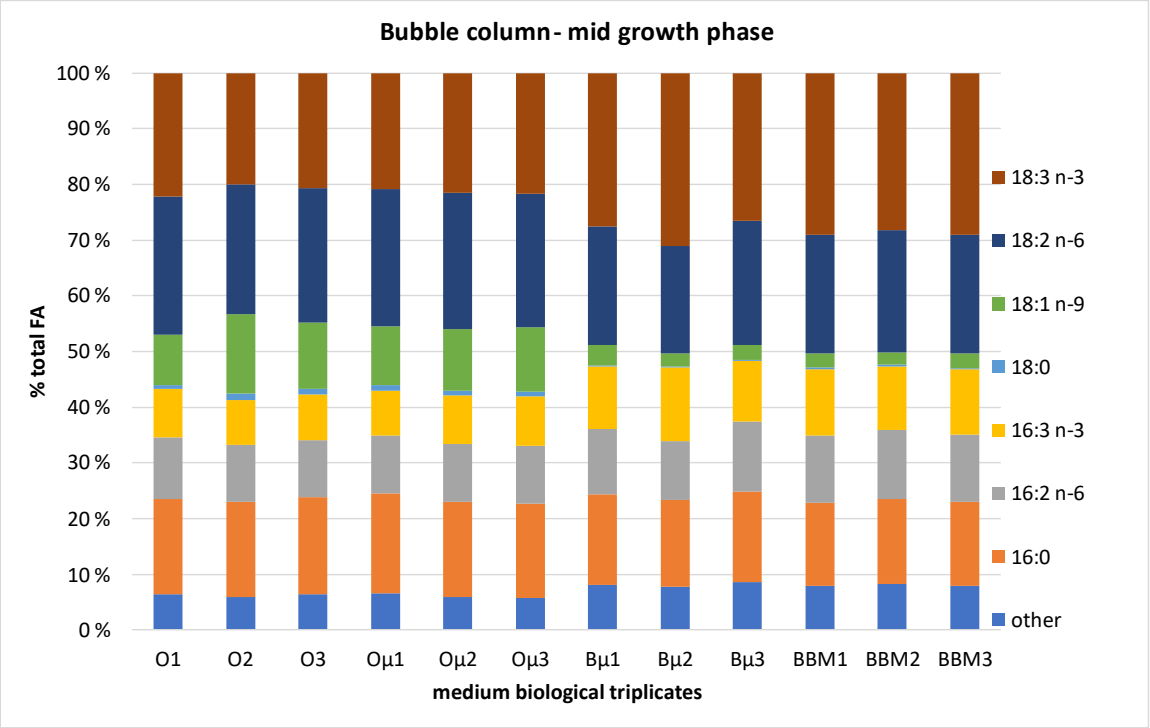


Figure 48 The fatty acid content of the various media in the bubble column experiment was analysed at mid growth stage. Samples from each biological triplicate was analysed (1-3). The quantities are presented as relative fatty acid content. O = original waste stream, μ = addition of micronutrients, B = waste stream with N:P ratio equal BBM, BBM = Bold's Basal Medium/control. The different colours indicate the different fatty acids as shown in the figure legend. Branched alcohol and unknown fatty acids have been combined under "other".