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Research article

Enhancement of targeted microalgae species growth using aquaculture sludge extracts

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ABSTRACT

Natural growth-promoting nutrients extracted from aquaculture sludge waste can be used to maximise microalgal growth. This study identified the influence of aquaculture sludge extract (SE) on four microalgae species. Conway or Bold's Basal Media (BBM) was supplemented with SE collected from a Sabak Bernam shrimp pond (SB) and Kota Puteri fish pond (KP), and tested using a novel microplate-incubation technique. Five different autoclave extraction treatment parameters were assessed for both collected SE, i.e., 1-h at 105 °C, 2-h at 105 °C, 1-h at 121 °C, 2-h at 121 °C, and 24-h at room temperature (natural extraction). Microalgae culture in the microplates containing control (media) and enriched (media + SE) samples were incubated for nine days, at 25 °C with the light intensity of 33.75 μmol photons m^{-2} s^{-1} at 12-h light/dark cycle. The total dissolved nitrogen (TDN) and total dissolved phosphorus (TDP) in KP SE were 44.0–82.0 mg L^{-1} and 0.96–8.60 mg L^{-1}. TDN (8.0%–515.0%) and TDP (105%–186 %) were relatively higher in KP SE compared to SB SE. The growth of microalgae species *Nannochloropsis ocenica* showed significant differences ($p < 0.05$) between the five extraction treatments from SB and the control. However, *Chlorella vulgaris*, *Neochloris conjuncta*, and *Nephroclamys subsolitaria* showed no significant differences ($p > 0.05$) in SB SE. *N. ocenica*, *C. vulgaris*, and *N. conjuncta* showed significant differences ($p < 0.05$) between five extraction treatments from KP and the control while *N. subsolitaria* showed no significant difference ($p > 0.05$). The specific growth rate (SGR) in the exponential phase of all microalgae species were relatively higher in SB SE compared to KP SE. While the organic matter content of KP SE was relatively higher, there were no significant differences in microalgae growth compared to SB SE. Nonetheless, modified SE did influence microalgae growth compared to the control. This study shows that modified SE could be used as enrichment media for microalgae cultivation.

1. Introduction

The aquaculture industry is growing faster than any other major food production sectors, with global aquaculture production accounting for 80.0 million tonnes of food fish and 30.1 million tonnes of aquatic plants, as well as 37,900 tonnes of non-food products in 2016 (Food and Agriculture Organization of the United Nations (FAO), 2018). Due to the increased demand for aquaculture products, more food is produced, causing increased pollution of soil and irrigation water (Turcios and Papenbrock, 2014). However, the growing footprint of aquacultures along the coast is drastically altering the ecosystem of the areas (Cao et al., 2007). For example, large volumes of aquaculture sludge are discharged from ponds, resulting in eutrophication and degradation of the regional environments. Nevertheless, aquaculture sludge often contains rich organic nutrients that can be recycled for primary production, such as in the mass culture of high-value microalgae species. In other words,

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aquaculture sludge has the potential to be an enrichment medium for primary production, and also acts as an alternative of utilising wastewater discharge (Khatoon et al., 2018).

Aquaculture sludge is a residue containing adsorbed and insoluble matter formed during the treatment of wastewater. The residual sludge also contains nutrient-rich organic materials. Recycling beneficial biosolids through land use and bioremediation has become a regular practice in several countries across the world (Madariaga and Marín, 2017). For example, 25%–89% of biosolids production was recycled as an alternative to disposal (Escudey et al., 2007). Further, mass cultivation of high-value microalgae species for the production of valuable products such as nutraceuticals, cosmeceuticals, pharmaceuticals, aquaculture feeds, and biofuels may also be conducted using aquaculture sludge. Previous studies have demonstrated that microalgae growth in aquaculture wastewater media is possible (Nasir et al., 2015; Michels et al., 2014; Guo et al., 2013).

Microalgae are an incredibly diverse group of eukaryotic organisms commonly found in marine and freshwater systems (Daneshvar et al., 2018). They can grow as single cells or in chains or small colonies (Postma et al., 2016), and play a significant role in marine ecosystems due to their photosynthetic capacity (Malcata et al., 2018). Besides essential elements like phosphorus, nitrogen, and iron, micronutrients are also required for microalgae growth. Microalgae have carbon-rich compounds that can be used in biofuels, pharmaceuticals, cosmetics, supplements, animal feeds, and more (Das et al., 2011). They also produce various bioproducts such as antioxidants, polysaccharides, bioactive compounds, proteins, vitamins, pigments, and lipids (Brennan and Owende, 2010).

Cell culture flasks are traditionally used to screen and test the growth of microalgae that can be expensive and time-consuming (Huesemann et al., 2016). In recent years, new screening methods are employed to cultivate selected microalgae using a microplate-incubation technique (Zhao et al., 2018). Microplates are an ideal option for high-throughput studies as they are reliable, fast, cost-effective, and do not require intensive labour (Van Wagenen et al., 2014). Previously, microplates were mostly used in clinical microbiology and not extensively used in environmental field studies. Dupraz et al. (2018) showed that microplates could be used for toxicity studies of antifouling compounds for three marine microalgae *Tisochrysis lutea*, *Skeletonema marinoi*, and *Tetraselmis suecica*. Van Wagenen et al. (2014) also utilised microplates for the high-throughput screening of microalgae growth. Thus, microplates can be an ideal platform for testing microalgae culture for medium to high-throughput screening purposes (Pacheco et al., 2013).

In this study, aquaculture sludge from two ponds, Sabak Bernam shrimp pond (SB) and Kota Puteri fish pond (KP), were sampled to determine their natural growth-promoting effects on microalgae. These two types of sludge are shown to have sufficient carbon, nitrogen and phosphorus that are important for microalgae, and the lack of any of these organic compounds may be a limiting factor in the growth of algae (Cruz et al., 2018). This work aims to evaluate the growth effects of aquaculture sludge extracts (SEs) on specific microalgae species. More specifically, our goal is to determine the enhancement potential of aquaculture wastes for mass culture of high-value microalgae.

2. Materials and methods

2.1. Sludge extracts (SEs)

Sludge was collected from two types of ponds, i.e., SB shrimp pond and KP fish pond. The collected sludge from each pond amounted to 2 kg mixture per site. Coarse particles such as pond snails, wood chips, and stones were removed by hand, and the samples were oven-dried at 60 °C for one week to remove moisture. The dried sludge was then ground using 700 g Swing Type Electric Herbal Powder Grinder (Weifang City, Shandong, China), sieved at 1 mm, and homogenised. The samples were collected at three (triangle) 1-m distance points.

Aqueous extraction treatment was carried out on the dried sludge samples. Milli-Q water or pure water was used as a solvent in the preparation of SE (Watanabe, 2005), as it dissolves a variety of substances compared to other liquids. Five autoclave extraction parameters were carried out on the sludge samples: 1-h at 105 °C, 2-h at 105 °C (twice), 1-h at 121 °C, 2-h at 121 °C (twice), and no autoclave, 24-h at room temperature. The selection for these parameters was based on autoclaved results from previous studies using similar temperatures such as 105 °C (Mercier et al., 2015) and 121 °C (Li et al., 2015) to sterilise the sludge samples. For each sludge sample, 20 g dried sludge was mixed with ultra-pure Milli-Q water (1:10) in 500 mL Schott bottles. For room temperature aqueous extraction, sludge samples were incubated in the dark for 24 h and then autoclaved at 121 °C for 1 h using the methods modified from Li et al. (2015). For high-temperature aqueous extraction, samples were autoclaved for 1 h at 105 °C and 121 °C. Two additional temperature treatments were adapted by conducting the autoclave for 1-h, twice at 105 °C and 121 °C for a total of 2-h each. These methods were modified from Mercier et al. (2015), where the sludge samples were autoclaved at 105 °C and 121 °C for 2-h each. Temperature-treated sludge samples were then centrifuged at 700 × *g* for 15 min using Allegra-30R centrifuge (Beckman, Indiana, United States). The supernatant (ca. 150 mL) of SE was filtered through a 0.7 μm glass fibre filter (GF/F, Whatman). The filtered samples were stored at 4 °C until further use.

Chemical analysis of all five temperature-treated SE filtrates from SB and KP were conducted. Total dissolved nitrogen (TDN) and total dissolved phosphorus (TDP) were analysed using MD600/MaxiDirect photometer system (Lovibond Tintometer, Amesbury, United Kingdom). TDN of SB and KP SE filtrates were analysed using a Vario Total Nitrogen LR Set, while TDP samples were analysed using a Vario Total Phosphate Reagent Set from Lovibond. A colourimetric assay was used to analyse TDN and TDP of SB and KP SE filtrates based on the manufacturer's instruction manual, where nitrogen and phosphorus levels were calculated in mg L^{-1}. The concentrations of the samples were positively correlated with colour intensity (Kawasaki et al., 2016). Each sample was measured in triplicate, and the average value was estimated.

2.2. Microalgae

The target microalgal species used in this study were *Nannochloropsis ocenica* (TRG 3A), *Chlorella vulgaris* (TRG 4C), *Neochloris conjuncta* (KDH3-C01), and *Nephroclamys subsolitaria* (KDH3-C05). *N. ocenica* and *C. vulgaris* were isolated from Kapas and Bidong islands at Terengganu, Malaysia, while *N. conjuncta* and *N. subsolitaria* were isolated from Tasik Dayang Bunting, Kedah, Malaysia. For marine microalgae (*N. ocenica* and *C. vulgaris*), Conway media was prepared from five basic solutions as described by Khatoon et al. (2016); mineral solution –100 g of $NaNo_3$, 45 g of disodium EDTA ($C_6H_{16}N_2O_8$), 33.6 g of H_3BO_3, 20 g of $NaH_2PO_4.4H_2O$, 1.3 g of $FeCl_3.6H_2O$, 0.36 g of $MnCl_2.4H_2O$, and 1 mL trace metal solution in 1 L of Milli-Q water; trace metal solution – 0.21 g of $ZnCl_2$, 0.2 g of $CoCl_3.6H_2O$, 0.09 g of $(NH_4)_6MO_7O_2.4H_2O$, and 0.2 g of $CuSO_4.5H_2O$ in 100 mL Milli-Q water; vitamin solution – 0.2 g of thiamine (B1), cyanocobalamin (B12) in 100 mL of Milli-Q water; silicate solution – 2 g of Na_2SiO_3 in 100 mL of Milli-Q water; and nitrate solution – 2 g of KNO_3 in 100 mL of Milli-Q water. The media were prepared by adding 1 mL of main mineral, silicate, and nitrate solution to the Schott bottle to prepare 1 L volume media. After autoclaving the prepared media, 1 mL of NH_4Cl and vitamin solution were added into the cooled medium to give a final medium concentration of 5.0×10^{-4} M. For freshwater microalgae (*N. conjuncta* and *N. subsolitaria*), Bold's Basal Media (BBM) was prepared (Bischoff, 1963). The 1000 μL of stock microalgae culture was inoculated into 50 mL sterilised media in an autoclaved conical flask. The cultures were grown at 25 ± 0.5 °C under a light intensity of 33.75 μmol photons m^{-2} s^{-1} on a 12 h light: 12 h dark cycle. The stock cultures were acclimatised to the experimental

conditions prior to the experiment before the strains were tested on sludge extracts.

Microplate-incubation technique was carried out for the four microalgal species in the five different extraction parameters of SE using 96-well microplates (Figure 1). Each well in the microplate can be filled up to 200 μL of solution. The border wells of the microplate were filled with 200 μL Milli-Q water to prevent evaporation during the study. Previous studies showed that the border wells of the microplates were not used during experiments as it exposes wells to strong currents of air, although microalgae growing in the border wells have more access to light and CO_2 (Blaise & Ferard, 2005; St-Laurent et al., 1992; Rojíčková et al., 1998). The remaining wells were filled with 195 μL of suitable media + 5 μL of 105 °C SE in the 2nd column (blank), and the 3rd column filled with 175 μL of suitable media + 5 μL of 105 °C SE + 20 μL of microalgae (experiment) as shown in Figure 1 to record the exponential phase of microalgae. The same steps were repeated in the 4th to 11th columns of a microplate with 105 °C twice (Column 5), 121 °C (Column 7), 121 °C twice (Column 9), and 24 h natural extraction (Column 11). For the control experiment, suitable media (Conway or BBM) without SE was used to test with the four different microalgae in another microplate. The microplates were sealed with parafilm after pipetting all the wells in the microplate to prevent evaporation by preserving the air humidity in the microplate wells and preventing external contamination prior to incubation. The microplates were incubated for nine days, and the biomass or growth of microalgae was determined by optical density (OD) at 680 nm for every 24 h using the microplate reader Infinite M200 PRO (Tecan, Austria). For every 24 h of OD measurement, each one of the wells containing controls and samples was mixed using 8-channel Eppendorf pipettor before measuring the OD to mix the microalgae suspended in the bottom well with the solution.

2.3. Data analysis

Three microplate replicates for each control and sample in a column were tested. The optical density (OD) of the control and sample was subtracted to get the net OD mean value. OD measurements were used in this study to determine microalgal biomass as it is simple, fast, and a commonly used technique to measure algal culture density (Sharma et al., 2016; Ding et al., 2015; Bohutskyi et al., 2015). The specific growth rate (μ) and the division rate (k) of microalgae were calculated as follows,

$$\mu = \frac{\ln(N_2 - N_1)}{(t_2 - t_1)} \quad (1)$$

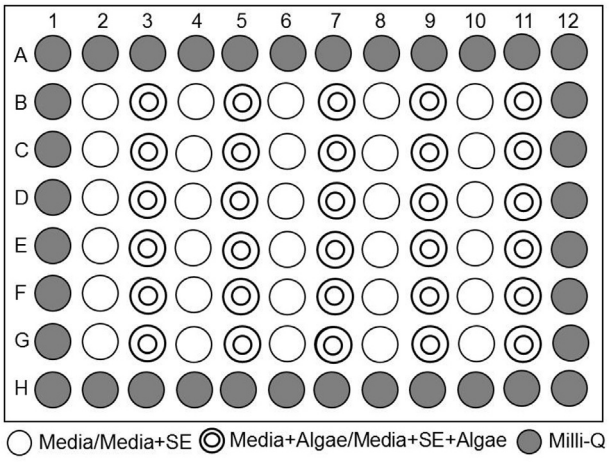

Figure 1. Microplate-incubation technique of media or media + soil extract (SE) and growth test (media + microalgae or media + SE + microalgae).

$$k = \frac{\mu}{ln2} \quad (2)$$

where N_2 and N_1 are the OD at times t_2 and t_1 respectively.

The TDN and TDP content, growth of microalgae, and maximum OD in respective temperature treatment parameters of SB and KP SE were analysed using independent samples t-test and one-way analysis of variance (ANOVA). Significant differences between the different extraction parameters were calculated at 95% confidence interval level. All statistical analyses were done using IBM SPSS (Statistical Package for the Social Sciences) statistics 20 software.

3. Results

3.1. Chemical analysis of aqueous extraction parameters from SB and KP SE

Potential natural growth-promoting materials from SB and KP SE were added together with suitable culture media to maximise microalgal growth. The organic matters in SE, especially TDN and TDP, may influence the growth of microalgae. KP SE showed increased organic matter in terms of TDN and TDP compared to SB SE in all temperature treatments, including the 24-h room temperature treatment (Table 1). Although the 105 °C treatment showed a higher percentage of TDN between SB and KP compared to the 121 °C treatment, the opposite was observed in the TDP extraction result. Notable differences between TDN and TDP in the SB and KP samples were observed when the autoclave treatment was conducted twice compared to once, except for TDP at 121 °C. The TDN and TDP content in SB and KP SE were significantly different ($p < 0.05$) under all extraction parameters.

3.2. Effects of modified SE on the targeted microalgae growth

All four microalgal species showed positive growth patterns under all extraction parameters tested. The growth of *N. ocenica* in the control experiment was lower compared to the five extraction treatments of modified SE (Figure 2A). *N. ocenica* shows significant differences ($p < 0.05$) between all modified SEs to the control. In KP SE, *N. ocenica* grew significantly higher ($p < 0.05$) in media + 121 °C twice compared to the control (Figure 2B).

The growth of *C. vulgaris* in all modified SB SE is approximately similar to the control with no significant results ($P > 0.05$; Figure 3A). *C. vulgaris* growth in KP SE media + 105 °C, media + 105 °C twice, media + 121 °C and media + 121 °C twice are higher compared to the control and media + 24 h (Figure 3B). This microalga shows significant differences ($p > 0.05$) between the five modified SEs tested and for the control.

The growth of *N. conjuncta* in modified SB SE and control is similar (Figure 4A), while growth in the control is lower than all modified KP SE (Figure 4B). Nevertheless, this microalga did not show significant differences ($p > 0.05$) between the five modified SE and control in SB SE but shows significant results ($P < 0.05$) in KP SE. Although *N. subsolitaria* growth in SB SE is not significantly different ($p > 0.05$) between the control and modified SB SE in all treatments, the growth in media + 121 °C twice was relatively higher compared to the others after day 3 until day 8 (Figure 5A). *N. subsolitaria* growth in media + 121 °C twice exhibits higher OD from day 3 day 8. This is mainly due to the rapid cell duplication of *N. subsolitaria* until day 8, and then the growth decreases on day 9. In KP SE, the biomass of *N. subsolitaria* is similar with all modified KP SE (Figure 5B), and no significant differences ($p > 0.05$) are observed between all SEs and the control.

The maximum OD observed for all four microalgae are different between the modified SEs and control (Table 2). Maximum OD of *N. ocenica* is observed in media + 121 °C twice in both SB SE and KP SE. *C. vulgaris* showed maximum OD in media + 105 °C twice and in media + 121 °C twice for SB SE, and media + 105 °C twice, media + 121 °C and media + 121 °C twice for KP SE, respectively. *N. conjuncta* showed higher OD in

Table 1. Total dissolved nitrogen (TDN) and phosphate (TDP) in five extraction parameters of soil extracts (SE) from *Sabak Bernam* shrimp pond (SB) and *Kota Puteri* fish pond (KP).

Extraction parameters	TDN (mg L^{-1})		Percent increase (%)	TDP (mg L^{-1})		Percent increase (%)
	SB SE	KP SE		SB SE	KP SE	
105 °C	25.5 ± 0.02^{d}	44.0 ± 0.01^{e}	73 ± 13.1	2.35 ± 0.01^{c}	5.15 ± 0.00^{d}	119 ± 1.98
105 °C twice	28.5 ± 0.02^{c}	72.0 ± 0.01^{b}	153 ± 30.8	2.10 ± 0.01^{d}	6.00 ± 0.01^{c}	186 ± 2.76
121 °C	60.0 ± 0.02^{b}	65.0 ± 0.01^{c}	8 ± 3.54	3.50 ± 0.01^{b}	8.50 ± 0.01^{b}	143 ± 3.54
121 °C twice	68.5 ± 0.02^{a}	82.0 ± 0.02^{a}	20 ± 9.55	4.20 ± 0.01^{a}	8.60 ± 0.02^{a}	105 ± 3.11
24 h	10.0 ± 0.01^{e}	61.5 ± 0.02^{d}	515 ± 36.4	0.46 ± 0.00^{e}	0.96 ± 0.00^{e}	109 ± 0.35

Note: Values shown are mean of three replicates with + SD.
[a-e] Mean value in same row with different superscripts are significant different ($P < 0.05$).

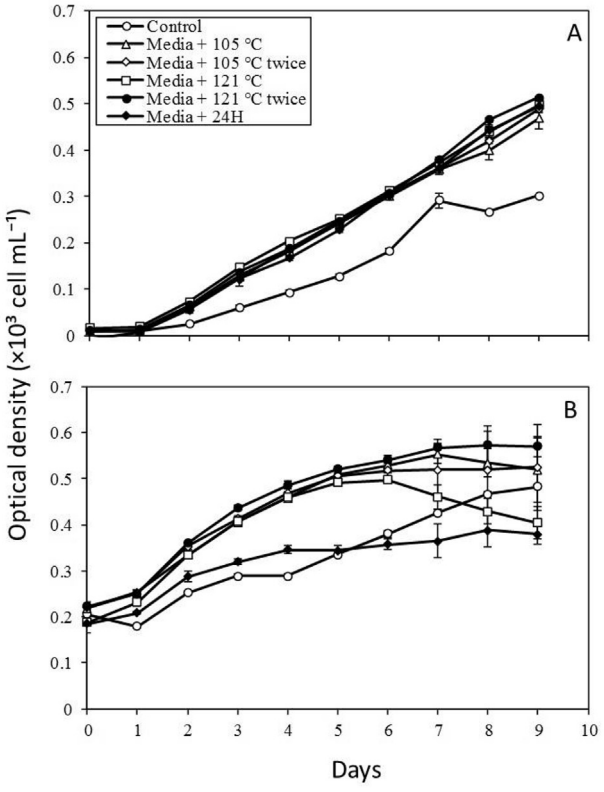

Figure 2. Optical Density at 680 nm of *N. ocenica* in control, media + 105 °C, media + 105 °C twice, media + 121 °C, media + 121 °C twice and media + 24 h at (A) SB SE and (B) KP SE. Error bars represent standard deviation ($n = 3$).

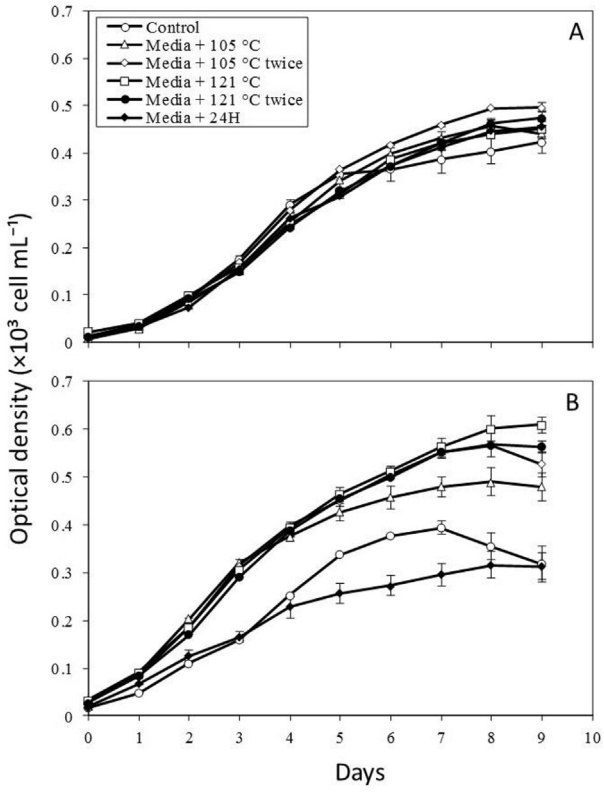

Figure 3. Optical Density at 680 nm of *C. vulgaris* in control, media + 105 °C, media + 105 °C twice, media + 121 °C, media + 121 °C twice and media + 24 h at (A) SB SE and (B) KP SE. Error bars represent standard deviation ($n = 3$).

SB SE media + 105 °C, while KP SE, media + 105 °C twice, media + 121 °C and media + 121 °C twice shows higher value. Meanwhile, *N. subsolitaria* maximum OD is observed in the control experiment for both SB and KP SE.

3.3. Specific growth rate (SGR, μ) and division rate (k) of targeted microalgae in modified SE

The SGR (μ) of all four microalgae in modified SB and KP SE varies depending on media parameterisation type (Figure 6A, B). The highest SGR observed in modified SB SE is 0.12 d^{-1} in media + 24 h for *N. ocenica*. The four microalgal species grown in modified KP SE showed *C. vulgaris* having the highest SGR at 0.12 d^{-1} in media + 105 °C twice and media + 121 °C. The SGR of *N. ocenica* is significantly different ($p < 0.05$) compared to other species, while the other three microalgae did not show any significant differences ($p > 0.05$) from modified SB SE to modified KP SE. The division rate (k) of the four microalgae in modified SB SE and KP SE are based on the SGR of the microalgae (Table 3). *N. ocenica* shows the highest division rate of 0.17 d^{-1} in media + 24 h of SB SE. In the KP SE treatments, *C. vulgaris* exhibits 0.17 d^{-1} as the highest division rate in media + 105 °C twice and media + 121 °C. According to the division rate, *N. ocenica* and *C. vulgaris* showed significant differences ($p < 0.05$) compared to *N. conjuncta* and N. *subsolitaria* in SB SE. Meanwhile, in KP SE, *N. ocenica* and *N. conjuncta* are significantly different ($p < 0.05$) compared to *C. vulgaris* and *N. subsolitaria*.

4. Discussion

The purpose of the present study was to determine the enhancement effects of aquaculture sludge extracts on the growth of specific microalgae species using a novel microplate incubation technique. The ultimate enhancement effect depends on the quality of the SE, particularly essential total nutrients such as nitrogen and phosphate. The TDN and TDP content of KP SE was significantly increased ($p < 0.05$) compared to SB SE. For TDN, the percentage increase of KP SE was highest compared to the others at 153% and 515% in the 105 °C twice and 24 h treatments,

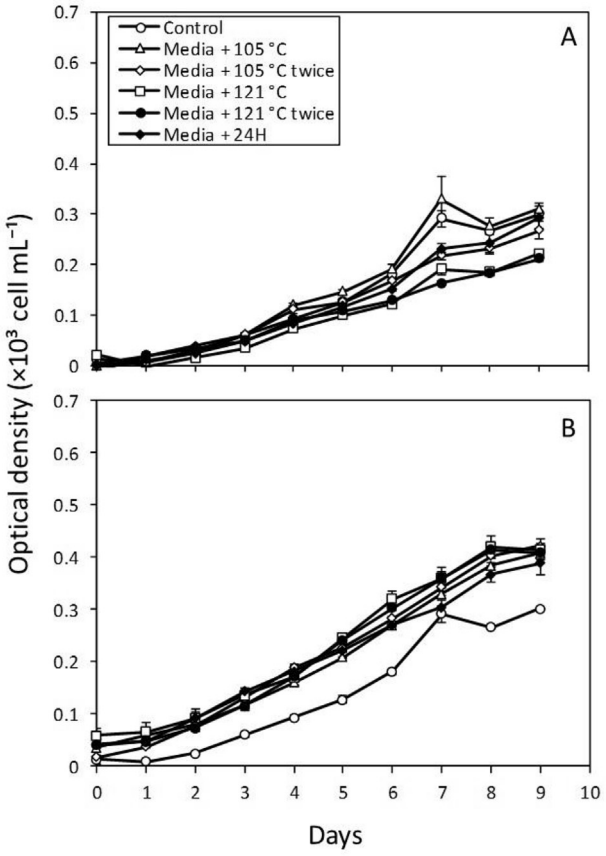

Figure 4. Optical Density at 680 nm of *N. conjuncta* in control, media + 105 °C, media + 105 °C twice, media + 121 °C, media + 121 °C twice and media + 24 h at (A) SB SE and (B) KP SE. Error bars represent standard deviation ($n = 3$).

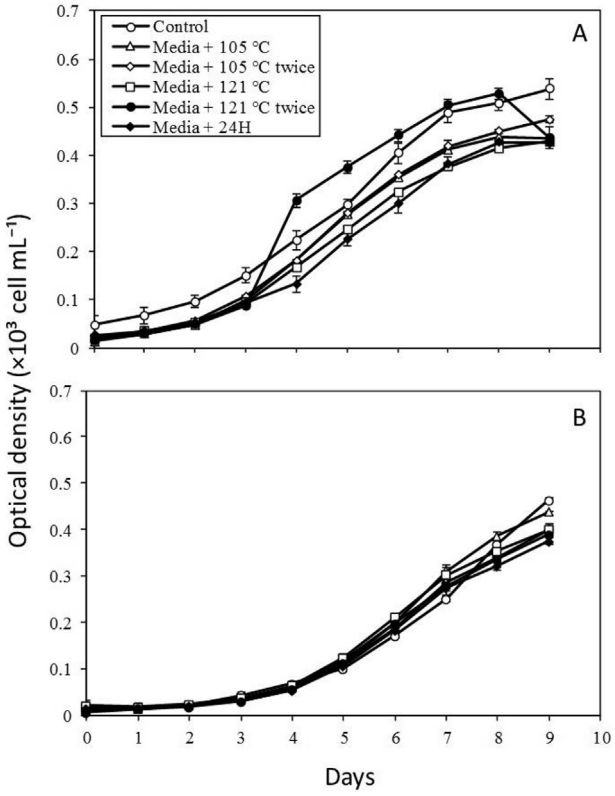

Figure 5. Optical Density at 680 nm of *N. subsolitaria* in control, media + 105 °C, media + 105 °C twice, media + 121 °C, media + 121 °C twice and media + 24 h at (A) SB SE and (B) KP SE. Error bars represent standard deviation ($n = 3$).

respectively. As for TDP, KP SE displayed high percentage increase of 186% in the 105 °C twice treatment. Previously, the nutrient composition found in sludge was nitrogen, phosphorus, and ions such as calcium, boron, and sulphur, which have beneficial effects on plant growth and physical conditions (Celis et al., 2008; Teuber et al., 2005, 2007). Tao et al. (2012) also noted that sludge compost contains high levels of organic matter, nitrogen, phosphorus, potassium, and other elements. Furthermore, Sinha et al. (2014) and Tyagi and Lo (2013) stated that sludge has substantial amounts of nitrogen (2.4%–5.0% total solid) and phosphorus (0.5%–0.7% total solid; Gong et al., 2015). Halfhide et al. (2015) also noted that nitrogen and phosphorus were sufficiently present in wastewater sources that could potentially be utilised for algae cultivation. The present findings showed similar increases in TDN and TDP in SE and indicated that the 105 °C twice treatment was an efficient extraction method for increased nitrogen and phosphate. Although it is not clear from the present study why the particular treatment was effective, a 2-h (twice) autoclaving approach showed better extraction than the 1-h (single) autoclave result. Autoclaving for an extended time at high temperature could eliminate bacterial and protozoal invaders, which may be limiting factors for microalgae growth (Marjakangas et al., 2015). Marjakangas et al. (2015) also noted that the final biomass of *C. vulgaris* was higher in sterilised wastewater compared to unsterilised wastewater. Future studies will need to focus on evaluating the potential time-dependent and temperature complexity of the extraction process (Mercier et al., 2015; Li et al., 2015).

Khatoon et al. (2018) recommended that aquaculture wastewater combined with commercial media be used as an alternative method for the production of microalgae to reduce overall costs rather than relying solely on commercial media. Previously, Valverde-Pérez et al. (2015) stated that wastewater might be the ideal nutrient source for balancing the culture medium for algae cultivation if the nutrients in wastewater are well-balanced. Wang et al. (2015) also stated that *C. vulgaris* growth in wastewater extract could generate sufficient lipid and carbohydrate by replacing nutrients in wastewater. In the present study, KP SE was believed to enhance microalgal growth relative to SB SE due to the former high organic matter content. However, the growth of some microalgae was similar in different extraction parameters of SE and the control. Similar results were observed in a previous study where SE treatments and control displayed similar growth pattern (Khatoon et al., 2018). In some cases, microalgae grew well in various extraction parameters compared to control due to high levels of nutrient coupled with specific extraction methods. It is also probable that specific extraction parameters reduce and precipitate the necessary vitamins and minerals present in the treated SE, in addition to the N and P concentrations. For example, Berns et al. (2008) reported that autoclaved soils exhibited more dissolved organic matters than in untreated soils through subsequent analysis. Thus, investigation or characterisation of chemical properties will be required in future studies to pinpoint the nature of the enrichment qualities of the SE (Kawasaki et al., 2016).

The maximum OD observed in KP SE was higher than SB SE for all microalgae species. More specifically, the maximum OD of *N. ocenica*, *C. vulgaris*, and *N. conjuncta* were higher in KP SE compared to SB SE due to the high TDN and TDP content. Previous studies have shown that microalgae can be an effective alternative method to reduce nitrogen and phosphorus from ponds (Aslan and Kapdan, 2006; García et al., 2006). Previous studies noted that algal production in wastewater was increased significantly in the centrate due to its higher levels of nitrogen, phosphorus, and chemical oxygen demand than other wastewaters (Wang et al., 2010; Xin et al., 2010). In the present study, higher TDN and TDP content increased microalgae growth. As for modified SEs, media + 105 °C twice, media + 121 °C, and media + 121 °C twice have the high values of maximum OD in KP SE. Autoclaving for an extended time at high

Table 2. The maximum OD of *N. ocenica, C. vulgaris, N. conjuncta* and *N. subsolitaria* on control, 105 °C, 105 °C twice, 121 °C, 121 °C twice and 24 h' soil extraction (SE) from *Sabak Bernam* shrimp pond (SB) and *Kota Puteri* fish pond (KP).

Types of SE	Microalgae	Control	Modified SE				
			Media + 105 °C	Media + 105 °C twice	Media + 121 °C	Media + 121 °C twice	Media + 24 h
SB SE	*N. ocenica*	0.30 ± 0.00[a]	0.47 ± 0.06[a]	0.49 ± 0.01[a]	0.50 ± 0.00[a]	0.51 ± 0.00[a]	0.49 ± 0.01[a]
	C. vulgaris	0.42 ± 0.04[a]	0.46 ± 0.00[a]	0.50 ± 0.01[a]	0.45 ± 0.07[a]	0.47 ± 0.02[a]	0.46 ± 0.01[a]
	N. conjuncta	0.30 ± 0.00[b]	0.33 ± 0.04[b]	0.27 ± 0.02[b]	0.22 ± 0.00[b]	0.21 ± 0.00[b]	0.29 ± 0.02[b]
	N. subsolitaria	0.54 ± 0.04[a]	0.44 ± 0.02[a]	0.47 ± 0.01[a]	0.43 ± 0.01[a]	0.53 ± 0.11[a]	0.43 ± 0.00[a]
KP SE	*N. ocenica*	0.48 ± 0.00[a]	0.55 ± 0.03[a]	0.53 ± 0.09[a]	0.50 ± 0.00[a]	0.57 ± 0.04[a]	0.39 ± 0.04[a]
	C. vulgaris	0.39 ± 0.02[a]	0.49 ± 0.20[a]	0.57 ± 0.00[a]	0.61 ± 0.02[a]	0.57 ± 0.03[a]	0.32 ± 0.2[a]
	N. conjuncta	0.30 ± 0.01[a]	0.41 ± 0.01[a]	0.42 ± 0.01[a]	0.42 ± 0.02[a]	0.42 ± 0.01[a]	0.39 ± 0.02[a]
	N. subsolitaria	0.46 ± 0.00[a]	0.44 ± 0.00[a]	0.40 ± 0.01[a]	0.40 ± 0.01[a]	0.39 ± 0.01[a]	0.38 ± 0.01[a]

Note: Values shown are mean of three replicates with + SD.
[a-b] Mean value in same row with different superscripts are significant different ($P < 0.05$).

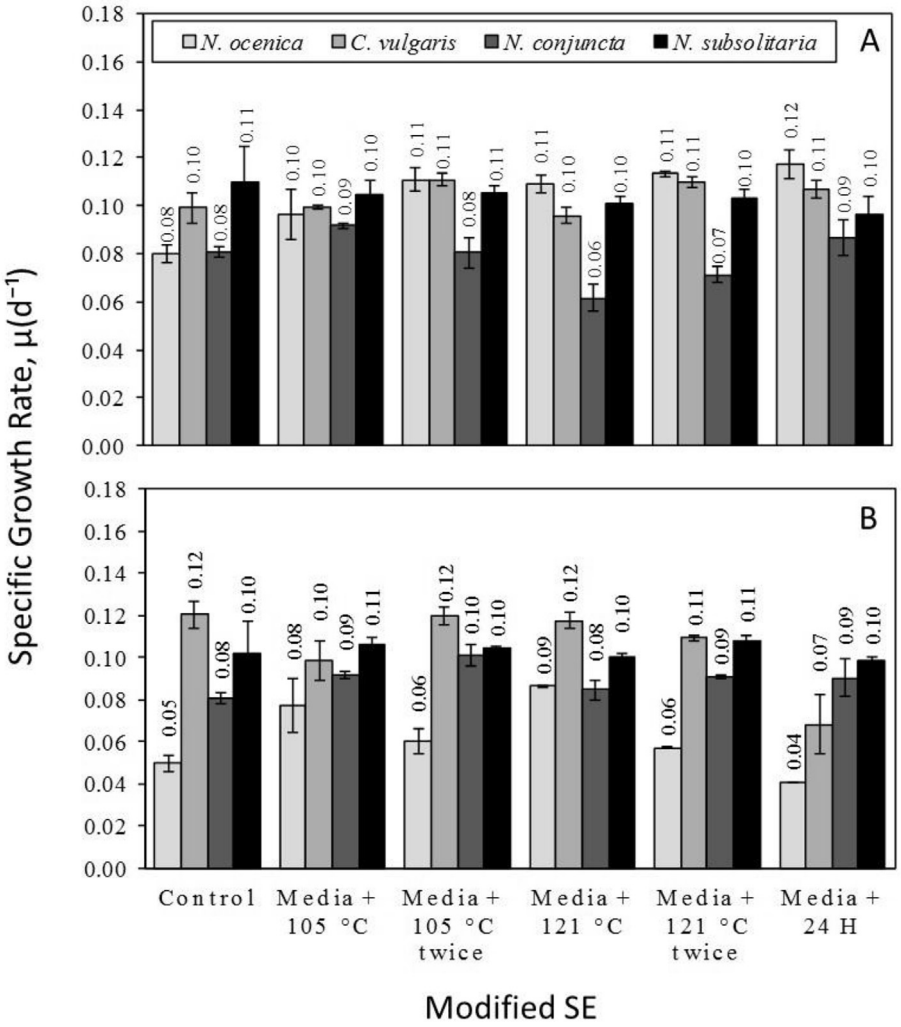

Figure 6. Specific growth rate, μ of *N. ocenica, C. vulgaris, N. conjuncta* and *N. subsolitaria* in control, media + 105 °C, media + 105 °C twice, media + 121 °C, media + 121 °C twice and media + 24 h at (A) SB SE and (B) KP SE. Error bars represent standard deviation ($n = 3$).

temperature could eliminate bacterial and protozoal invaders, which can limit microalgal growth (Marjakangas et al., 2015). Marjakangas et al. (2015) also noted that the final biomass of *C. vulgaris* was higher in sterilised wastewater than in unsterilised wastewater. Further, autoclaved soils revealed more dissolved organic matters than in untreated soils through subsequent analysis (Berns et al., 2008). Thus, investigation or characterisation of chemical properties will be required in future studies to pinpoint the nature of the enrichment qualities of the SE (Kawasaki et al., 2016).

The SGR of microalgal species varied based on the types of SE, the method of modification or extraction, and culture media used in the control experiment. The SGR of *N. ocenica* increased relative to the control in modified SB SE (Figure 6). The relatively higher SGR suggests that the extraction parameters and additional nutrients present in the

Table 3. The division rate, *k* of *N. ocenica, C. vulgaris, N. conjuncta* and *N. subsolitaria* on control, 105 °C, 105 °C twice, 121 °C, 121 °C twice and 24 h' soil extraction (SE) from *Sabak Bernam* shrimp pond (SB) and *Kota Puteri* fish pond (KP).

Types of SE	Microalgae	Control	Modified SE				
			Media + 105 °C	Media + 105 °C twice	Media + 121 °C	Media + 121 °C twice	Media + 24 h
SB SE	*N. ocenica*	0.12 ± 0.00[b]	0.14 ± 0.01[b]	0.16 ± 0.00[b]	0.16 ± 0.00[b]	0.16 ± 0.00[b]	0.17 ± 0.01[b]
	C. vulgaris	0.14 ± 0.01[b]	0.14 ± 0.00[b]	0.16 ± 0.00[b]	0.14 ± 0.00[b]	0.16 ± 0.00[b]	0.15 ± 0.00[b]
	N. conjuncta	0.12 ± 0.00[a]	0.13 ± 0.00[a]	0.12 ± 0.01[a]	0.09 ± 0.01[a]	0.10 ± 0.00[a]	0.12 ± 0.01[a]
	N. subsolitaria	0.16 ± 0.02[a]	0.15 ± 0.01[a]	0.15 ± 0.00[a]	0.15 ± 0.00[a]	0.15 ± 0.00[a]	0.14 ± 0.01[a]
KP SE	*N. ocenica*	0.07 ± 0.00[b]	0.11 ± 0.01[b]	0.09 ± 0.01[b]	0.12 ± 0.00[b]	0.08 ± 0.00[b]	0.06 ± 0.00[b]
	C. vulgaris	0.17 ± 0.01[a]	0.14 ± 0.01[a]	0.17 ± 0.00[a]	0.17 ± 0.00[a]	0.16 ± 0.00[a]	0.10 ± 0.01[a]
	N. conjuncta	0.12 ± 0.00[b]	0.13 ± 0.00[b]	0.15 ± 0.00[b]	0.12 ± 0.00[b]	0.13 ± 0.00[b]	0.13 ± 0.01[b]
	N. subsolitaria	0.15 ± 0.00[a]	0.15 ± 0.00[a]	0.15 ± 0.00[a]	0.14 ± 0.00[a]	0.16 ± 0.00[a]	0.14 ± 0.00[a]

Note: Values shown are mean of three replicates with + SD.
[a-b]Mean value in same row with different superscripts are significant different ($P < 0.05$).

modified SE were critical for the increase. Gao et al. (2016) stated that the growth of *C. vulgaris* and *Scenedesmus obliquus* in aquaculture and treated sewage wastewater were much lower than those obtained in other wastewaters containing higher nutrients, such as urban sewage and pig feedlot wastewater, suggesting that the amount of nutrients in aquaculture wastewater is inadequate to sustain high productivity of algal biomass in batch culture mode. While KP SE has a relatively higher nutrient content, it did not influence the SGR of microalgae to the maximum levels attained in SB SE, suggesting that there are some species dependence and variability. Latiffi et al. (2017) noted that too many microalgal populations might cause suffocation of the nutrients available in wastewater to support the production of microalgae, resulting in no increase in total biomass concentrations over time. Moreover, Shuler (2002) also noted that a high concentration of microalgae in the medium could inhibit growth rates simply due to population concentration. Thus, since the microplate incubation was used in the present study, some growth limitations might have been caused due to low volume (micro--wells) and eventual high concentration of the microalgae biomass on day 9. In other words, some growth enhancement could have been limited due to the low incubation volume of the microplate wells.

The successful mass cultivation of microalgae requires adequate light, temperature, and nutrients such as nitrogen and phosphorus along with a variety of microelements (Markou et al., 2014; Peccia et al., 2013). Industrial cultivation of microalgae is restricted by the high cost of nutrients for the production of these organisms (Zuliani et al., 2016). Koller et al. (2012) stated that wastewater and its high nutrient content appear to be a potential solution for the acquisition of low-cost nutrients for microalgae cultivation. Previous research documented the possibility of using urban wastewater with anaerobic digestion for *Nannochloropsis gaditana* (Ledda et al., 2015) or *Nannochloropsis salina* (Sheets et al., 2014; Cai et al., 2013) production. Thus, aquaculture sludge extract as an enrichment medium for microalgae growth is possible, and can enhance the growth to maximum levels compared with the artificial culture medium.

5. Conclusion

In conclusion, the study showed the promise for enhanced (marine and freshwater) microalgal growth with the addition of supplemental enrichment from treated aquaculture sludge extract. The study shows that SE enrichment increases TDN and TDP compared to growth media, but is dependent on sludge (source) type, extraction technique, and the type of microalgae (species-specific). In other words, the quality of the SE and the type of microalgae studied will determine the outcome of any enrichment experiment, and potential future application to mass culture. In terms of extraction parameters, 121 °C twice showed high TDN and TDP content in the present study. Our study shows that autoclaving at high temperatures helps to recover nutrients and potentially reduces pathogens. However, all the extraction parameters of SB and KP SE enhanced microalgae growth. Regarding the use of the novel microplate incubation technique, the study determined that the method is exceptionally effective as a high throughput method to screen microalgae response to enrichment experiments. However, our findings also indicate that the small volume of the microplate wells may limit the maximum growth rate and biomass increase from overpopulation of cells. Artificial culture medium alone is costly and insufficient; thus, natural growth-promoting materials were explored from aquaculture ponds to enhance the microalgae productivity. Enrichment of mass cultures with SE will reduce the total cost of producing microalgae and also improves the microalgae growth and nutritional content.

Declarations

Author contribution statement

Kasturi Arumugam: Conceived and designed the experiments; Wrote the paper.

Mohd Fadzli Ahmad: Performed the experiments.

Nor Suhaila Yaacob: Performed the experiments; Wrote the paper.

Wan Muhammad Ikram, Maegala Nallapan Maniyam, Hasdianty Abdullah: Analyzed and interpreted the data.

Tomoyo Katayama, Kazuhiro Komatsu, Victor S. Kuwahara: Contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

References

Aslan, S., Kapdan, I.K., 2006. Batch kinetics of nitrogen and phosphorus removal from synthetic wastewater by algae. Ecol. Eng. 28 (1), 64–70.

Berns, A.E., Philipp, H., Narres, H.D., et al., 2008. Effect of gamma-sterilization and autoclaving on soil organic matter structure as studied by solid state NMR, UV and fluorescence spectroscopy. Eur. J. Soil Sci. 59 (3), 540–550.
Bischoff, H.W., 1963. Phycological Studies. IV. Some Algae from Enchanted Rock and Related Algal Species, 6318. Univ. Texas Publ., p. 95
Blaise, C., Férard, J.F., 2005. Overview of contemporary toxicity testing. Small-Scale Freshwater *Toxicity Investigations*. Springer, Dordrecht, pp. 1–68.
Bohutskyi, P., Liu, K., Nasr, L.K., et al., 2015. Bioprospecting of microalgae for integrated biomass production and phytoremediation of unsterilized wastewater and anaerobic digestion centrate. Appl. Microbiol. Biotechnol. 99 (14), 6139–6154.
Brennan, L., Owende, P., 2010. Biofuels from microalgae—a review of technologies for production, processing, and extractions of biofuels and co-products. Renew. Sustain. Energy Rev. 14 (2), 557–577.
Cai, T., Park, S.Y., Racharaks, R., et al., 2013. Cultivation of *Nannochloropsis salina* using anaerobic digestion effluent as a nutrient source for biofuel production. Appl. Energy 108, 486–492.
Cao, L., Wang, W., Yang, Y., et al., 2007. Environmental impact of aquaculture and countermeasures to aquaculture pollution in China. Environ. Sci. Pollut. Res. Int. 14 (7), 452–462.
Celis, J., Sandoval, M., Barra, R., 2008. Plant response to salmon wastes and sewage sludge used as organic fertilizer on two degraded soils under greenhouse conditions. Chil. J. Agric. Res. 68, 274–283.
Cruz, Y.R., Aranda, D.A., Seidl, P.R., et al., 2018. Cultivation systems of microalgae for the production of biofuels. Biofuels: State Develop. 199.
Daneshvar, E., Zarrinmehr, M.J., Hashtjin, A.M., et al., 2018. Versatile applications of freshwater and marine water microalgae in dairy wastewater treatment, lipid extraction and tetracycline biosorption. Bioresour. Technol. 268, 523–530.
Das, P., Aziz, S.S., Obbard, J.P., 2011. Two phase microalgae growth in the open system for enhanced lipid productivity. Renew. Energy 36 (9), 2524–2528.
Ding, G.T., Takriff, M.S., Salihon, S., et al., 2015. Feasiblity of the optical density (OD) in the determination of the microalgal biomass using palm oil mill effluent (POME) as medium. In: Proceedings of 50th the IIER International Conference, Zurich, Switzerland, 26th December 2015.
Dupraz, V., Stachowski-Haberkorn, S., Ménard, D., et al., 2018. Combined effects of antifouling biocides on the growth of three marine microalgal species. Chemosphere 209, 801–814.
Escudey, M., Förster, J.E., Becerra, J.P., et al., 2007. Disposal of domestic sludge and sludge ash on volcanic soils. J. Hazard Mater. 139 (3), 550–555.
Food and Agriculture Organization of the United Nations (FAO), 2018, October 29. The State of World Fisheries and Aquaculture 2018. http://www.fao.org/3/i9540en/i9540en.pdf.
Gao, F., Li, C., Yang, Z.H., Zeng, G.M., Feng, L.J., Liu, J.Z., et al., 2016. Continuous microalgae cultivation in aquaculture wastewater by a membrane photobioreactor for biomass production and nutrients removal. Ecol. Eng. 92, 55–61.
García, J., Green, B.F., Lundquist, T., et al., 2006. Long term diurnal variations in contaminant removal in high rate ponds treating urban wastewater. Bioresour. Technol. 97 (14), 1709–1715.
Gong, C., Jiang, J., Li, D.A., 2015. Ultrasound coupled with Fenton oxidation pre-treatment of sludge to release organic carbon, nitrogen and phosphorus. Sci. Total Environ. 532, 495–500.
Guo, Z., Liu, Y., Guo, H., et al., 2013. Microalgae cultivation using an aquaculture wastewater as growth medium for biomass and biofuel production. J. Environ. Sci. 25, S85–S88.
Halfhide, T., Dalrymple, O.K., Wilkie, A.C., et al., 2015. Growth of an indigenous algal consortium on anaerobically digested municipal sludge centrate: photobioreactor performance and modeling. BioEnergy Res. 8 (1), 249–258.
Huesemann, M., Crowe, B., Waller, P., et al., 2016. A validated model to predict microalgae growth in outdoor pond cultures subjected to fluctuating light intensities and water temperatures. Algal Res. 13, 195–206.
Kawasaki, N., Kushairi, M.R.M., Nagao, N., et al., 2016. Release of nitrogen and phosphorus from aquaculture farms to Selangor River, Malaysia. Int. J. Environ. Sustain Dev. 7 (2), 113.
Khatoon, H., Banerjee, S., Syakir Syahiran, M., et al., 2016. Re-use of aquaculture wastewater in cultivating microalgae as live feed for aquaculture organisms. Desalinat. Water Treatm. 57 (60), 29295–29302.
Khatoon, H., Haris, H., Rahman, N.A., et al., 2018. Growth, proximate composition and pigment production of *Tetraselmis chuii* cultured with aquaculture wastewater. J. Ocean Univ. China 17 (3), 641–646.
Koller, M., Salerno, A., Tuffner, P., et al., 2012. Characteristics and potential of micro algal cultivation strategies: a review. J. Clean. Prod. 37, 377–388.
Latiffi, N.A.A., Mohamed, R.M.S.R., Apandi, N.M., Tajuddin, R.M., 2017. Preliminary assessment of growth rates on different concentration of microalgae scenedesmus sp. in industrial meat food processing wastewater. In: MATEC Web of Conferences, 103. EDP Sciences, p. 6010.
Ledda, C., Villegas, G.R., Adani, F., et al., 2015. Utilization of centrate from wastewater treatment for the outdoor production of *Nannochloropsis gaditana* biomass at pilot-scale. Algal Res. 12, 17–25.
Li, Y.P., Feng, Y.L., Chen, Y.J., et al., 2015. Soil microbes alleviate allelopathy of invasive plants. Sci. Bull. 60 (12), 1083–1091.
Madariaga, S.T., Marín, S.L., 2017. Sanitary and environmental conditions of aquaculture sludge. Aquacult. Res. 48 (4), 1744–1750.
Malcata, F.X., Pinto, I.S., Guedes, A.C., 2018. Marine Macro-And Microalgae: an Overview. CRC Press. https://content.taylorfrancis.com/books/download?dac=C2014-0-34031-9&isbn=9781498705349&format=googlePreviewPdf.
Marjakangas, J.M., Chen, C.Y., Lakaniemi, A.M., et al., 2015. Simultaneous nutrient removal and lipid production with *Chlorella vulgaris* on sterilized and non-sterilized anaerobically pretreated piggery wastewater. Biochem. Eng. J. 103, 177–184.
Markou, G., Vandamme, D., Muylaert, K., 2014. Microalgal and cyanobacterial cultivation: the supply of nutrients. Water Res. 65, 186–202.
Mercier, A., Michel, C., Joulian, C., et al., 2015. Decrease of the level of extractable polychlorinated biphenyls in soil microcosms: influence of granular activated carbon and inoculation by natural microbial consortia. Int. Biodeterior. Biodegrad. 105, 127–136.
Michels, M.H., Vaskoska, M., Vermuë, M.H., et al., 2014. Growth of *Tetraselmis suecica* in a tubular photobioreactor on wastewater from a fish farm. Water Res. 65, 290–296.
Nasir, N.M., Bakar, N.S.A., Lananan, F., et al., 2015. Treatment of African catfish, *Clarias gariepinus* wastewater utilizing phytoremediation of microalgae, Chlorella sp. with *Aspergillus niger* bio-harvesting. Bioresour. Technol. 190, 492–498.
Pacheco, A., Hernández-Mireles, I., García-Martínez, C., et al., 2013. Microplates as a microreactor platform for microalgae research. Biotechnol. Prog. 29 (3), 638–644.
Peccia, J., Haznedaroglu, B., Gutierrez, J., et al., 2013. Nitrogen supply is an important driver of sustainable microalgae biofuel production. Trends Biotechnol. 31 (3), 134–138.
Postma, P.R., 't Lam, G.P., Barbosa, M.J., et al., 2016. Microalgal biorefinery for bulk and high-value products: product extraction within cell disintegration. Handbook of electroporation, pp. 1–20.
Rojíčková, R., Dvořáková, D., Maršálek, B., 1998. The use of miniaturized algal bioassays in comparison to the standard flask assay. Environ. Toxicol. Water Qual. Int. J. 13 (3), 235–241.
Sharma, A.K., Sahoo, P.K., Singhal, S., et al., 2016. Impact of various media and organic carbon sources on biofuel production potential from Chlorella spp. 3 *Biotech* 6 (2), 116.
Sheets, J.P., Ge, X., Park, S.Y., et al., 2014. Effect of outdoor conditions on *Nannochloropsis salina* cultivation in artificial seawater using nutrients from anaerobic digestion effluent. Bioresour. Technol. 152, 154–161.
Shuler, M.L., 2002. Bioprocess Engineering Basic Concepts, second ed. Prentice Hall PTR, New Jersey, U.S.A.
Sinha, A., Singh, A., Kumar, S., et al., 2014. Microbial mineralization of struvite: a promising process to overcome phosphate sequestering crisis. Water Res. 54, 33–43.
St-Laurent, D., Blaise, C., MacQuarrie, P., et al., 1992. Comparative assessment of herbicide phytotoxicity to *Selenastrum capricornutum* using microplate and flask bioassay procedures. Environ. Toxicol. Water Qual. 7 (1), 35–48.
Tao, J., Wu, S., Sun, L., et al., 2012. Composition of waste sludge from municipal wastewater treatment plant. Proc. Environ. Sci. 12, 964–971.
Teuber, N., Alfaro, M., Salazar, F., et al., 2005. Sea salmon sludge as fertilizer: effects on a volcanic soil and annual ryegrass yield and quality. Soil Use Manag. 21, 432–434.
Teuber, N., Salazar, F., Alfaro, M., et al., 2007. Effect of different rates of cage salmon sludge on potato crop and its residual effect on annual ryegrass. Agric. Tec. (Santiago) 67, 393–400.
Turcios, A.E., Papenbrock, J., 2014. Sustainable treatment of aquaculture effluents—what can we learn from the past for the future? Sustainability 6 (2), 836–856.
Tyagi, V.K., Lo, S.L., 2013. Sludge: a waste or renewable source for energy and resources recovery? Renew. Sustain. Energy Rev. 25, 708–728.
Valverde-Pérez, B., Ramin, E., Smets, B.F., et al., 2015. EBP2R–an innovative enhanced biological nutrient recovery activated sludge system to produce growth medium for green microalgae cultivation. Water Res. 68, 821–830.
Van Wagenen, J., Holdt, S.L., De Francisci, D., et al., 2014. Microplate-based method for high-throughput screening of microalgae growth potential. Bioresour. Technol. 169, 566–572.
Wang, L., Min, M., Li, Y., et al., 2010. Cultivation of green algae Chlorella sp. in different wastewaters from municipal wastewater treatment plant. Appl. Biochem. Biotechnol. 162 (4), 1174–1186.
Wang, Y., Guo, W., Yen, H.W., et al., 2015. Cultivation of Chlorella vulgaris JSC-6 with swine wastewater for simultaneous nutrient/COD removal and carbohydrate production. Bioresour. Technol. 198, 619–625.
Watanabe, M.M., 2005. Algal culturing techniques. In: Andersen, R.A. (Ed.), Freshwater Culture, p. 13.
Xin, L., Hong-Ying, H., Ke, G., et al., 2010. Effects of different nitrogen and phosphorus concentrations on the growth, nutrient uptake, and lipid accumulation of a freshwater microalga Scenedesmus sp. Bioresour. Technol. 101 (14), 5494–5500.
Zhao, Q., Chen, A.N., Hu, S.X., et al., 2018. Microalgal microscale model for microalgal growth inhibition evaluation of marine natural products. Sci. Rep. 8 (1), 10541.
Zuliani, L., Frison, N., Jelic, A., et al., 2016. Microalgae cultivation on anaerobic digestate of municipal wastewater, sewage sludge and agro-waste. Int. J. Mol. Sci. 17 (10), 1692.