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EFFECTS OF VARIOUS SOIL EXTRACT FRACTION TYPES ON THE GROWTH OF MARINE MICROALGAE

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Abstract: Organic matter from terrestrial and aquatic sources plays a major role in microalgae cultivation; however, its complementary effects on microalgal growth are almost unknown to date. In this study, we investigated the effects of dissolved organic matter from forest soil and its fractions on naturally isolated marine microalgae: Green microalgae (*Chlorella sorokiniana* and *Oocystis heteromucosa*) and diatom (*Thalassiosira weissflogii*) from Malaysia. The soil was extracted with ultrapure water at two different temperatures: 105°C and 121°C. These two soil extracts were fractionated into humic, non-humic, low-molecular-size, and high-molecular-size fractions. Microalgal cultivation was conducted using microplates that were added with Conway medium, soil extracts and their fractions. Results showed higher concentrations of all tested chemical elements in soil extracts from 121°C extraction than 105°C. Both specific growth rates of *C. sorokiniana* and *T. weissflogii* were found to be around 20% higher in soil extracts at 105°C than in the control. Simultaneously, the humic and low-molecular-size fractions were found to have improved growth rates of 11-17% and 15-31% respectively from their soil extracts at 105°C. The specific growth rate of *O. heteromucosa* only increased marginally by 7% when compared with the control and with soil extracts at 121°C. The humic and low-molecular-size fractions increased around 10-37% relative to these soil extracts. The humic and low-molecular-size fractions were consistently associated with greater growth-enhancing effects; meanwhile, others were less effective or ineffective.

Keywords: Humic, molecular size, non-humic, organic matter, phytoplankton.

Introduction

Microalgae biomass has gained significant importance over the last few decades because of its carbon-rich compounds that can be used profitably in biofuels, cosmetics, animal feed, and other related industries (Das *et al*., 2011; Arumugam *et al*., 2020). Microalgae also produce a wide range of highly valuable products including lipids, pigments, proteins, polysaccharides, and phycotoxin which have potential as foods and medicines for human consumption (Soares *et al*., 2019). In order to obtain these high-value add compounds commercially, it is necessary to produce microalgae biomass on a large scale (Bermejo *et al.,* 2021). According to Sanchez-Bayo *et al*. (2020), adequate supplements are the key to generating a relevant quantity of high-quality microalgal biomass. However, the consumption of chemical nutrients and strict, high energy maintenance procedures is the most challenging hurdle for many microalgae-related industries to overcome. This makes harvesting microalgal biomass in sufficient quantities at a consistently high quality more costly than traditional crop production (Blair *et al*., 2014). Therefore, there is a critical need for uncovering the natural and cost-effective nutrient supplements that have circular economy values to sustain these large-scale industries in the future.

The use of soil extracts in a natural seawater cultured medium was introduced in the 1910s to

enhance marine microalgae growth (Harrison & Berges, 2005). Terrestrial soil generally consists of several elements, such as carbon (C), hydrogen (H), oxygen (O), magnesium (Mg), and small quantities of sulfur (S), nitrogen (N), potassium (K), phosphorus (P) and calcium (Ca) (Bot & Benites 2005; Neto *et al.,* 2012; Yaacob *et al.,* 2021). The use of its 'extract in culture methodology' was established and recognised as the famous Erdschreiber's medium (ESM) (Schreiber, 1927) and contains only soil extract, nitrogen, and phosphorus; which leads to the growth of various planktonic and benthic microalgae species. A new version of the ESM medium was then developed with the addition of other elements such as vitamins (biotin and thiamine) and iron (Provasoli *et al*., 1957) that is suitable for many species of marine and brackish water microalgae.

Comprehensive soil extraction methods were necessary to produce effective soil extracts for enhancing microalgae growth, thus, many methods with various extraction temperatures were applied in previous studies. However, most of these soil extraction methods were seemingly based on arbitrary decisions, regardless of either soil types or extraction temperatures. It was believed that any high temperature, combined with sufficient incubation time after the extraction, would be able to conserve more solubilised soil components relevant to microalgae growth (Provasoli *et al*., 1957; McLachlan, 1973; Tompkins *et al*., 1995; Glazer *et al*., 1997). Moreover, several different studies in soil chemistry proved that the quality of solubilised carbon, phosphorus, and nitrogen in the extracted soil solutions was different between low and high temperatures of extraction methods (Razavi & Lakzian, 2007; Serrasolses *et al*., 2008; O'Brien *et al.,* 2018; Arumugam *et al.,* 2020). However, the association between the quality of soil extracts at different extraction temperature ranges and the growth effects on microalgae was seemingly not established and well-documented. Only a few comparative studies of soil extraction methods that establish the effects of its extracts specifically on the growth of marine microalgae could be found (Watanabe, 2005).

Soil extracts proved effective in stimulating the growth of marine microalgae by the influence of their distinctive constituents mainly comprising of humic and non-humic substances (Lee *et al*., 2009; Kovachik *et al.,* 2018; Popa *et al.,* 2022). Humic substances have the potential to act as chelating agents for copper, iron, and zinc; making these important trace metals bioavailable for uptake by microalgae (Sweeney, 1954; Prakash & Rashid, 1968; Nagai *et al*., 2006; Pokora *et al.,* 2014; Kovachik *et al.,* 2018). Due to this potential, many studies were found and focused on the manipulation of humic substances as indicators for toxic metal contamination (Rovira *et al.,* 2010; Martin *et al*., 2014; Wen *et al.,* 2019) and algae bloom phenomenon (Gagnon *et al*., 2005; Cawley *et al*., 2013; Wang *et al.,* 2020) in the river and lake environments rather than in benefiting the microalgae cultivation.

In addition, few reports were found about the significant characteristics of non-humic substances beneficial in microalgae cultivation, compared with the humic substances. Since these constituents were underpinned by complex soil organic matter, their specific roles remain obscured (Chen *et al*., 2013; Zhou *et al.,* 2020). They were probably not only directly acting as a source of nutrients but also indirectly enhancing the bioavailability of inorganic nutrients that stimulated the growth of microalgae (Lee *et al*., 2009; Sanchez-Marin & Beiras, 2011; Popa *et al.,* 2022). Thus, clarifying the distinctive roles of respective fractions of soil organic matter in microalgae cultivation is necessary. This aspect is undeniably challenging because the constituents of soil organic matter are complex and naturally intractable. The soil organic matter can be chemically fractionated into humic and non-humic fractions (Imai *et al*., 2001; 2002; Komatsu *et al*., 2019) and physically fractionated into low-and high-molecular-size fractions (Tulonen *et al*., 1992; Fagerberg *et al*., 1992; Kim *et al*., 2017). Therefore, fractionating soil extract into its respective fractions is one of the efforts of this study to clarify the relationship with the growth of marine microalgae. In this study, we focused on

and accounted for, different soil extracts with their respective fractions to explain this relationship.

Materials and Methods

Preparations of Soil Extracts

Forest soils more commonly known as podzolic soils from Chini Forest near the Berang River in Pahang, Malaysia (Figure 1) were used in this study and the extract solutions of these soil samples were provided by University Selangor (Unisel), Malaysia. Before sampling the soils, any dried leaves, twigs and other matter were removed. Soils were collected at a depth of between 15 and 30 cm, from the first soil horizon, known as the topsoil (United States Department of Agriculture, 2014). The soil was then extracted with ultrapure water (Milli-Q SP TOC, Merk-Millipore, USA) at a volume ratio of 1:10 (Provasoli *et al*., 1957). An autoclave machine was used to facilitate the extraction process at two different temperatures: 105°C (based on the modification of soil extraction temperature used in the ESM media preparation) and 121°C (based on the autoclaving temperature). After 1 hour of extraction, the obtained solution was cooled for about 30 minutes, before being centrifuged at 2500 rpm for 15 minutes. After centrifuging, the supernatant was filtered using a glass fibre filter (Whatman GF/F), and the filtered solution was stored at -20°C before analyses and fractionations.

Figure 1: The soil sampling site at Chini Forest, Pahang (3°23'N 102°53'E)

Fractionations of Soil Extracts

Two types of soil extract fractions (humic-non-humic fractions and low-high molecular size fractions) from the two different soil extraction temperatures (105°C and 121°C) were involved in this study. An auto-fractionation system developed by the National Institute of Environmental Studies, and Nikkyo Technos, Co., Ltd in Japan, was used to separate the soil extract into humic and non-humic fractions through a polymethyl methacrylate resin adsorbent; SupeliteTM DAX-8 (Supelco, Sigma-Aldrich Co., German). The column-capacity factor (k') at 50 was set up for separating both fractions through the resin column (Leenheer, 1981). The separation between the humic and non-humic fractions was achieved when 50% of the hypothetical Dissolved Organic Matter (DOM) or soil extracts with k′ = 50 was absorbed into the resin in accordance with this Equation 1:

$$k'(50) = V_{el}/2V_0 \quad (1)$$

where V_{el} is the sample volume and V_0 is the void volume of resin which is 65% of the bulk column volume. In this study, we used 9 mL of DAX-8 resin and 600 mL of soil extract. Before fractionation, the soil extract was accordingly diluted, acidified to pH 2 with hydrochloric acid, and pumped through the resin. Based on their affinity to absorb onto the resin, the humic and non-humic fractions were recognised as absorbed fractions and non-absorbed fractions, respectively (Leenheer, 1981; Thurman & Malcolm, 1981; Imai *et al*., 2002). At the first cycle of fractionation, the non-humic fraction (non-adsorbed fraction) was primarily collected, while the humic fraction was eluted from the resin with 0.1 M sodium hydroxide. The Dissolved Organic Carbon (DOC) concentrations of both fractions were measured after the test was completed.

The separation between low-molecular-size fraction (< 10 kDa) and high-molecular-size fraction (> 10 kDa) of the soil extract was facilitated by a combination of methods from the filtration, using an ultra-membrane filter (MicrocepTM, Pall Co., USA) and filter (10-kDa cutoff) and centrifugation at 3,300 rpm (Komatsu, 2020). Both soil extracts were diluted by referring to their actual DOC concentrations recorded from the chemical characterisation experiments. The filter tube was filled with soil extract and centrifuged for 30 minutes. The supernatant that passed through the membrane filter was recognised as a low-molecular-size fraction. The fraction trapped inside the filter tube was recognised as a high-molecular-size fraction. The low-molecular-weight fraction was collected while a series of filtration and centrifugation of the high-molecular-size fraction with ultrapure water was performed to eliminate impurities. Finally, the DOC concentration of the fractions was measured.

Chemical Characterisations of Soil Extracts and Fractions

The characteristics of the soil extracts and fractions were evaluated based on the concentrations of DOC, Total Dissolved Nitrogen (TDN), Total Dissolved Phosphorus (TDP), and the ratio of ultraviolet absorbance at 254 nm (UVA_{254nm}) to DOC concentration (Imai *et al*., 2002; Komatsu *et al*., 2019). The carbon double bonds and benzene rings of soil DOM can be detected by this specific ultraviolet absorbance at 254 nm (UVA_{254nm}) (Imai *et al*., 2001). The concentrations of TDN and TDP from the soil extracts were analysed using a spectrophotometer (Lovibond MD 600D, German). A total organic carbon analyser (Shimadzu TOC-5000, Japan) with Pt catalyst on quartz wool was used to measure DOC concentrations and potassium hydrogen phthalate (Kanto Chemical Co., Tokyo, Japan) was selected as a standard. A UV–Vis spectrometer (Shimadzu UV-4500, Japan) was used to measure the UVA_{254nm}. The diluted samples were placed in a quartz cell with a 1-cm path length; Milli-Q water was used as a blank.

Microalgae Isolation and Stock Culture Preparation

Two green microalgae strains, *Chlorella sorokiniana* (SLG-4-13), and *Oocystis heteromucosa* (TRG10-p102), and a diatom, *Thalassiosira weissflogii* (TRG10-p105) were tested in this study (Figure 2). All stock cultures of these microalgae were provided by the culture collection of the University of Tokyo, Japan. Previously, all the microalgae was isolated from the various aquaculture shrimp ponds located in Malaysia with different pH and salinity. *C. sorokiniana* (SLG4-13) were isolated from the water samples collected from the pond in Selangor with a pH of 7.3 and salinity of 30 ppt. (Figure 3). *O. heteromucosa* (TG10-p102) and *T. weissflogii* (TRG10-p105) was isolated from the water samples collected from the ponds at the same location in Terengganu (Figure 4). The

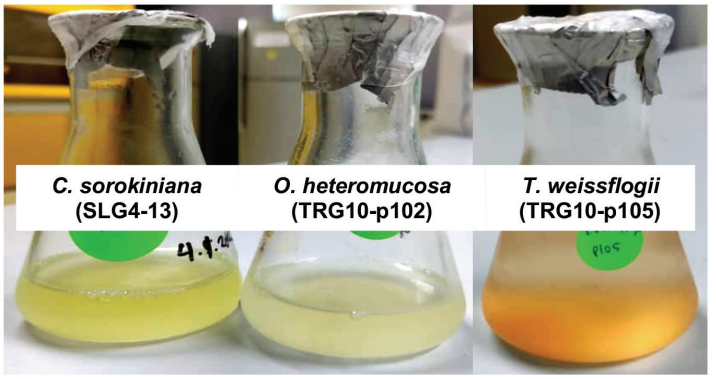

Figure 2: The isolated marine microalgae from various shrimp ponds

Figure 3: The sampling site for isolated *Chlorella sorokiniana* (SLG4-13) in Selangor (3°21'N 101°15'E)

Figure 4: The sampling site for isolated *Oocystis heteromucosa* (TRG10-p102) and *Thalassiosira weissflogii* (TRG10-p105) in Terengganu (5°38'N 102°45'E)

pH and salinity of the pond for *O. heteromucosa* (TG10-p102) were recorded at 7.8 and 20 ppt. and for *T. weissflogii* (TRG10-p105) at 7.8 and 30 ppt.

The water samples from the particular ponds were filtered through a 60 µm nylon mesh soon after collection to remove zooplankton and debris (Katayama *et al.,* 2020). Capillary pipetting (liquid medium) and streak plating (solid medium) techniques were applied for the microalgae isolation (Hoshaw & Rosowski, 1973). Using a glass capillary aided by a microscope, the collected microalgae were pipetted and isolated individually. These isolated marine microalgae were cultured in a Conway medium (Walne 1970) and incubated in a temperature-controlled incubator (TG-300WLED-3LE, NK System, Japan) with a light and dark cycle ratio of 12:12 hours at 30°C and under the visible light intensity of 140 µmol-photons m^{-2} s^{-1}. Conway medium with natural seawater was used in this study to cultivate all these strains of marine microalgae since numerous studies showed its significant effects in marine microalgae cultivation (Lananan *et al*., 2013; Praba *et al*., 2016; Maharajan *et al*., 2020).

Major chemicals that were formulated in 1 L Conway medium for isolation by capillary pipetting were included: 0.1 g of $NaNO_3$, 0.02 g of KNO_3; 0.02 g of $NaH_2PO_4 \cdot 4H_2O$; 0.02 g of Na_2SiO_3; 0.045 g of $Na_2H_2EDTA \cdot 2H_2O$; 1.3 mg of $FeCl_3 \cdot 6H_2O$; 0.0021 mg of $ZnCl_2$; 0.36 mg of $MnCl_2 \cdot 4H_2O$; 0.002 mg of $CoCl_3 \cdot 6H_2O$; 0.002 mg of $CuSO_4 \cdot 5H_2O$; 0.0009 mg of $(NH_4)6Mo_7O_{24} \cdot 4H_2O$; 0.0336 g of H_3BO_3; 0.2 mg of thiamine HCl and 0.01 mg of cyanocobalamin. Silicate was further added in the medium only for *T. weissflogii*. Natural seawater at a salinity value of 30 ppt. was prepared in the media for *T. weissflogii* and *C. sorokiniana*, and 20 ppt. for *O. heteromucosa*. As a final preparation, the pH of the media was accordingly adjusted to 8.0 ± 0.01. For the isolation of microalgae by streak plating, a solid Conway medium under the same conditions as described above was prepared with an addition of 2% agar. Individual colonies grew on the solid medium after 3 to 6 weeks and were streaked onto new plates with their respective medium. All isolated marine microalgae from these two isolation techniques were maintained in a liquid-Conway medium as stock cultures. These stock cultures were stored in the collection of the Laboratory of

Aquatic Biology and Environmental Science, The University of Tokyo, Tokyo, Japan, before being used.

Experimental Design

A schematic diagram of the experimental design of this study was shown in Figure 5. The study started with the soil sampling and continued with the soil extractions using water at different temperatures: 105°C and 21°C. Chemical characterisations based on TDC, TDN, TDP and UV_{254nm} contents of these varied soil extract solutions were then analysed before being fractionated into humic-non-humic fractions and low-high-molecular-size fractions. The growth tests for three species of microalgae were separately conducted using a 96-microplate (Stem Corporation, Japan). As preparations, the volumes of soil extracts and fractions were primarily adjusted to achieve 1 mg/L of their total DOC concentration before conducting the growth test. The soil extracts and fractions were filtered with a 0.22 µm glass fibre filter (Millex-GV, Merck, German) before being added to the microplate well. A blank solution that contained medium and without microalgae was prepared as a control in this growth test. The total capacity or final volume of all added samples in the microplate wells was 200 µL. The border wells of the microplate were also filled with 200 µL MQ water to prevent evaporation.

According to Rojickova *et al.* (1998), the border wells of the microplates were not used during experiments as it exposes the wells to strong air currents. The microplate wells were horizontally divided into two sections with triplicate wells respectively. The upper three-well columns were used as a blank and its bottom three-well column was used to test soil extract/soil fraction samples. Conway medium with 200 µL was added at the first upper three-well column (as a blank), meanwhile, Conway medium with 180 µL and tested microalgae with 20 µL were added at its bottom three-well column. The next upper three-well column was filled with 195 µL Conway medium + 5 µL of 105°C soil extract (as a blank) and its bottom three-well column was filled with 175 µL Conway medium + 5 µL of 105°C soil extract + 20 µL of tested microalgae.

Similar steps were repeated in the remaining wells for 105°C humic fraction, 105°C non-humic fraction, 105°C low-molecular-size fraction, 105°C high-molecular-size fraction, 121°C soil extract, 121°C humic fraction, 121°C non-humic fraction, 121°C low-molecular-size fraction and 121°C high-molecular-size fraction. The microplates were incubated for 12 days to reach the stationary phase and the microalgae growth was spectrometrically measured with the optical density at a wavelength of 680 nm wavelength (SpectraMax® M2, Molecular Devices, USA). The specific growth rate of the microalgae was calculated using the following Equation 2 (Wood *et al.,* 2005):

$$\mu = \ln(N_2/N_1)/(t_2 - t_1) \quad (2)$$

where μ is the specific growth rate and N_1 and N_2 are the biomass at time 1 (t_1) and time 2 (t_2), respectively.

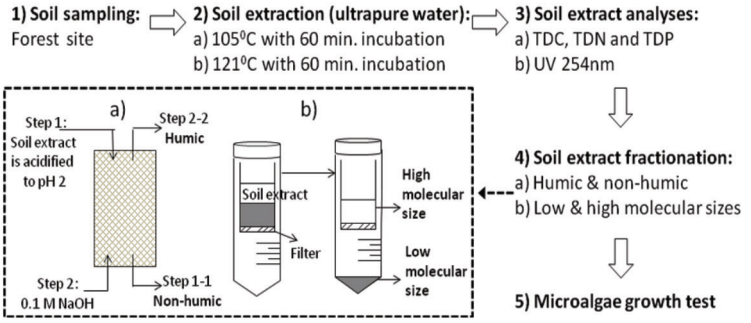

Figure 5: Experimental design of the study from soil sampling to the microalgal growth test

Data Analysis

Results of soil extracts and fractions in chemical characterisations, kinetic growths of microalgae, and specific growth rates of microalgae were analysed using one-way Analysis of Variance (ANOVA) and Duncan post-hoc analysis. Significant differences between the tested parameters were calculated at a 95% confidence interval level. All statistical analyses were conducted using IBM SPSS (Statistical Package for the Social Sciences) Statistics 20 software.

Results and Discussion

Results showed the DOC concentration of the soil extracted at 121°C was almost twice as high as that of the soil extracted at 105°C (Table 1). The concentration of its TDP was higher, although the concentration of TDN was just slightly higher than the soil extracted at 105°C. Furthermore, the ratio of UVA_{254nm} to DOC concentration for the aromaticity index exhibited similarly higher rates in the solution extracted at 121°C than that extracted at 105°C. The total DOC concentrations for both fraction types of soil extract showed a slight difference after the fractionation, regarding the distinctive principles and limitations in each fractionation method (Table 2 and Table 3). However, the total DOC recovery percentage for both fraction types was somehow found high (> 80%) after fractionation, which is close to the concentration before fractionation (Table 1). These results suggested that the best extraction methods for the highest recovery of Dissolved Organic Carbon (DOC), Total Dissolved Nitrogen (TDN), Total Dissolved Phosphorus (TDP), and DOC for all tested soil fractions are the autoclaved method at 121°C. Since the rates of recovery are high and the soil extract solution itself originated from the pristine forest soil, it is believed that its impacts on the growth performances of marine microalgae are also parallel when supplemented in the growth media formulation.

On top of these, however, only 1 mg/L from the total DOC concentration of soil extract and soil fractions was standardised and used for the growth tests of marine microalgae. Therefore, concentrations of nitrogen and phosphorus that were adjusted to 1 mg/L of DOC were approximately estimated between 0.01 to 0.1 mg/L concentration in soil extracts and fractions compared to the concentration of dissolved carbon. In this circumstance, both nitrogen and phosphorus from the soil extract showed very low concentrations when added to the Conway medium. Their potential as macronutrients that contributed to the major growth-promoting factors in marine microalgae was seemingly irrelevant and ignored in this study. Thus, the potential of enhancing effects from the dissolved carbon sources of soil extract solution on the microalgal growth was a major concern.

The growth curve (Figure 6) of each tested marine microalgae was plotted and observed for confirming the growth phases and determining the specific growth rates (μ) (standardised value). The specific growth rate of *C. sorokiniana* [Figure 7 (a)] was found to be the highest in the soil extracted at 105°C (1.20). This rate was estimated at between a 7%-20% increase from that 121°C (1.12) and the control (1.00), respectively. In humic and low-molecular size fractions, the specific growth rates were estimated at between an 11% and 17% increase

Table 1: Mean in triplicates (n = 3) with standard deviations of concentrations of Dissolved Organic Carbon (DOC), Total Dissolved Nitrogen (TDN), Total Dissolved Phosphorus (TDP), UVA_{254nm} value, and ratio of UVA_{254nm}/DOC of soil extract solutions at 105°C and 121°C

Soil Extract	DOC	TDN	TDP	UVA_{254nm}	UV/DOC
	(mg /L)			(mAbs. cm^{-1})	
105°C	308.3 ± 2.5	22.7 ± 2.08	0.22 ± 0.20	4198.0 ± 2.0	13.6 ± 0.11
121°C	527.7 ± 1.5	37.3 ± 0.58	0.77 ± 0.06	10519.7 ± 2.5	19.9 ± 0.06

Table 2: Mean in triplicates (n = 3) with standard deviations of concentrations of Dissolved Organic Carbon (DOC) of humic and non-humic fractions from the soil extract solutions at 105°C and 121°C

Soil Extract	DOC Concentration (mg/L)		
	Total	Humic	Non-humic
105°C	274.3 ± 1.5	72.0 ± 2.0	202. 3 ± 2.5
121°C	428.0 ± 2.0	113.3 ± 2.5	314.7 ± 2.5

Table 3: Mean in triplicates (n = 3) with standard deviations of concentrations of Dissolved Organic Carbon (DOC) of low-molecular-size and high-molecular-size fractions from the soil extract solutions at 105°C and 121°C

Soil Extract	DOC Concentration (mg/L)		
	Total	Low Molecular-size	High Molecular-size
105°C	303.3 ± 2.5	203.7 ± 0.6	99.0 ± 3.6
121°C	438.3 ± 1.5	307.7 ± 4.0	129.7 ± 3.5

from the soil extracted at 105°C in their humic (1.34) and low-molecular-size (1.41) fractions. For the non-humic and high-molecular size fractions of both soil extracts (105°C and 121°C), the specific growth rates showed a significant decrease from their respective controls. In the *O. heteromucosa* culture [Figure 7 (b)], the increased specific growth rate was found to be insignificant ($p > 0.05$) with control from both soil extract solutions of 105°C (1.05) and 121°C (1.07). However, humic, and low-molecular size fractions from both soil extracts satisfactorily increased the specific growth rates by between approximately 10% and 37% from their non-fractionated soil extracts. The decreased effects in the specific growth rate were also observed from the non-humic and high-molecular size fractions of both soil extracts (105°C and 121°C) when compared with their respective controls. The specific growth rate of *T. weissflogii* was found highest in the soil extracted at 105°C (1.22) and this rate was estimated at a between 10% and 22% increase from that 121°C (1.11) and control [Figure 7 (c)]. In low-molecular size and humic fractions, the specific growth rates were found at 1.41 and 1.60, respectively, which increased by around 15% and 31% from the soil extracted at 105°C. The non-humic and high-molecular-size fractions from both soil extracts also decreased in the specific growth rate from their control; similar to results from the green microalgae.

Soil organic matter is generally depicted as a complex mixture of macromolecular substances including humic and non-humic substances (Lam *et al*., 2007; Cawley *et al*., 2013). Most of the soil's organic matter is present in a dissolved form. Its major constituent, humic substances, has always been regarded as refractory products resulting from the bio-geochemical degradation of detritus from biomass (D'Andrilli *et al*., 2013; Derrien *et al*., 2017). These refractory structures are adequately suitable to be weakened or disturbed to make them bioavailable to other living organisms (Leenheer *et al*., 1998). According to Truhlar, (1978) and Steinweg *et al*. (2013), high energy derived from the high temperature that exceeds its activation energy is essential for breaking down the chemical bonds of any component before they can be solubilized. These reports were in agreement with our results that soil extraction temperatures of 105°C and 121°C can adequately conserve the solubilised soil organic matters which are sufficient to stimulate microalgal growth in this study. The stimulation effect can be observed

from the enhancement of the specific growth rates in all tested microalgae from the soil extracts at different extraction temperatures.

Generally, organic components found in the soil are from the hydrophilic acids group (carbohydrates, proteins, and amino acids) that are referred to as non-humic substances and hydrophobic acids group referred to as humic substances (Imai *et al*., 2001; 2002). Humic substances are mainly made up of fulvic and humic acids, and the molecular size of these substances ranged from a few hundred to millions of daltons (Perminova *et al*., 2003; Rodriguez & Nunez, 2011; Derrien *et al*., 2017). The humic and non-humic fractions in this study were separated by the resin adsorbent DAX-8 method, which has been an original and widely used method for fractionating aquatic humic substances for a long time (Christl *et al*., 2000; Abbt-Braun *et al*., 2004). Despite this, our results for the concentrations of humic and non-humic fractions, however, showed relatively high recovery yield with unexpectedly low concentrations of humic fractions (Table 2). Many studies have reported that the organic components that build up the entire

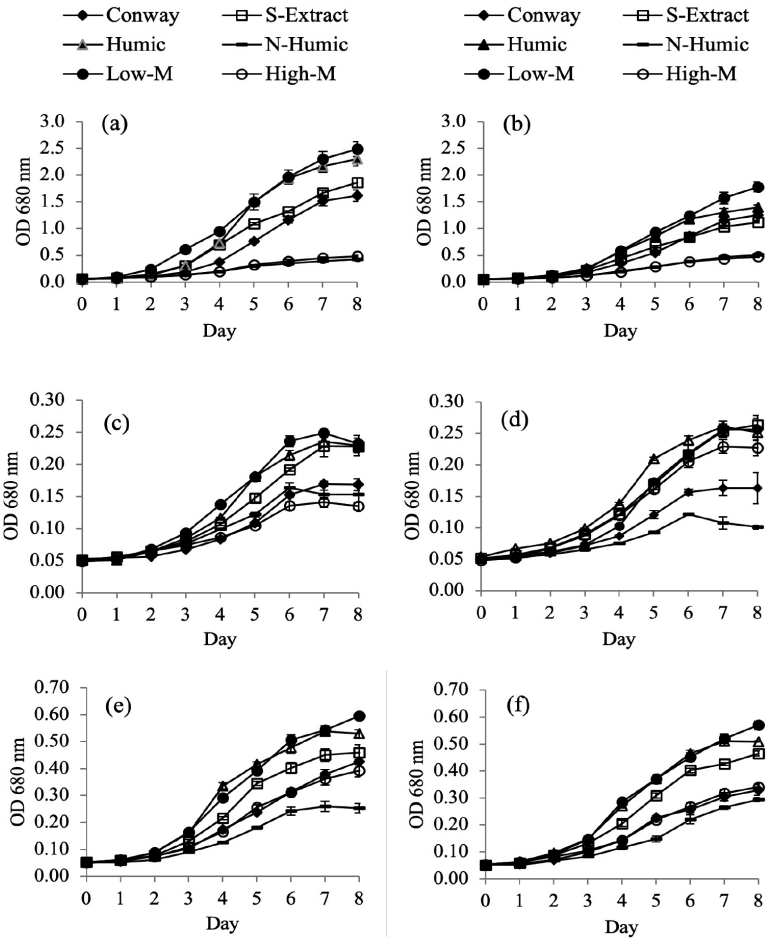

Figure 6: Kinetic growths of *Chlorella sorokiniana* (a and b), *Oocystis heteromucosa* (c and d) and *Thalassiosira weissflogii* (e and f) based on optical density value at 680 nm with additions of soil extract (S-Extract), humic, non-humic (N-Humic), low-molecular-size (Low-M) and high-molecular-size fractions (High-M) at different extraction temperatures. Error bars represent standard deviation in triplicates (n = 3)

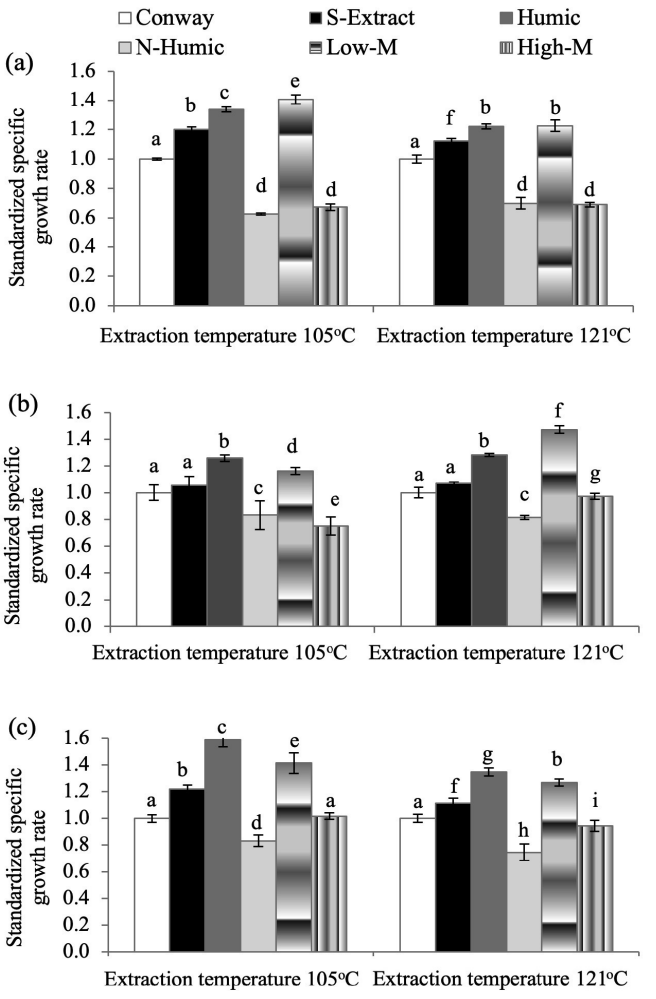

Figure 7: Mean of the standardized specific growth rates of *Chlorella sorokiniana* (a), *Oocystis heteromucosa* (b) and *Thalassiosira weissflogii* (c) with additions of soil extract (S-Extract), humic, non-humic (N-Humic), low-molecular-size (Low-M) and high-molecular-size fractions (High-M) at different extraction temperatures. Error bars represent standard deviation in triplicates (n = 3). Significant differences in specific growth rates between the tested samples are shown by different alphabets on the top of each bar (ANOVA, Duncan-MRT *post-hoc* test, $p < 0.05$)

soil organic matter and characterize the soil type are dependent on prevailing sources and environmental fate, such as leaching and runoff (Alken, 1984; Hur & Kim, 2009; Rodriguez & Nunez, 2011; Weber *et al*., 2018; Olk *et al*., 2019), even its major counterpart, humic substances are considerably enriched with condensed molecules and lignin (Ertel & Hedges, 1984; Stenson *et al*., 2003; Derrien *et al*., 2017). As mentioned earlier, humic substances were always defined as heterogeneous mixtures of partially biodegradable matter, and standard protocols for purification and extraction to recover them from the whole soil organic matter before fractionation was almost non-existing (Malcolm, 1990; Muscolo *et al*., 2013; Weber *et al*., 2018). This might be the most relevant reason for justifying the differences in the concentration of our soil extract fractions; however, this explanation may need to be

further confirmed using an extensive number of soil samples from various environments.

The humic fractions consistently showed higher specific growth rates and more competence than the non-humic fractions; based on the enhancement percentages observed from this study. The significant effects of these fractions after being separated from their non-fractionated soil extracts were greater in both green microalgae and diatom species. Therefore, humic fractions in this study were the most conceivable factor that directly responds to growth stimulation and enhancement in microalgae probably by their chelation reactions with trace elements (Anderson & Morel, 2005; Gagnon *et al*., 2005; Cawley *et al*., 2013). Iron is recognised as the most essential trace element required in microalgae cultivation which is involved in several enzymatic reactions, photochemistry, and chlorophyll synthesis (Naito *et al*., 2005; Terauchi *et al*., 2005; Rizwan *et al*., 2017). In this study, iron is supplemented through $FeCl_3.6H_2O$ with its chelating agent $Na_2H_2EDTA.2H_2O$ in the Conway medium at a low volume that is suited for a microplate well final capacity of 200 µL.

Although no further tests were done for observing and confirming the precipitation of trace elements in the microplate well, it is generally believed that without chelating and solubilising them specifically, they would otherwise present and precipitate in the insoluble or colloidal form (Hoeffner & Manahan, 1980; Lee *et al*., 2009; Sanchez-Marin & Beiras, 2011; Botebol *et al*., 2014; Rizwan *et al*., 2017). Thus, chelated iron is necessary because these precipitation and solubility limitations can trigger iron deficiency that makes the availability of iron insufficient to stimulate microalgae growth (Hoeffner & Manahan, 1980; Botebol *et al*., 2014; Rizwan *et al*., 2017). This anticipation is consistent with the results of specific growth rates in this study that are comparable between the medium with soil extracts; as an external chelating agent and control in the absence of an external source of the chelating agent. Furthermore, in these trace elements, microalgae also tend to have particular preferences for iron sources and vary from species to species (Liu *et al*., 2008; Botebol *et al*., 2014; Rizwan *et al*., 2017).

In the study done by Rizwan *et al*. (2017), the fastest cell growth of green microalgae, *Dunaliella tertiolecta* at an early stage was achieved with the presence of ferric chloride ($FeCl_3$) compared with ferric EDTA ($C_{10}H_{12}FeN_2O_8$) and ferric ammonium sulphate ($FeNH_4(SO_4)_2$). Another study by Botebol *et al*. (2014), however, found that iron was much more available to the cells when provided as ferric citrate ($C_6H_5FeO_7$) as compared with ferric EDTA in a few strains of marine microalgae: Green microalgae (*Ostreococcus tauri*), diatom (*Phaeodactylum tricornutum*), and coccolithophorid microalgae (*Emiliania huxleyi*). This evidence showed that the cellular uptakes of iron might be influenced by the differences between microalgae species and the ionic parts of ferric compounds, thus, appropriate chelation of iron would be helpful to maintain its availability. Our results in the specific growth rate of each tested marine microalgae from humic substances also varied. We expect that the growth-stimulation impact may reflect from the iron chelation and preferences matters. However, the details on those matters would be certainly investigated in future research since we are now in the early stages of establishing the extraction and fractionation of organic matter in soil samples.

Apart from the effects on trace element complexation properties, humic substances can act as intermediate components in the cellular metabolic processes of microalgae, probably to the growth mechanisms that have been suggested for terrestrial plants (Rashid *et al*., 1971; Hoeffner & Manahan, 1980; Nardi *et al*., 2002; Liu *et al*., 2008). Other unanticipated factors that contributed to growth-stimulation might be related to the different growth activity and adaptation mechanisms among the marine microalgae (Prakash & Rashid, 1968; Watanabe, 2005; Martin *et al.,* 2014). To understand this situation, a study by Yu *et al*. (2018) showed that

microalgae can naturally excrete extracellular substances or extracellular waste during their growth metabolism, which might act as a growth suppressor. This extracellular waste can also lead the oxidative damage and restrain all cell activities by increasing the reactive oxygen species (Zhang *et al*., 2018). However, these would be dependent on the microalgae cell systems and mechanisms to encounter and respond to these problems for their survival. Furthermore, since the humic fractions have the potential as antioxidants to alleviate cellular damage (Lee *et al*., 2009; Yu *et al*., 2018; Zhang *et al*., 2018) the microalgal growth rates would be expected to be higher than in the non-humic fractions.

The capacity of the growth stimulations from the non-humic fractions was incomparable and inconsistent with that of the humic fractions. Moreover, the growth effects that resulted from these fractions which mainly consisted of hydrophilic acids components (polysaccharides, proteins, and lipids) were found not well-documented as compared to the former fraction types. Nonetheless, they also showed promising potential. Their significant effect on the growth of microalgae was not surprising since the components of these fractions may directly function as sources of nutrients for microalgae (Prakash, 1967; Prakash & Rashid, 1968). Previous studies from Hassler and Schoemann (2009) additionally reported that polysaccharides also have the potential to increase iron uptake rates in microalgae species from the Southern Ocean, including a diatom, *Thalassiosira antartica* although the mechanism remains unknown.

The classical view of humic substances reported that they are complex polydisperse macromolecules and high in molecular sizes: From a few hundred to millions in Dalton unit (Dell'Agnola & Nardi, 1987; Muscolo *et al*., 2013). These findings are somewhat contradictory to some studies that hypothesised they were less polydisperse and low in molecular sizes, from a few hundred to thousand in Dalton unit (Thurman, 1985; Chin *et al.*, 1994; Swift, 1996; Muscolo *et al.*, 2013) likely what has been anticipated and proved through this study. The higher specific growth rates in our tested marine microalgae were observed from both fractions of humic and low-molecular-size fractions (< 10,000 Da) compared to non-humic and high-molecular-size fractions (> 10,000 Da) (Figure 7). Despite these contradictions, there is evidence from previous studies that prove that marine microalgae, specifically diatoms, prefer to utilise low-molecular-size substances from the aquatic organic matter as a source of nutrients (Kaplan & Bott, 1983; Flynn & Butler, 1986; Antia *et al*., 1991; Martin *et al*., 2014).

The marine diatom *Gonyaulax sp.* can respond well in the media with the addition of the low-molecular-size fraction from the terrestrial (forest soil) and aquatic (marine sediment and river water) organic matter sources (Prakash, 1967). Another study by Prakash, (1967) and Prakash *et al*. (1973) showed some species of marine diatoms that generated maximum growth rate in a low-molecular-size fraction and the concentration of humic substances extracted from the mangrove soil. Our results revealed that the low-molecular-size fractions from the forest soil are effective for both diatom species (*T. weissflogii*) and the green microalgae species (*C. sorokiniana* and *O. heteromucosa*).

Conclusion

Overall, we investigated the effects of different soil extracts and their fractions from the forest soil on the growth of selected marine microalgae. The selection of appropriate temperatures for soil extraction is essential since it can affect the quality of the solution and its fractions on the growth of marine microalgae. However, the soil extracts with different extraction temperatures before being fractionated were found inconsistent in their growth effects and are strain-dependent. Soil extracts at an extraction temperature of 105°C were found to be most effective for the green microalgae strain (*C. sorokiniana*) and diatom strain (*T. weissflogii*); meanwhile, soil extraction at an extraction temperature of 121°C is seemingly

effective for other strains of green microalgae; *O. heteromucosa*. After the soil extracts were fractionated into their respective fractions, the main contributor and potential of each fraction to the growth effects on marine microalgae managed to be proven and preliminary clarified in this study. The humic and low-molecular-size fractions of soil extracts were consistently shown as the major contributors to the growth enhancement of the tested marine microalgae strains. The non-humic and high-molecular-size fractions also showed promising potential, nonetheless, their growth-enhancing effects were not persistent and unpredictable. The growth stimulation and enhancement observed in this study might be associated with the greater potential of humic fraction constituents (humic and fulvic acids) in the iron-chelation reactions, nutrient sources and combinations with the microalgal growth responses and adaptation mechanisms. The underlying mechanisms of physiological stimulation by the soil extract fraction and the pathways to the successful growth enhancement in marine microalgae are not adequately understood and offer great opportunities for critical inquiry and further establishment.

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