Pertanika J. Sci. & Technol. 33 (5): 2177 - 2205 (2025)

PERTANIKA JOURNALS

SCIENCE & TECHNOLOGY

Journal homepage: http://www.pertanika.upm.edu.my/

Screening and Isolation of Microalgae Collected from Tin Mining at Bangka Belitung Province with Remarks on Their UV-C Absorbance and Lead Remediation

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ABSTRACT

Microalgae inhabit mining sites, performing specific adaptations to survive the stress conditions. This study aimed to investigate the effect of growth media on the microalgae isolated from water bodies at abandoned tin mining sites and analyse their sensitivity to ultraviolet-C (UV-C) spectrum and lead resistance. Samples from six locations were enriched in Bold Basal Medium (BBM) (pH 6.8) and Blue Green-11 (BG-11) (pH 7.4) media, and the grown cultures were kept in a cold room at a temperature of 21°C, provided with continuous light (1,600 lux). The UV-C sensitivity of the cultures was observed with spectrophotometry at λ=230 nm, followed by growth rate measurement. The isolate was subjected to lead concentrations of 0, 10, 100, and 200 ppm. The results showed that the diversity of the microalgae in the BBM was higher than in BG-11, with 7 and 3 species, respectively. Most microalgae grown in BBM were coccoid green algae, while diatoms and cyanobacteria were in BG-11. All cultures developed from enrichment step showed UV-C absorption, and the fastest growth was performed by Ulu River (UL) culture. Isolate coccoid green algae UL4 had sensitivity to the UV-C spectrum and survived high lead concentrations up to 200 ppm. This character, plus the capability to survive in high lead concentration, made UL4 a potential candidate for a bioremediation agent. Phylogenetic analysis based on the 18S rRNA

ARTICLE INFO

Article history:
Received: 22 October 2024
Accepted: 29 April 2025
Published: 28 August 2025

DOI: https://doi.org/10.47836/pjst.33.5.07

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e-ISSN: 2231-8526

gene showed that the strain UL4 is nested within the clade of *Chlorococcum* spp. It is of interest for future research to investigate the involvement of secondary metabolites as a mechanism for UL4 survival in stress conditions.

Keywords: Algal media, Bangka Belitung, green algae, lead remediation, UV-C tolerance

INTRODUCTION

Microalgae are autotrophic microorganisms which are found in aquatic, terrestrial, and aerial environments (Sahoo & Baweja, 2015). The potential of the microalgae in various fields has been acknowledged. Species such as *Chlorella* and *Spirulina* have been widely developed as health and beauty supplements because of their active ingredients, like polyunsaturated fatty acid (PUFA) and β-1,3-glucan (Koyande et al., 2019). Another species like *Scenedesmus obliquus* and *Chlamydomonas reinhardtii* are developed for biofuel due to their high lipid content. In waste treatment, *Chlorella* sp. is used for bioremediation agent to reduce ammonia and palm oil waste (Shuba & Kifle, 2018). The application of algae in many fields is a long journey from sampling, isolating, and culturing potential species, producing biomass, until algal productions.

Muntok City, the capital of West Bangka Regency, Bangka Belitung Islands Province, has been established as a tin mining site since 1707 (Swastiwi et al., 2017). Due to the expansion of the tin mining areas, Haryati and Dariah (2019) estimated the production of carbon dioxide emissions in Bangka Belitung would reach 36,908,808 t carbon dioxide (CO_2) in 2040. The high CO_2 emission thinned the ozone layer, thereby increasing ultraviolet (UV) radiation on the Earth's surface. The excessive exposure to the UV radiation, especially the UV-C radiation, which has high energy, could affect the survival ability, growth rate, distribution, motility, and even death of the microorganisms (Tekiner et al., 2019). Therefore, the microalgae that live in extreme areas, such as tin mining sites, must be able to adapt to the UV-C radiation.

Several studies reported that the microalgae survived and thrived in mining areas. Gomes et al. (2021) reported the discovery of *Spirogyra, Mouegotia*, *Chlorella*, and diatoms, like *Pinnularia*, *Navicula*, and *Eunotia*, in the copper and silver mining area at Sao Domingo, Portugal. Another research by Senhorinho et al. (2018) discovered several species, including *Chlorococcum*, *Desmodesmus*, and *Cocomyxa*, in an abandoned mining site in Canada. Water sample observations conducted around the tin mining sites in West Bangka Regency by Rachman (2019) revealed that the microalgae diversity was dominated by the diatoms such as *Thalassiothrix*, *Chaetoceros*, and *Rhizosolenia*. Besides diatoms, Rachman (2019) also found dinoflagellates such as *Protoperidinium* and *Pyrophacus*. Previously, Bidayani (2017) found *Chroococcus* and *Uroglena* as the dominant microalgae in the reuse of a tin mining pit.

Several studies showed that the UV-C light-adapted microalgae could produce advantageous metabolites. For example, *Dunaliella salinea*, *Chlorella vulgaris*, and *Pavlova lutheri*, which produced high sterols and lipids that are useful for biofuel production (Ahmed & Schenk, 2017; Carino & Vital, 2022; Ghezelbash et al., 2023). Another potential of the microalgae living in extreme conditions was investigated by Gauthier et al. (2020) and Senhorinho et al. (2018). Both reported that the microalgae which were exposed to heavy metals, ultraviolet-B (UV-B) radiation, and high salinity had the potential to generate advantageous metabolite compounds such as antibacterials and antioxidants.

The microalgae that have been living in the Muntok tin mine since the mining's existence more than a hundred years ago could develop a strategy to adapt to the UV exposure. One of the strategies is producing specific metabolites that not only protect cells from UV radiation but also reduce other environmental stresses, such as high concentrations of heavy metals. The metal-binding protein metallothioneins have roles for transporting toxic metals from the cytosol into other organelles (Balzano et al., 2020). Metallothionein-2 transplastomic *Chlamydomonas reinhardtii* showed higher resistance to the UV-B exposure than the wild type. The addition of metals (zinc, cadmium, and copper) to the growth medium of MT2 transgenic *C. reinhardtii* is related to the survival of the cells (Zhang et al., 2005).

The water bodies in the Bangka Belitung tin mining area were contaminated with heavy metals, dominated by lead. Some research, such as Miranda et al. (2018), reported the lead content in the sediment of River Pakil was 12.96 mg/kg, while Tawa et al. (2019) found lead content in the waters of Kelabat Bay was 0.2624-0.5713 mg/L. Exploration of the lead-tolerant microalgae in the tin mining area of Bangka Belitung becomes important to get information on the diversity of indigenous microalgae and their potential. Moreover, isolation of the UV-C and lead-tolerant microalgae could be viewed as one of the solutions to the environmental problems.

Algal growth media play an important role in the success of algal isolation and culture. Differences in nutrients of the algal growth media influence the kind of microalgal species, their growth rate, and metabolite production. Synthetic media, such as BBM and BG-11, are widely used as both medium enrichment and growth medium for an established culture (Andersen, 2005; Badr & Fouad, 2021).

Bold Basal Medium was introduced by Bischoff and Bold in 1963 for isolating soil microalgae. Several characteristics of the BBM include high metal element content, pH 6.4-6.8, and a lack of vitamins (Andersen, 2005). The BBM is a generic medium for the green microalgae, and many species have been cultured in the BBM, such as *Chlorella* spp. (Blair et al., 2014), *Scenedesmus* spp. (Habibi et al., 2019), *Micractinium* spp., and *Chlamydomonas* spp. (Lloyd et al., 2021).

On the other hand, the BG-11 medium was developed by Allen and Stanier in 1968. This medium was developed from the artificial medium No. 11 created by Hughes et al. in 1958. The BG-11 medium was created for culturing the freshwater, saltwater, and soil

cyanobacteria (Andersen, 2005; Rippka, 1979). Cyanobacteria, such as *Synechococcus elongatus* PCC7942 (Yang et al., 2015) and *Synechocystis* sp. (Yan et al., 2014), can be cultured in the BG-11.

The aims of this research were to investigate the effect of growth media and the existence of UV-C-tolerant microalgae communities from Muntok tin mining. Besides, we also evaluated the potential of a microalgal isolate for lead resistance.

MATERIALS AND METHODS

Sample Collection and Enrichment

Samples were collected from fresh and brackish waters around the abandoned tin mining site located in the Muntok Subdistrict, West Bangka Belitung Regency, Bangka Belitung Province, Indonesia, in January and February 2022. Six sampling sites were selected based on a purposive sampling method, which were Pait (PA) and Ketok (KE) abandoned mining sites, Belo River (BE), Pancur River (PC), Pantai Baru Beach (PB), and Ulu River (UL) (Figure 1). From each location, a 100 ml water sample was collected approximately 5 cm below the surface and transferred into polyethylene terephthalate (PET) plastic

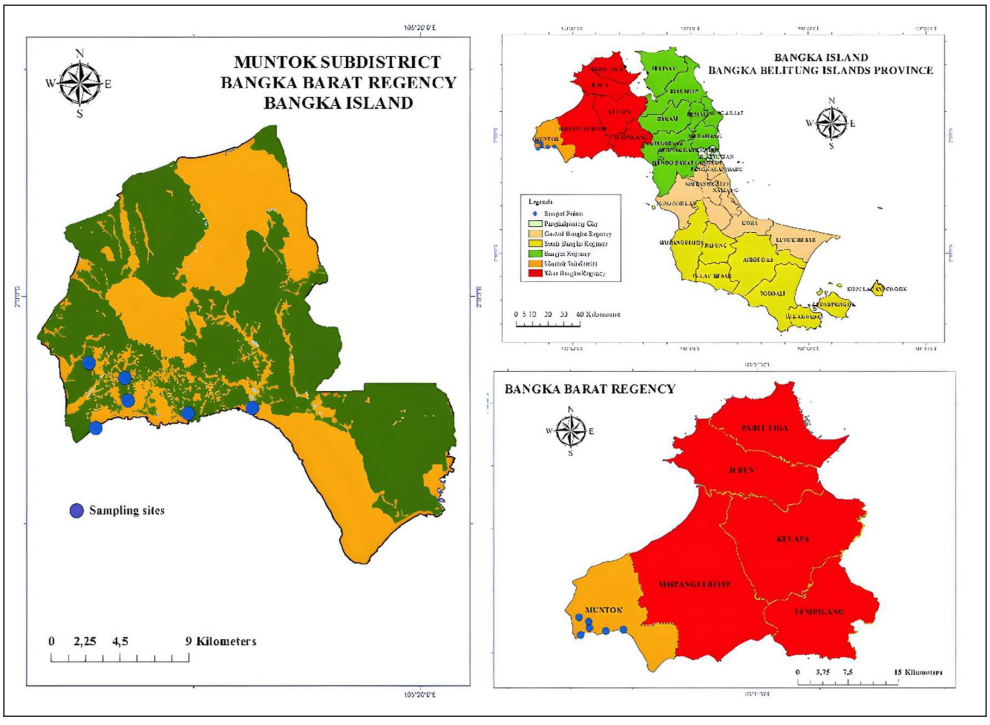

Figure 1. Research locations map. The coordinate points of Pait (PA): -2.06826, 105.19686; Belo River (BE): -2.06491, 105.23462; Ketok (KE): -2.03884, 105.13849; Ulu River (UL): -2.06079, 105.16154; Pancur River (PC): -2.04750, 105.15974; and Pantai Baru Beach (PB): -2.07589, 105.14210

bottles. The collected samples were stored in a refrigerator (4°C) before being transported to the Microalgae Culture Preparation Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Indonesia, West Java Province. At the laboratory, samples were immediately subjected to the enrichment process.

All samples were enriched using the BBM and BG-11 media in a 48-well microplate. The BBM and BG-11 media were prepared according to Andersen (2005) and Stanier et al. (1971), respectively. The pH value was adjusted to 6.8 for the BBM and 7.4 for the BG-11 by adding 0.1 M sodium hydroxide (NaOH, Merck, USA). The media were sterilised using an autoclave (TOMY SX 700, Japan) at a temperature of 121°C, with an air pressure of 1 atm. The enriched samples in a well microplate were placed on a culture storage rack (Figure 2) at a temperature of approximately 21°C, under continuous light for two to three weeks. Observations were carried out once a week. Any growing microalgae from the enrichment step were re-cultured with addition of fresh BBM (50 ml volume per 100 ml Erlenmeyer flask; triplicate) and observed for 14 days. At the end of the observation, the most stable culture was selected and used for the UV-C absorption experiment. The stable culture was designated qualitatively based on the homogeneity of biomass and colour as determined by the Hexadecimal (HEX) Colour Codes (https://www.colour-hex.com/). The entire process was carried out in an aseptic process.

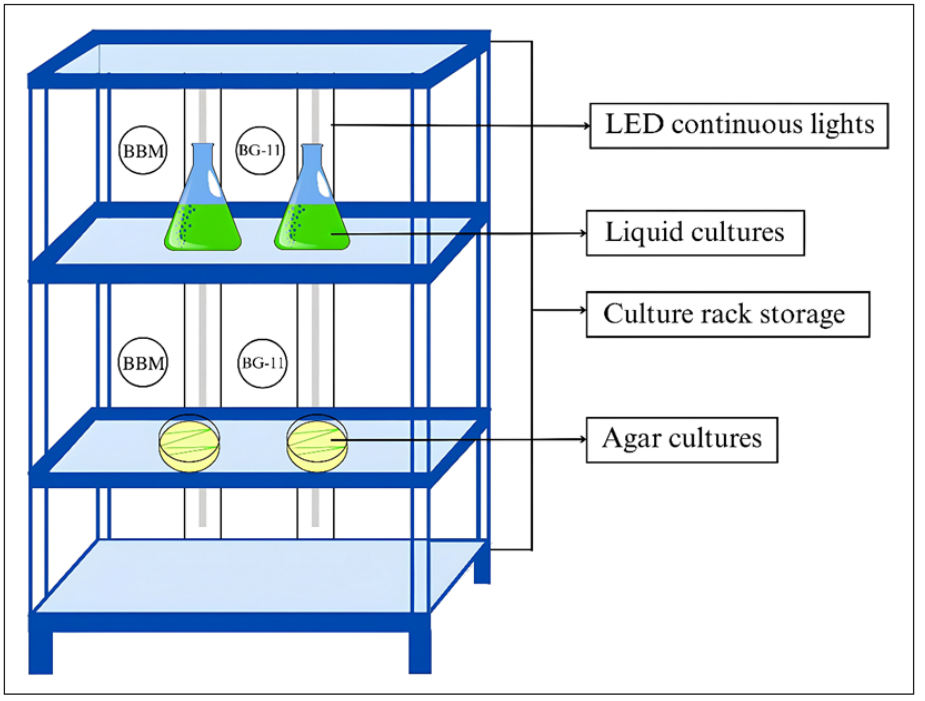

Figure 2. Culture storage setup for the microalgae enrichment and cultivation
Note. BBM = Bold Basal Medium; BG-11 = Blue Green-11; LED = Light emitting diode

Microalgal Observation

The observation of the microalgae was conducted using a binocular light microscope (Leica DM 500 Microscope, Germany) equipped with the LAS EZ software (version 3.4.0) for documentation. Culture sampling for the morphological observation was carried out in a transfer box following an aseptic procedure. Representative samples were taken from the liquid cultures using a sterilised Pasteur pipette, placed on a glass slide, and covered with a coverslip (Govindan et al., 2021). For the agar culture, one colony was taken using a sterilised loop and then placed on glass slides with a single drop of sterilised aquabidest. Morphological characters were observed, including the cell shape and size, colour, chloroplast type, number of pyrenoids, presence of flagella and sheath, and other cells' ornaments. Identification was performed using a determination key (Rosen & Mareš, 2016; Wehr et al., 2014) and other references (Komárek et al., 2014; Puilingi et al., 2022; Tan et al., 2016). Information from the AlgaeBase was also used (https://www.algaebase.org/).

Measurements of UV-C Sensitivity and Microalgal Growth Rate

For the growth rate experiment, a total of 20% of the inoculum from the stock of the selected culture was transferred at the exponential phase to different 500 ml flasks, each containing 200 ml BBM or BG-11 medium (Blanco-Vieites et al., 2022). Experiments were performed with three replications. The experiment cultures were then placed in the culture rack previously described. Daily manual agitation was performed to ensure homogeneous distribution of nutrients.

Growth rate observations were conducted using the optical density measurement method with a spectrophotometer (Genesys 105 UV-VIS Spectrophotometer, Thermo Fisher Scientific, USA). The wavelength was determined using the culture absorbance sensitivity, which ranged from 200 to 800 nm. After repeated observations, the wavelength of 230 nm (OD_{230}) was selected for growth measurement. The experiment was carried out for 20 days and the microalgal culture growth was observed every 2 days (Susanti et al., 2021). The specific growth rate was calculated using the formula as follows:

$$Specific\ growth\ rate\ (\mathrm{K}')(\mu/day) = \frac{Ln\ (\frac{N2}{N1})}{t2 - t1}$$

where, Ln = Constant (3.22); N1 = Biomass time t1; and N2 = Biomass at time t2 (Krzemińska et al., 2014).

Isolation and DNA Extraction, Amplification, and Sequencing

Isolation and purification of the microalgae were carried out using the streak and serial dilution methods (Sandeep et al., 2019) using the BBM and BG-11 liquid and agar media (2.5% Bacto™ Agar, Becton Dickinson, Canada). The biomass of the isolate was collected by centrifugation, and 1 mg was subjected to DNA extraction using the DNeasy Plant Mini Kit (Promega, USA) according to the manufacturer's instructions. Concentration and purification of extracted DNA were measured with a nanophotometer (Implen, Germany).

The amplification of the 18S rRNA gene was carried out using the thermal cycler (Techne TC-3000G, USA) with forward primer of 18SF (5'-CCTGGTTGATCCTGCCAG-3') and 18SR (5'-WTGATCCTTCYGCAGGTTCA-3') (Wan et al., 2011). The reaction was performed in a total volume of 25 µl, having 12.5 µl of Dream Taq Green (ThermoFisher, Germany) master mix, 1 µl of each primer, 2 µl of DNA template, and 8.5 µl of nuclease-free water. Polymerase chain reaction (PCR) was initiated with denaturation (94°C, 3 min), followed by 30 cycles of denaturation (94°C, 1 min), annealing (59°C, 1 min), elongation (72°C, 1 min), and finished by terminal extension (72°C, 10 min).

The amplicons were sent to Macrogen (Singapore) for sequencing. The result sequenced forward, and reverse fragments were checked using the software package Chromas 2.6.2 (Technelysium.com). The 18S rRNA gene sequences obtained were run through the Basic Local Alignment Search Tool nucleotide (BLASTn) programme from GenBank to check species identity with reference sequences before reconstructing the phylogenetic tree.

Lead Resistant Experiment

A total of 1.34 g of lead (II) chloride ($PbCl_2$) (Merck, USA) was dissolved in 1 L of distilled water (Zehra et al., 2018). The lead solution is then sterilised using an autoclave before use. To prevent lead precipitation due to the sterilisation process, the lead solution was filtered using a filter paper (Whatman 934-AH, diameter 47 mm). The lead medium was diluted to concentrations of 10, 100, and 200 ppm. The growth rate of the microalgae at various lead concentrations was measured using a wavelength of 680 nm (OD_{680}).

The culture biomass dry weight was measured to observe the influence of lead. At the end of the growth observation, the culture biomass was harvested using centrifugation at 4,427 x *g* for 15 min. The wet biomass was placed in an evaporating basin and dried using an oven (Jisico, Korea) until a constant weight was achieved. The dried biomass was measured using an analytical balance (Precisa XT-200, Swiss). The weight difference between the empty evaporating basin and the dish with dry biomass measured the biomass weight.

Data Analysis

Data analysis was conducted both qualitatively and quantitatively. The qualitative analysis was performed for morphological observations and colour, while the quantitative analysis

was performed for optical density, specific growth rate, and dry biomass. All data were transformed into tables and graphics using Microsoft Excel 2021. The statistical analysis was run using a *t*-test in comparing the growth rates of culture in the BBM and BG-11 media, while analyses of variance (ANOVA) ($P<0.05$) was used to compare the growth rate of the isolate at various lead concentrations. The correlation analysis (r) was conducted to measure the relationship between lead concentration and dry biomass.

The phylogenetic tree was inferred by using the maximum likelihood method and the Tamura-Nei model (Tamura & Nei, 1993). The tree with the highest log likelihood (-5576.57) is shown. The percentage of trees on which the associated taxa clustered together is shown above the branches. Initial tree(s) for the heuristic search were obtained automatically by applying the Neighbour-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model and then selecting the topology with the superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 22 nucleotide sequences. There was a total of 1,656 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura et al., 2021).

RESULTS

Observation of Microalgal Growth in the BBM and BG-11 Media

Most microalgae grown in KE, BE, PC, and PB samples were unicellular coccoid green microalgae, while in the UL sample, filamentous cyanobacteria were present. Diatoms appeared in three samples: PA, BE, and UL. Overall, the BE sample had the most diverse microalgae. Diatom, coccoid green algae, and filamentous cyanobacteria were found in both the BBM and BG-11 (Figure 3 and 4). However, coccoid cyanobacteria were the only population found in the BBM. Most microalgae grown in the BBM were unicellular coccoid green algae (Figure 4 F, G, H, I, J, L, M), while in the BG-11 were diatoms (Figure 3 A, B, C). The growth of algae in the BBM flourished more than in the BG-11 (Table 1).

Table 1
List of species grown in the BBM and BG-11 as a result of enrichment

Medium	List of species	Morphology characteristics	References
Blue Green No. 11 (BG-11)	*Nitzschia* sp. (Figure 3 A-C)	Two chloroplasts with a yellow-brown shape at the cell edge. Lancet and sometimes rectangular. Solitary and sometimes a colony. Cell size varied. L: 38–110 µm and W: 9–11 µm.	Puilingi et al., (2022); Tan et al., (2016)
	Pseudanabaena sp. (Figure 3 E-F)	Cylindrical septate without a heterocyst. Cell size varied. L= 7.04-12.83 µm.	Rosen and Mareš (2016); Yusof et al. (2017)

Table 1 *(continue)*

Medium	List of species	Morphology characteristics	References
	Chlorella sp. (Figure 3 D)	Cup-shaped chloroplast with or without pyrenoid Elliptical or rounded with cell size varying from 2-10 µm.	Shubert and Gartner (2015)
Bold Basal Medium (BBM)	*Chlorella* sp. (Figure 4 A, G, L, M)	(See *Chlorella* in BG-11)	
	Pseudanabaena sp. (Figure 4 A, C)	(See *Pseudanabaena* in BG-11)	
	Nitzschia sp. (Figure 4F)	(See *Nitzschia* in BG-11)	
	Nostoc sp. (Figure 4 E, N)	Long filament with heterocyst and akinete. Cell size varied from 5-10 µm.	Rosen and Mareš (2016); Videau and Cozy (2019)
	Pinnularia sp. (Figure 4 B, C)	Linear shaped with curved end. Cell size varied. L= 28-64 µm; W = 5-12 µm.	Xu et al. (2024)
	Chlorococcum sp. (Figure 4 F)	Cell spherical and solitary. Parietal with one pyrenoid. Cell size varied from 5-15 µm.	Hodač et al. (2015); Shubert and Gartner (2015)
	Coccomyxa sp. (Figure 4 H)	Cell ellipse to cylindrical. Parietal chloroplast with pyrenoid.	Hodač et al. (2015); Shubert and Gartner (2015)

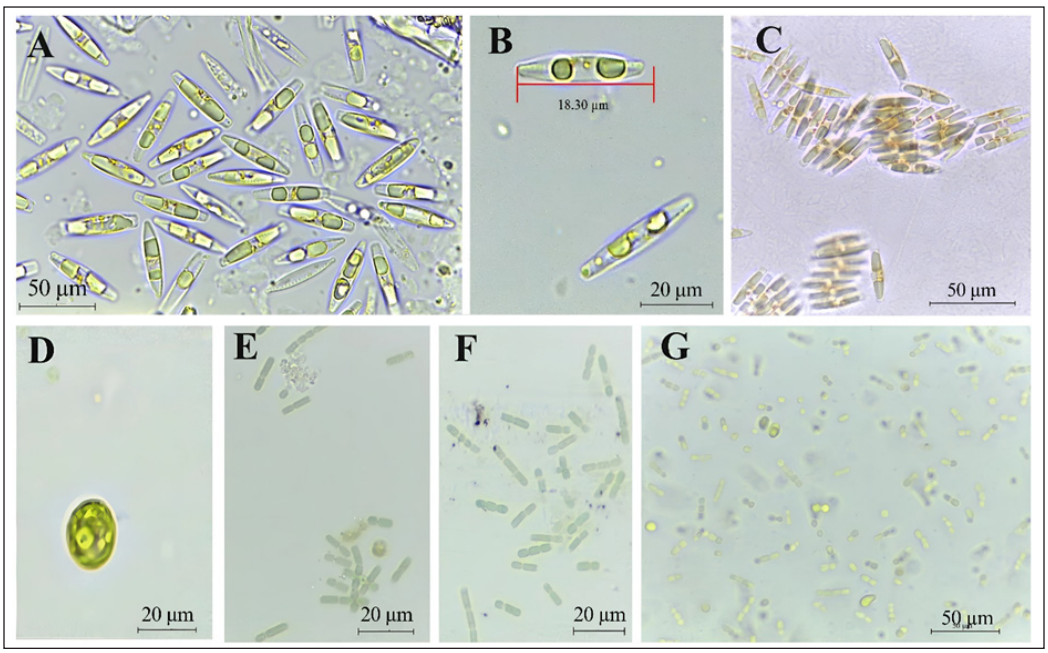

Figure 3. Diversity of the microalgae from six locations in the Blue Green-11 medium
Note. Diatom (A, B, C), coccoid green algae (D), and filamentous cyanobacteria (E, F, G)

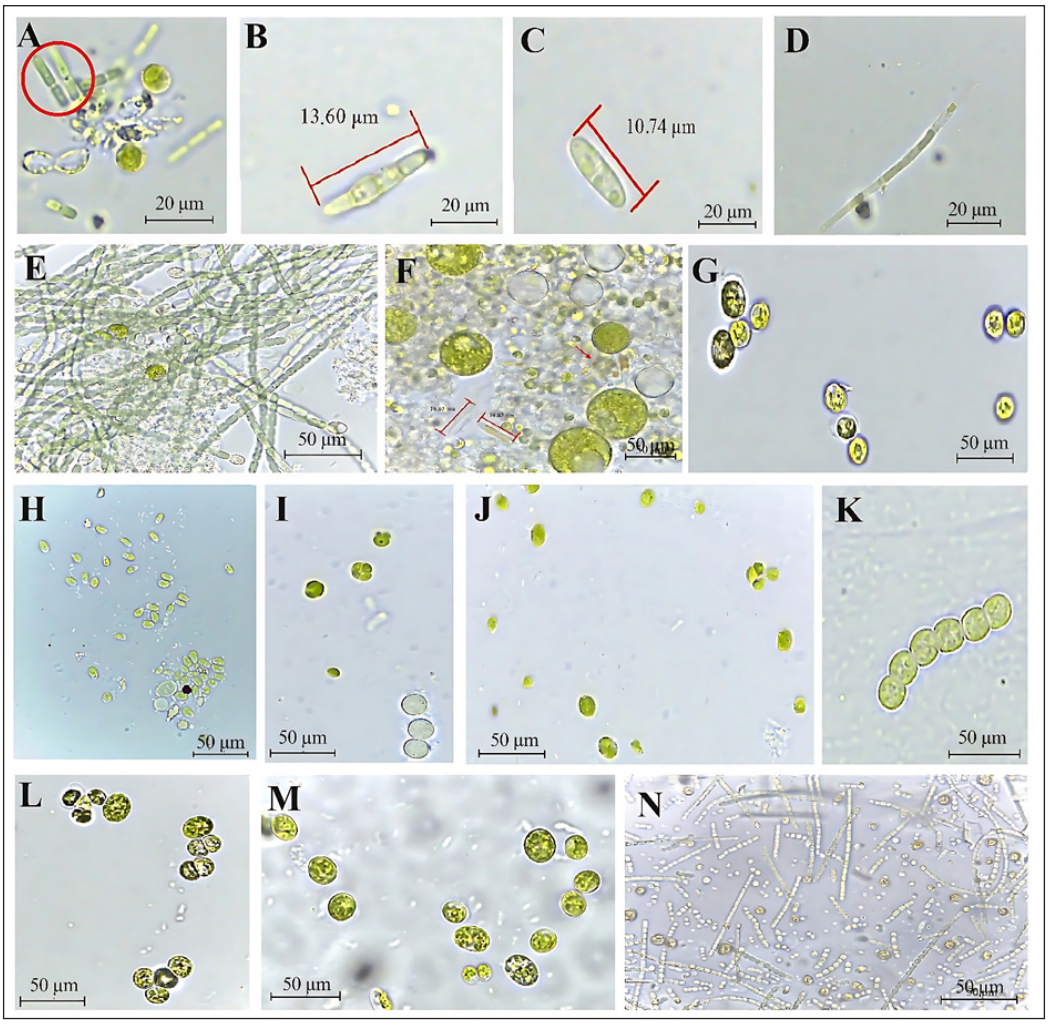

Figure 4. Diversity of the microalgae from six locations in the Bold Basal Medium
Note. Coccoid green algae (A, F, G, I, J, L, M), filamentous cyanobacteria (A, D, E, K, N), unicellular microalgae (B, C), and diatoms (F)

UV-C Spectrum Sensitivity and Growth Rate

Colour changes of six (mix) cultures during incubation were systematically documented (Figure 5). The colour began to change at day 12. The UL and KE cultures changed into yellow-green (HEX #9ACD32), while the others were light green (HEX #90EE90). At day 15, the colours of the UL, PA, and PC cultures changed to avocado green (HEX #A568203). At the end of the experiment (day 21), the colour of UL and KE cultures changed to golden brown (HEX #996515), and the biomass clumped.

Figure 5. Colour change of six cultures at day 15. The first day (top) and after 15 days (bottom). PA, UL, PC culture biomass changed to avocado green (HEX #A568203) and the KE culture biomass changed to yellow green (HEX #9ACD32)
Note. PA = Pait; UL = Ulu River; PC = Pancur River; KE = Ketok

The UV sensitivity analysis was conducted on the UL (mix) culture because, under standard laboratory conditions, the culture showed optimum growth compared to other cultures. Spectrum analysis from T0 up to T10 days old culture showed that different peaks were observed in culture media BBM and BG-11 (Table 2) (Figure 6). However, the peak

Table 2
Wavelength peaks of UL (mix) culture in the BBM and BG-11

Time observation (d)	Wavelengths (nm)	
	BBM	BG-11
T0	230, 340, 640	230
T1	220, 240, 440, 680	230
T2	220, 240, 360, 680	230, 340
T3	240, 440, 680	230, 360, 680
T4	210, 230, 360, 440, 680	230, 360, 680
T5	230, 440, 680	230, 340, 440, 680
T6	230, 440, 680	230, 360, 440, 680, 740
T7	230, 360, 440, 480, 680	230, 340, 440, 680, 740
T8	230, 440, 680	230, 440, 680, 760
T9	230 320, 440, 680,780	230, 440, 480, 680
T10	230, 440, 600, 680	230, 440, 680, 760

Note. UL = Ulu River; BBM = Bold Basal Medium; BG-11 = Blue Green-11

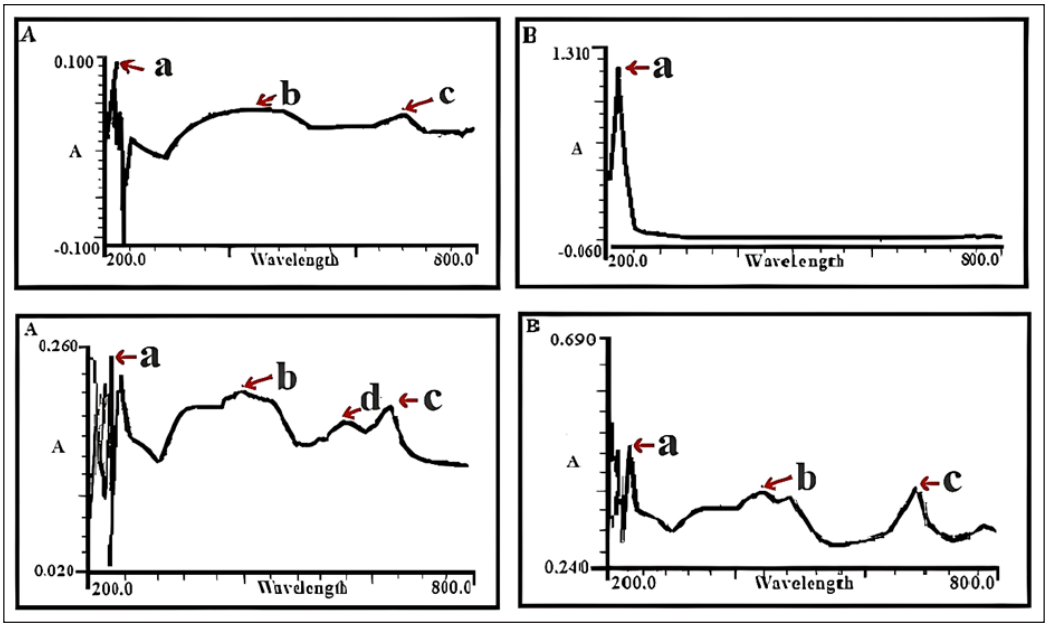

Figure 6. Absorbance wavelength sensitivity test of the UL culture at T0 (top) and T10 (bottom)
Note. (A) Bold Basal Medium (BBM); (B) Blue Green-11 (BG-11) medium; (a) wavelength 230 nm, (b) wavelength 440-480 nm, (c) wavelength 680 nm, and (d) wavelength 600 nm

at λ=230 nm was considered as an indicator for the UV sensitivity (International Agency for Research on Cancer [IARC], 2012). The peak was consistently observed from T0 up to T10 in media growth BG-11, while the same peak was observed consistently from T4- T10. Moreover, this peak indicated the presence of the UV-tolerant microalgae in the UL culture. The growth of the UV-tolerant microalgae in the UL culture on the BBM was predicted to be faster compared to the BG-11, as indicated by the colour change (Figure 7A). The BBM culture entered the stationary phase on day 16 and steadily decreased at day 20. Meanwhile, the BG-11 culture was still in log phase until the end of the observation. The average specific growth rate of the UL culture on the BBM media was 0.027 μ/day, while on the BG-11 was 0.018 μ/day (Figure 7B).

Morphological and Molecular Identification of the Isolate UL4

The morphological observations showed that the cell of the isolate (coded as UL4) was unicellular, round in shape, 6-14 μm in length, and non-motile. The shape of the chloroplast was cup-shaped, and a single pyrenoid was located at the periphery of the cell. The cell size ranged from 6 to 12 μm. The vegetative cell reproduced by autospore formation, but sometimes cell division occurred (Figure 8).

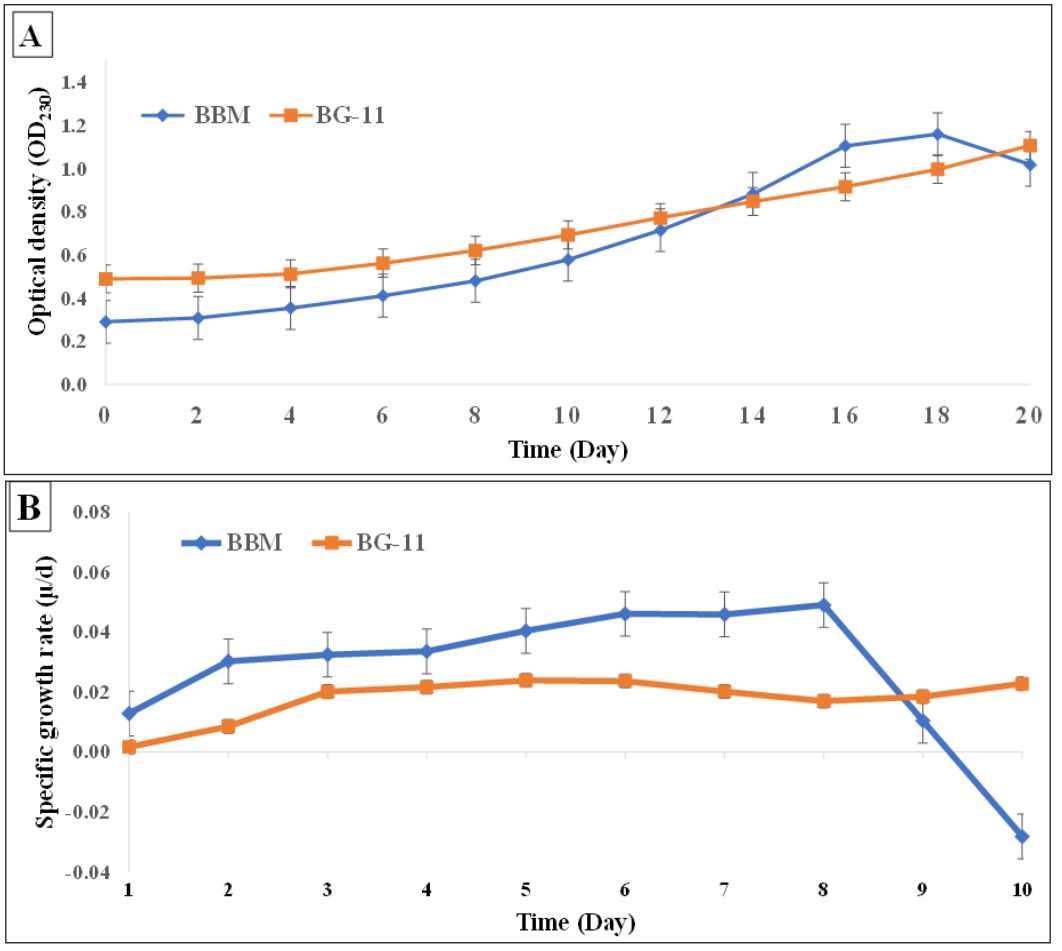

Figure 7. The growth curves (A) and specific growth rate (B) of the UL culture in Bold Basal Medium (BBM) and Blue Green-11 (BG-11) at 230 nm wavelength.
Note. *T*-test analysis on the mean of specific growth rate data showed there was no significant difference between growth on the BBM and BG-11 (p>0.05). However, *t*-test analysis on day 20 (T10) showed a significant difference in growth rate between the BBM and BG-11 (p = 0.02)

The length of the UL4 forward and reverse sequences was 491 and 533 bp, respectively. The whole sequence of the 18S rRNA gene using 18F-18R primers should be 1,500 bp (Figure 9), but the capability of the sequence reading machine was limited to 500-600 bp. Therefore, there was a gap between the F- and R-sequences after the sequence's continuity. The phylogenetic analyses of the isolate UL4 were conducted using the partial sequence (1,024 bp). Tree reconstructions were performed using the neighbour joining (NJ), maximum parsimony (MP), and maximum likelihood methods. *Neospongiococcum macropyrenoidosa* and *Neospongiococcum vacuolatum* were used as outgroups. Bootstrap of 1,000 was set up for all trees. The isolate UL4 was always placed within the big

clades, consisting of *Chlorococcum* spp., *Neospongiococcum gelatinosum*, *Pleurastrum rubrioleum*, and *Macrochloris radiosa*. The nodes in NJ/MP/ML trees that separated the UL4 clade from other aforementioned OTUs were supported by the 93/100/99 bootstrap values. Only ML phylogenetic trees are shown here (Figure 10). Other tree analyses can be seen upon request.

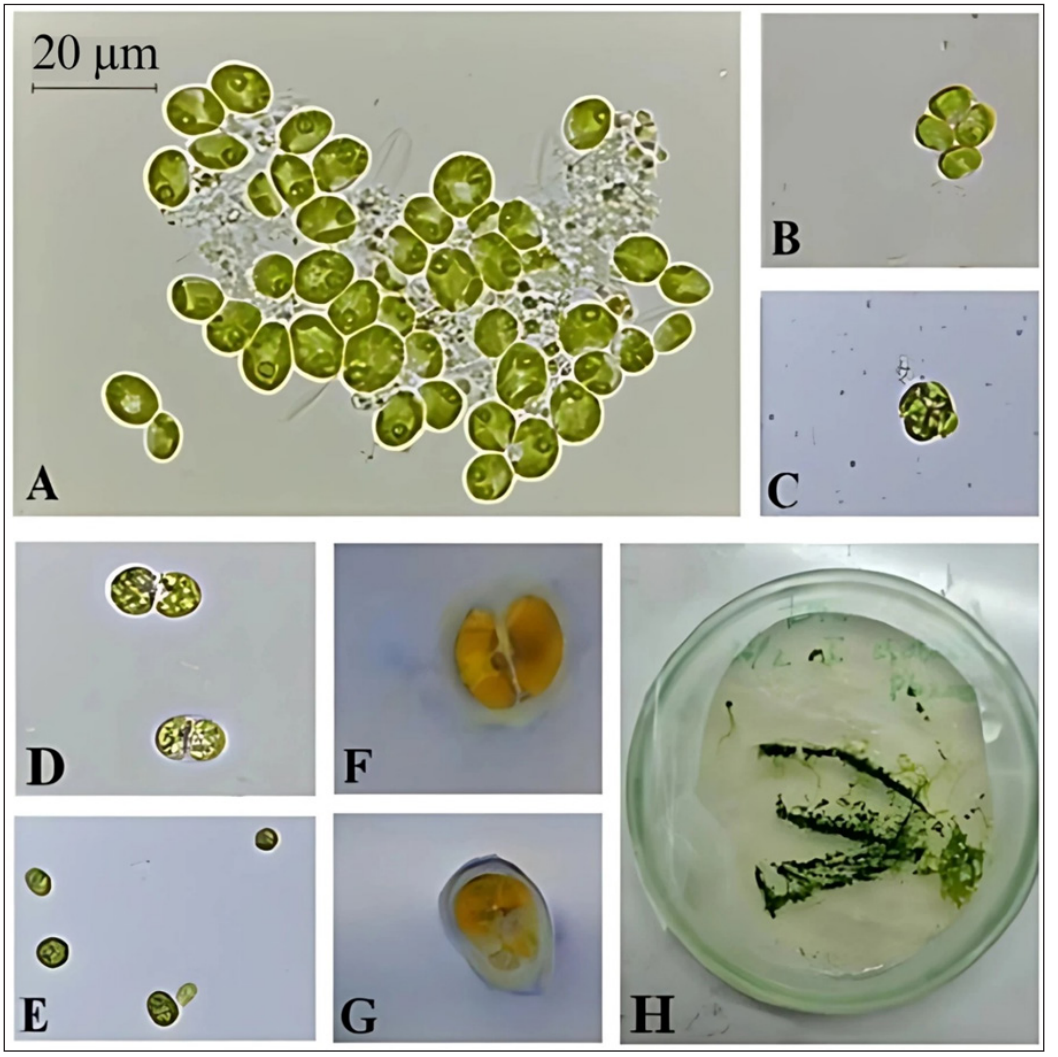

Figure 8. The morphology of the UL4 in different life stages. (A) Mature cells (18 days old); (B-C) Autospores; (D) Cell division; (E) Young cells (2-7 days old); (F-G) Old cells (age ± 245 days); (H) The isolate UL4 in the Bold Basal Medium agar
Note. Figures B-H without scale. All documentation was taken under a light microscope with 1,000× magnification

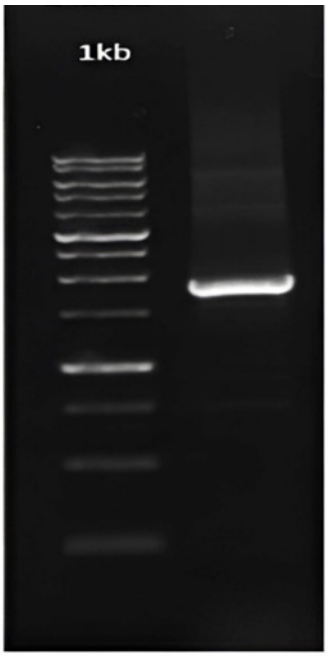

Figure 9. Polymerase chain reaction amplification of the UL4

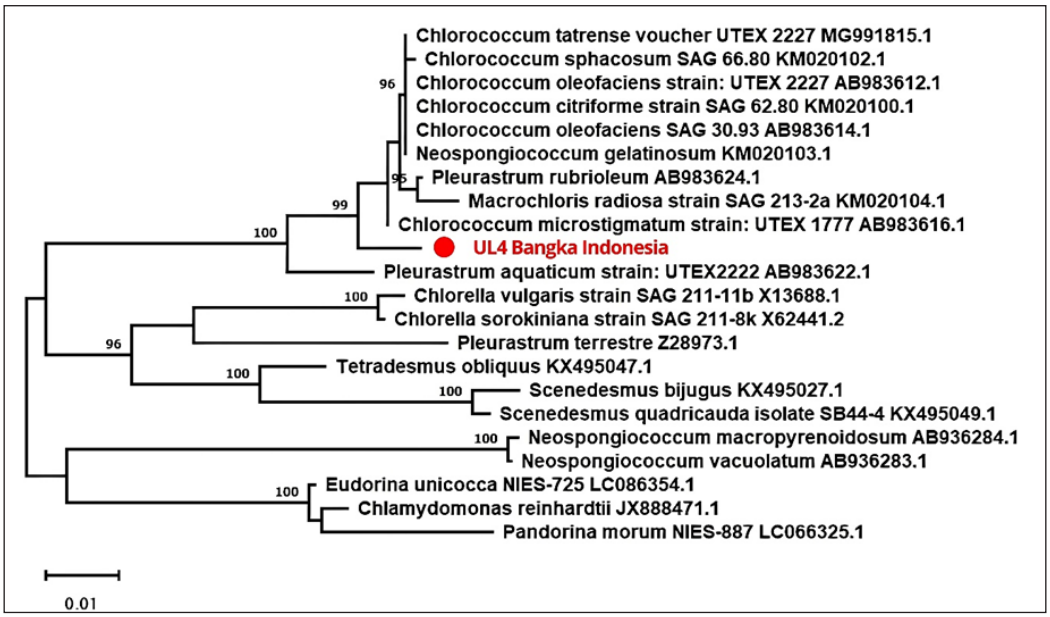

Figure 10. Phylogenetic tree of the UL4 inferred from the Maximum Likelihood method

Effects of Various Lead Concentrations

The morphology of the UL4 grown in various lead concentrations showed changes in the cell shape and size (Figure 11). Compared to the control, the cell on 10-200 ppm lead either enlarged or decreased (Table 3).

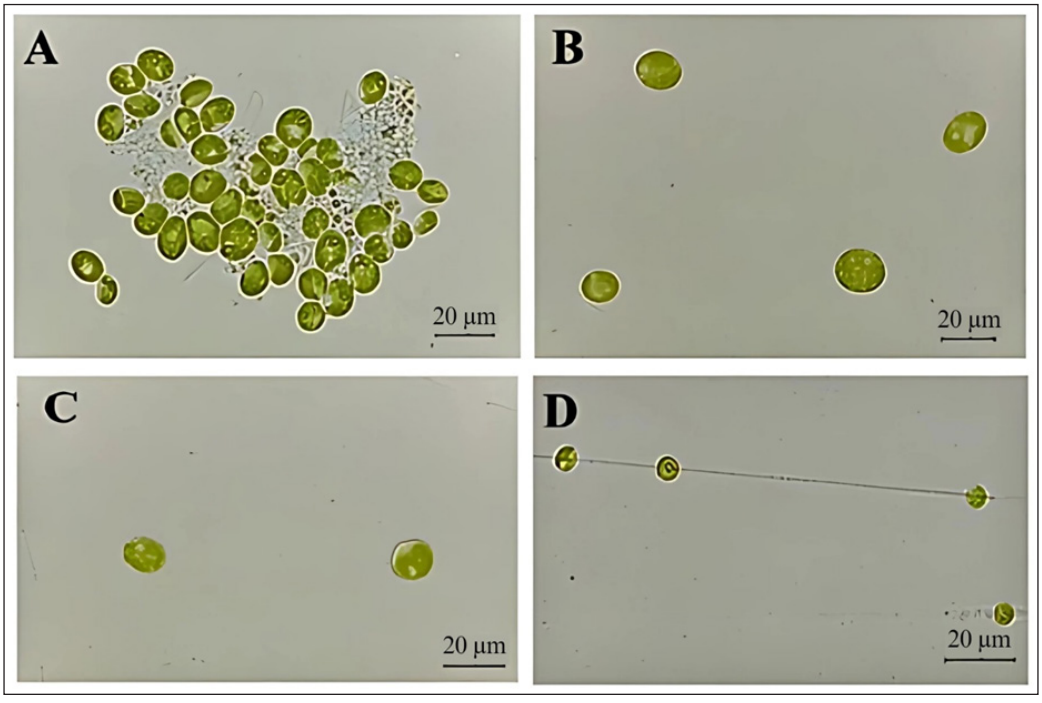

Figure 11. The mtorphological observations of the UL4 isolate under various lead concentrations after 22 days of incubation (T8): (A) 0 ppm, (B) 10 ppm, (C) 100 ppm, and (D) 200 ppm

Table 3
Effect of lead on total cell count and cell size of the isolate UL4

Lead concentrations (ppm)	Total cell (unit)	Cell size (µm)
0	273	11.26
10	87	13.86
100	63	5.02
200	34	4.58

The lead also changes the colour of the culture. The control culture showed avocado green (HEX#3A5702), while at 10 and 100 ppm, the cultures appeared olive green (HEX#446702) and dark green (HEX#568203) respectively, and at 200 ppm, the colour changed to yellowish green (HEX#9DC209) (Figure 12).

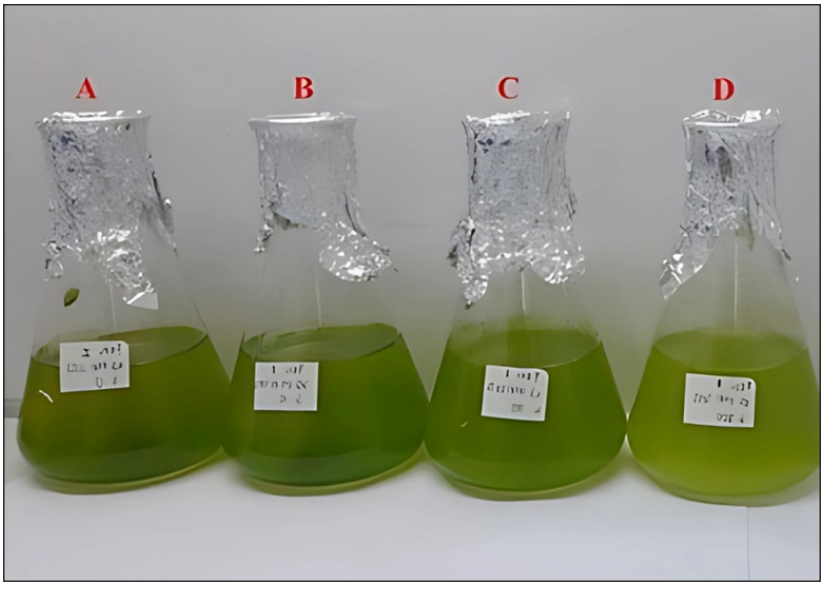

Figure 12. Colour differences of the UL4 cultivation in various lead concentrations at day 18
Note. (A) 0 ppm, (B) 10 ppm, (C) 100 ppm, and (D) 200 ppm

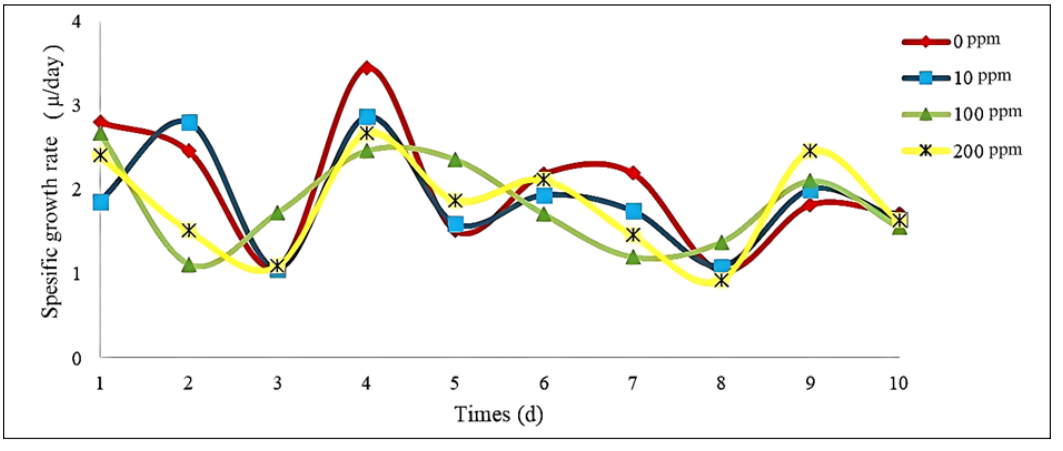

Figure 13. Specific growth rates of the UL4 culture based on optical density values. The optical density value was taken at a wavelength of 680 nm (OD_{680})
Note. Analyses of variance test showed $P > 0.05$

Lead also affected the growth of the UL4. The specific growth rates (µ/day) for the control (K0), 10 ppm (K10), 100 ppm (K100), and 200 ppm (K200) were 2.027 ± 0.75, 1.860 ± 0.60, 1.827 ± 0.54, and 1.814 ± 0.59, respectively. Although the growth rates varied among different lead concentrations, these variations were not statistically significant (ANOVA, *P*>0.05) (Figure 13).

Furthermore, the average of the dry biomass data showed that the lead affected the production of the UL4 biomass (Table 4). The highest biomass production was in the control group (1.7877 g/ml), whereas the lowest was in the 200-ppm lead treatment (0.3608 mg/L). These results indicated that increased lead concentrations caused a decrease in the biomass. The correlation analysis (r) showed an r value of -0.8. This indicated a strong negative relationship between concentration and dry weight; as the concentration of the metal increased, the production of dry weight decreased.

Table 4
Comparison of dry biomass weight of the UL4 isolates

Concentrations (ppm)	Dry biomass weight (g/mL)		
	Sample 1	Sample 2	Average
0	1.5903	1.9851	1.7877 ± 0.2
10	0.8323	1.3869	1.1096 ± 0.3
100	0.4695	0.9809	0.7252 ± 0.3
200	0.2993	0.4223	0.3608 ± 0.08

Note. Data were shown as mean ± standard deviation ($P > 0.05$) ($r = -0.8$)

DISCUSSION

The Different Microalgae Communities in the BG-11 and BBM

The BG-11 and BBM media could be used as the enrichment media to initiate the growth of the microalgae from the tin mining sites in West Bangka, Bangka Belitung. The coccoid green algae, filamentous green algae, and coccoid cyanobacteria, and diatoms were germinated after three weeks of incubation. Unfortunately, the initial observations of fresh samples could not be carried out because of the long lapse (4-6 weeks) from the time the sample was taken until the sample arrived at the laboratory. More microalgae exist in their natural habitat than those that germinate in the laboratory. While considering the lack of initial observation, the enrichment results in this study could provide a (limited) picture of the microalgae that exist in West Bangka. The presence of the green algae and diatoms in the mining sites has been reported by Gomes et al. (2021), who found *Chlorella* sp., *Chlamydomonas* sp., *Pinnularia* sp., and *Navicula* sp. in river flows around the copper and silver mining sites in São Domingos, Portugal. Additionally, the occurrence of filamentous and coccoid cyanobacteria in the mining sites was also documented by Damatac II and Cao (2022) in the mining sites around Acupan, Philippines.

The growth of many microalgae (7 species) on the BBM, while only three in the BG-11, can be predicted considering that the nutrient composition in the BBM was richer than the BG-11, especially the phosphorus content. Badr and Fouad (2021) reported that more

microalgae from Nile River water samples grew in the BBM (27 species) compared to the BG-11 (25 species). According to Badr and Fouad (2021), the high phosphorus (P) nutrient in the BBM supported a broader range of microalgal species compared to the BG-11. In this study, the phosphorus concentration in the final BBM medium was 1.711 mM, which was derived from 17.5 g/L potassium phosphate (KH_2PO_4, Merck, USA) and 7.5 g/L di-potassium hydrogen phosphate (K_2HPO_4, Merck, USA) stock solutions, respectively, whereas the BG-11 contained 0.229 mM phosphorus, which was derived from a 2 g/500 ml K_2HPO_4 stock solution. Previous studies also reported that high phosphorus could increase the algal diversity due to high nutrient sources, which supported the microalgae growth and reduced competition among species (Felisberto et al., 2011; Smith & Schindler, 2009). Furthermore, Felisberto et al. (2011) also discovered that high phosphorus supported the green algae diversity because the green algae could assimilate and accumulate P faster.

Although the BG-11 was specifically designed for cyanobacteria (Rippka, 1979), other species such as the diatoms and green algae could also grow in this medium. This study found the diatom *Nitzschia* sp. and a coccoid green alga. The cultivation of the diatoms in the BG-11 was reported previously by Sahin et al. (2019), who cultivated the marine diatom *Nanofrustulum shiloi*, and by Machado et al. (2021), who cultured the freshwater diatom *Nitzschia palea*. Additionally, the culture of the green algae in the BG-11 was documented by Purkayastha et al. (2017), who cultured *Chlorella elipsodea*.

The Culture Selection and Absorption Scanning

The selection of the UL cultures for the UV-C wavelength absorption experiments uses colourimetry. The use of colour to identify the culture conditions and microalgae growth has been extensively studied. For example, Salgueiro et al. (2022) used a Red–Green–Blue (RGB) colour palette to identify the growth of *Chlorella vulgaris*, while Castillo et al. (2023) used HEX colour pellets to observe the astaxanthin pigmentation in *Haematococcus pluvialis*. In this study, HEX colour pellets were used for the qualitative observation of the microalgal growth.

The variations of the culture colours indicated some cell conditions, such as pigment composition (Sarrafzadeh et al., 2015) and cell death (Indrayani et al., 2023). The green colour implied the dominance of the chlorophyll pigment, which reflected cell growth (Tang et al., 2023). The yellow colour indicated nutrient depletion (Fierdaus et al., 2015) and pigment alterations due to the metabolic processes (Alkhamis et al., 2022). Meanwhile, the brown colour indicated cell deterioration due to the nutrient deficiencies or contamination (Rinawati et al., 2020). Based on the colour, we documented that the UL culture grew faster and therefore chose it as the testing culture.

The ultraviolet visible (UV-Vis) scanning of the UL culture revealed absorption peaks at various wavelengths, including the UV-C range (200-280 nm). The appearance

of absorption peaks in the UV-C range indicated the presence of molecules that absorbed light, including the pigment molecules. The discovery of the UV wavelength absorption peaks was aligned with the study by Post and Larkum (1993), who found that maximal absorption occurred in the macroalga *Prasiola crispa* at a wavelength in the UV-B range (295 nm). According to Post and Larkum (1993), the UV-B absorption peaks were related to the presence of pigments such as chlorophyll and carotenoids as protective agents. Another pigment, such as sporopollenin, mycosporine-like amino acid (MAAs), and syctonemin, were also detected in the range of 280-315 nm (Dionisio-Sese, 2010).

Furthermore, Post and Larkum (1993) stated that the high UV-absorbing pigments appeared due to the high UV exposure in their habitat. Tin mining activities had increased the risk of UV exposure due to the reduction of vegetation and excavation activities. Excessive exposure to UV radiation led to the destruction of chloroplasts, mitochondria, and DNA (Holzinger & Lütz, 2006). Some of the species discovered in this study, such as *Anabaena* and *Nitzschia* from PA, UL, and BE samples, were recognised to survive the UV exposure. *Anabaena* produces syctonemin, mycosporine-glycine, porphyra, and shinorine to adapt to the UV-C exposure (Dionisio-Sese, 2010; Singh, 2008). On the other hand, *Nitzschia* adapted by changing the antioxidant, protein, and pigment levels.

Besides chlorophyll and carotenoid, the microalgae adapted to the UV radiation by forming xanthophyll and phycobiliprotein (Dionisio-Sese, 2010). According to Croce and van Amerongen (2014), phycobiliproteins were detected in the range of 560-680 nm, while xanthophyll was detected in the range of 400-450 nm. In this research, the absorption peaks at 400-480, 600, and 680 nm were documented (Table 2). The presence of these peaks at different wavelengths in the UL culture indicated the diversity of pigments contained in the various microalgal species in the UL culture.

The Absorbance of the UL Culture in the UV-C Spectrum

The absorbance observations of the UL cultures at the UV wavelengths showed differences in the maximum absorbance values between the BG-11 and BBM media. The cultures grown in the BBM medium exhibited a higher average absorbance value. A high absorbance value indicated a high concentration of substances that absorb light (Pratiwi & Nandiyanto, 2022). Although the specific types of substances or pigments at a wavelength of 230 nm were not investigated in this study, several studies reported that the pigments from the phenolic group, such as anthocyanins (Aleixandre-Tudo & du Toit, 2018), the photosynthetic pigments like scytonemin, tetramethoxyscytonemin, and dimethoxyscytonemin (Simeonov & Michaelian, 2017), as well as the MAAs (Garcia-Pichel & Castenholz, 1993), were absorbed at a wavelength of 230 nm.

The differences in the concentration of substances might have been caused by the variations in the growth rates of the microalgae or differences in the types of microalgae

present in the two media (Dionisio-Sese, 2010). Growth rate observations indicated that the microalgae grew more optimally in the BBM medium compared to the BG-11 medium. We also found the different microalgae that are present in the BBM and BG-11. For example, in the BBM, the microalgae were dominated by the green algae, while in the BG-11, the microalgae were dominated by the diatoms.

The higher growth in the BBM medium compared to the BG-11 medium might have been due to differences in the concentrations of the components of the media. The slower growth of microalgae in the BG-11 medium was also reported by Idenyi et al. (2016), who compared the growth of *Chlorella* in BG-11 and Bristol's Modified Medium (BMM). Idenyi et al. (2016) stated that high nitrogen concentrations resulted in slower growth. High nitrogen content could have decreased growth for some microalga species due to stress, which triggered a response leading to decreased protein levels affecting the photosynthesis process (Courchesne et al., 2009; Ördög et al., 2012). Furthermore, Ördög et al. (2012) explained that high nitrogen concentrations also triggered a decrease in the amount of chlorophyll a and b pigments.

Although there was no UV light treatment during the cultivation process, the UV-absorbing substances showed increased and decreased absorbance values. Variations in the absorbance values might be caused by the microalgae's population and adaptation mechanisms to nutritional changes and laboratory conditions. This result suggested that the UV-absorbing substances not only functioned to absorb UV radiation but also served other roles such as the abiotic stress and even involved in the remediation. Dionisio-Sese (2010) and Simeonov and Michaelian (2017) mentioned that the MAAs not only played a photoprotective role but also helped against thermal stress. Another UV absorption pigment, the phycocyanin, also played roles as a chelator for heavy metal remediation (Kalita & Baruah, 2023).

The Effect of Various Lead Concentrations on the UL4 Isolate

Lead influenced cell size, growth, and biomass, ultimately causing the death of the microalgae. This study discovered that the addition of metals could have increased the cell size. The increase in cell size after metal addition was also reported by Nishikawa et al. (2003), who discovered the increase in *Chlamydomonas acidophila* after being exposed to 20 μM cadmium. The increases in cell size were attributed to several factors, including the deposition of metal ions in the vacuole, which increased the vacuole size, cytoplasmic vesiculation, and an increase in starch as a response to stress protection conditions (Bauenova et al., 2021; La Rocca et al., 2009).

High lead concentration led to a decrease in the cell size. The decrease in cell size after the metal treatment was previously reported by Qiu et al. (2006), who cultivated *Chlorococcum AZHB* at copper and cadmium concentrations ranging from 0 to 200 ppm. The result showed that at a 200-ppm concentration of copper and cadmium, the cell size

of the microalgae decreased. The decrease in the cell size due to metal addition was also reported for *Koliella antarctica* (La Rocca et al., 2009). The cells of *K. antarctica* experienced loss of chloroplast shape, irregular thylakoid distribution, and cell lysis due to the addition of 5 ppm cadmium (La Rocca et al., 2009). The irregular cell shape and cell death of the UL4 in 200 ppm lead treatment were observed.

Besides affecting the cell morphology, lead influenced the growth of the microalgae. The growth of the microalgae decreased in alignment with the high concentrations of lead. The reduction in growth was indicated by decreases in the growth rate and dry weight. According to Dao and Beardall (2016), lead (Pb^{2+}) ions produce greater stress, resulting in alterations in antennae and central reactions in photosystem II, which inhibit photosynthesis, thereby decreasing the growth and biomass production of the algae.

The decrease in the specific growth rate of the UL4 isolate due to lead exposure was also reported by Teoh and Wong (2018), who cultured *Chlorella* sp. at a lead concentration of 0-100 mg/L. Their results showed that higher lead concentrations led to a lower specific growth rate. A decrease in the growth rate of the green algae due to an increase in lead concentration was also previously reported in *Chlorella sorokiniana* (Carfagna et al., 2013). Although the specific growth rate measurements in this research did not show a significant difference, there was still a difference in growth rates between the treatment and control. The control had the highest growth rate (2.027 μ/days), whereas at the 200-ppm lead concentration, the growth rate was 1.814 μ/days.

Additionally, Nanda et al. (2021) reported a decreased biomass production following lead treatment in *Chlorella sorokiniana UNIND6*. Their results showed that *C. sorokiniana* produced 0.78 g/L of dry biomass, whereas the control produced 1.2 g/L. Similarly, this research also observed less dry biomass after lead treatment. The control produced an average dry biomass of 1.787 g, whereas the 200-ppm lead treatment yielded an average of 0.608 g. According to Zeng et al. (2024), the decrease in the biomass was caused by lead metals binding with various compounds that disrupted the microalgal metabolite production, including proteins, thereby inhibiting growth.

CONCLUSION

There were different microalgae communities between the BG-11 and BBM. In the BG-11, the microalgae were dominated by diatoms and cyanobacteria, while in the BBM, there was a greater variety of green algae. The microalgae exhibited various absorbance peaks, demonstrating their ability to grow under the UV-C wavelengths. Lead treatment affected the microalgae cell size, organelle, and growth. The various absorbance peaks indicated that the microalgae may produce advantageous metabolites as an adaptive mechanism to a severe environment. Further investigation was needed regarding the species' molecular analysis, types of UV-C wavelength compounds, and their roles in lead bioremediation.

ACKNOWLEDGEMENT

This research was funded by Hibah Riset Universitas Indonesia in 2023 (Grant no: PENG-001/UN2.RST/PPM.00.00/2023).

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