Vietnam Journal of Marine Science and Technology 2025, 25(3) 341–354

Evaluation of heavy metals in green algae *Caulerpa lentillifera* from Van Phong Bay and the bioaccumulation of copper in different concentrations in the laboratory

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Received: 26 November 2024; Accepted: 15 January 2025

ABSTRACT

In this study, morphological observation and genetic marker were applied to compare two clones, including natural Vietnamese and introduced clones. 11 elements of heavy metals in the algae collected from three different farms in Van Phong Bay were analyzed. In the laboratory, *Caulerpa lentillifera* was cultivated in different levels of Cu concentrations to evaluate the ability of Cu bioaccumulation and phytochelatin production of the algae. The results show that the morphological traits of erect frond and stalk between natural Vietnamese clones and introduced clones from Japan revealed significant differences but not ramuli diameter. Based on the *tuf*A gene, there is no genetic differentiation between two clones, and *Caulerpa lentillifera*, *C. germinata*, *C. cactoides*, and *C. bartoniae* are sister species. The concentrations of Cd, Hg, and Pb from algae in Van Phong Bay were 0.10 (±0.017), 0.12 (±0.015), and 0.58 (±0.02) $\mu g.g^{-1}$, repectively. In the seawater with 10 µM, algae showed the highest accumulation with 29.02 $\mu g.g^{-1}$ in day 6, indicating the potential impact of the findings on understanding of heavy metal accumulation in marine algae.

Keywords: Cu, *Caulerpa lentillifera*, heavy metals, phylogency.

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https://doi.org/10.15625/1859-3097/20343

INTRODUCTION

Marine macroalgae are plant-like organisms consisting of three major groups as follows: Chlorophyta or green algae; Phaeophyceae, or brown algae; and Rhodophyta, or red algae [1]. They are widespread in the coastal areas of the oceans and the world's seas. In Vietnam, there are 881 species of marine macroalgae [2]. Among cultivated species such as *Kappyphycus* spp., *Eucheuma* spp., and *Gracilaria*/*Hydropuntia* spp., the only green lage *Caulerpa lentillifera* J.Agardh, is widely cultivated [3].

Some heavy metals (HMs), including Fe, Cu, Zn, and Pb are trace elements for normal biological functions and are beneficial in plant growth and development [4]. However, increased amount of HMs within the plant tissue displays direct and indirect toxic impacts [5]. Some seaweeds exhibit a high affinity for heavy metals [6]. Therefore, they were used as HM indicators worldwide [7]. For food safety, the standards of IAEA (2005) for HMs in marine macroalgae are Cu < 23.2, Zn < 128, Fe < 497, and Pb < 0.574 mg/kg. Besada et al., [6] reported that the brown algae *Sargassum fusiforme* (Harvey) contained the highest total and inorganic As among nine analyzed species; the authors also mentioned that most Cd concentrations exceeded the French Legislation. In New England's sugar kelp (*Saccharina latissima*), total arsenic concentrations frequently exceeded EU seaweed standards, especially since samples collected near a sewage outfall showed higher As than at other sample sites [8]. Plants have various ways to respond to heavy metal toxicity [9]. Among them, phytochelatins (PCs) are essential to the metal detoxifying mechanisms [10]. Faizan et al., [10] indicated that higher PC contents can reflect the higher HM concentration in the environment. The aquatic plant *Hydrilla verticillata* (L.f.) Royle showed a high ability to absorb As in the water column; therefore, it was used as phytoremediation [12]. Some Chlorophyta species, such as *Tetraselmis* sp. and *Dunaliella tertiolecta* Butcher, can tolerate high copper concentrations at 15 µM.L^{-1} [13].

In Vietnam, *Caulerpa lentillifera* was described by Pham-Hoang [14]. It was found in the offshore islands such as Phu Quy [15, 16], Ly Son [17], Con Dao [18], Phu Quoc [14]. Based on some seedlings from Japan, the Institute of Oceanography, VAST cultivated this species in the grass-tank in the laboratory in 2003 and in the shrimp pond in 2004 [19]. *Caulerpa lentillifera* farms are currently found in several South Vietnam provinces [20]. Therefore, two putative clones of *Caulerpa lentillifera*, including "domestic" *Caulerpa lentillifera* are found in off-shore islands and originate from Japan. The elongation factor Tu gene (*tuf*A) was considered a DNA barcode for the genus *Caulerpa* [21], leading to the hypothesis that genetic differentiation between two clones is based on genetic markers.

For example, *Ulva compressa* Linnaeus showed higher Cu accumulation in the marine green algae at copper-polluted sites than at the control sites [22]. Bonanno et al., [23] also revealed a similar trend in *Ulva lactuca* Linnaeus. Interestingly, the growth of *Ulva compressa* in different Cd concentrations (10, 25, and 50 µM) indicated that intracellular cadmium increased on day 1, no change until day 5, and the level of phytochelatins PC_2 increased on day 5 [24]. There are several studies on the bioaccumulation of *Caulerpa* spp. [24, 25]. Unfortunately, Cu accumulation involves the synthesis of and PCs of *Caulerpa,* which is very limited.

This present study aims to answer three research questions: (1) Is there genetic differentiation between *Caulerpa lentillifera* collected in the offshore islands of Vietnam (Vietnamese clone) and introduced *Caulerpa lentillifera* (Japanese clone)? (2) How much is HM concentration in cultivated *Caulerpa lentillifera* at Van Phong Bay? and (3) Cu and PCs in *Caulerpa lentillifera* may increase when Cu concentration is increased in seawater.

MATERIALS AND METHODS

Sample collection, morphological observation, and molecular data acquisition

The herbarium voucher specimens included specimens collected in Ly Son in February 2019, Phu Quy Island [14], introduced from Japan [16], and samples cultivated in Van Phong Bay (Fig. 1). For morphological observation, there

are three characters, including the erect frond (cm), ramuli diameter (µm), and stalk (µm). Erect fronds were directly measured in herbarium voucher specimens. At the same time, ramuli diameter and stalk were examined under an Olympus ZS-PT microscope (Olympus, Tokyo, Japan) with a Q-imaging digital camera (Burnaby, BC, Canada) connected to a computer at the VAST Key Lab on Food and Environmental Safety (Central Vietnam). Ten natural samples collected in Ly Son Island and ten collected in seaweed farms at Van Phong were used for comparison.

For molecular analysis, the herbarium voucher specimens, including two specimens collected in Ly Son (VMO.190345; VMO.190346) in February 2019, one sample collected in Phu Quy Island (VMO.231103) [14], one sample introduced by Japan (VMO.040316) [16], and one sample in Van Phong Bay (VMO.231102) were used. The dried samples were rinsed in sterilized water before homogenizing with a bead mill MM400 (Retsch, Germany) at 22 Hz for 2 min. 100 mg powder of each was used for DNA extraction by The Quick-DNA^{TM} Miniprep Plus Kit (Zymo Research, CA, USA): Primers and PCR conditions followed by Kazi et al., (2013) [18]. The 1ST BASE (Selangor, Malaysia) directly sequenced the PCR products. The consensus sequence was achieved by Clone Manager 9 (Sci-Ed, Cary, NC, USA).

The *tuf*A sequences included five newly generated sequences obtained in this study, and 52 sequences of known *Caulerpa* species retrieved from GenBank (https://www.ncbi.nlm.nih.gov/genbank/) (Appendix 1) were used for phylogenetic analysis. The dataset was aligned by the MAFFT algorithm with the selection of the q-ins-i option. Phylogenetic analyses were performed using Maximum Likelihood (ML) in RAxML version 8.1 with 1,000 bootstrap replications, and Bayesian Inference (BI) (Metropolis-coupled Markov chain Monte-Carlo method) in MrBayes v.3.2.2.

Collection of *Caulerpa lentillifera* in Van Phong Bay

The fresh *Caulerpa lentillifera* samples (2 kg) were collected from three farms (two Vija farms and one Tri Tin) in Van Phong Bay, Khanh Hoa province in April 2022. Three different samples per farm (9 samples in total) were used for analysis. In the field, samples were washed by seawater, cleared to remove the sediment and epiphytes commonly attached to the thallus, stored in the cool box (4^{o}C), and transferred to the Institute of Oceanography, VAST, on the same day. The samples were washed in the laboratory with deionized water before homogenizing in small mortars and pestles with liquid nitrogen. Samples were refrigerated at -20^{o}C until later analysis in the laboratory.

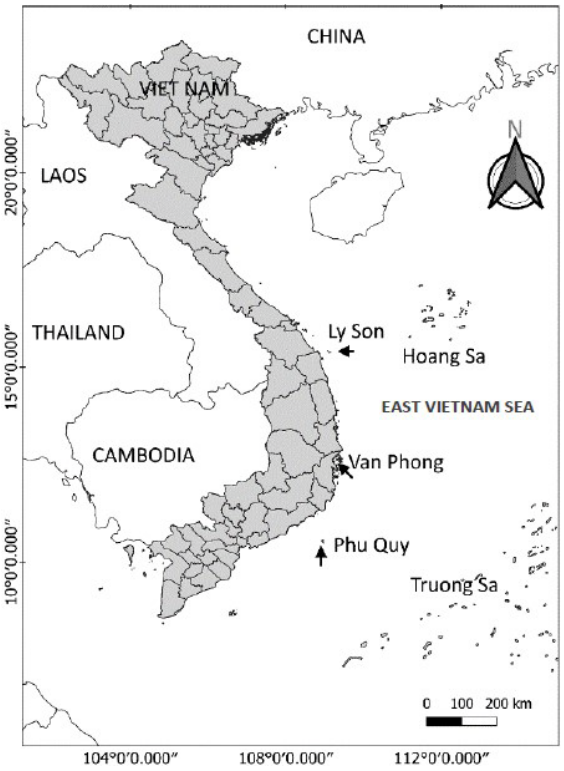

Figure 1. Map of Vietnam showing the locations where samples were collected (arrows)

Caulerpa lentillifera cultivation in different levels of Cu concentration

The experiments presented in this present study were carried out from March to May 2022 at the laboratory facilities of the Institute of Oceanography, VAST. The stock *Caulerpa lentillifera* (300 g) was attached to the hard plastic net (20 × 30 cm) and put into the

bottom of 10 little glass tanks. The algae was grown using natural seawater (Cu^{2+} is around 4 µm.L^{-1}) in an outdoor shadowed area with natural solar irradiances (30–70 µmol photons $m^{-2}.s^{-1}$). Seawater temperatures were 26-28°C. There are four sets of Cu concentrations by adding 2.5, 5, 7.5, and 10 µm $L^{-1}.Cu^{2+}$; each concentration was three replicates. Cu^{2+} was supplied by $CuCl_2.2H_2O$ (Merck, Germany). Samples were separately collected at 0, 1, 3, 6, 9 and 12 days. Therefore, 15 samples (two erect fronds of each tank), including control samples, were collected each time. The culture medium was changed every 48 hours following the protocol of González et al., (2021) [24]. Samples were washed two times with 50 mM Tris–HCl (pH 7) -10 mM EDTA for 20 min to remove Cu ions attached to cell walls. The collected materials were used separately for analysis of Cu bioaccumulation.

Heavy metal analysis in *Caulerpa lentillifera*

Samples of algae collected in the farms were dried to constant weight at 60°C, and a 0.1 g quantity of samples was used for digestion. The samples were placed into a Teflon digestion vessel (CEMAnalytical Matthews, NC, USA). After adding 10 mL of 1:1 (v/v) HNO_3 (65%, Merck, Darmstadt, Germany) into each vessel, they were sealed and placed into a microwave reaction system (MARS, CEMAnalytical Matthews, NC, USA). The samples were heated to 130°C for 60 min and cooled for 10 minutes. Then 5 mL 30% H_2O_2 (Merck) was added and the mixture was heated to 130°C for 60 minutes for complete digestion. After cooling, the digested samples were filtered with filter papers (Whatman No. 1441-110, Sigma-Aldrich, Darmstadt, Germany), and rinsed with 5% HNO_3 to release the residues in the digested samples. The samples were then filled to 100 mL with deionized water (Merck). The concentrations of the metals, including As, Cd, Co, Cr, Cu, Fe, Hg, Mn, Ni, Pb, and Zn were determined by inductively coupled plasma mass spectrometry (ICP-MS) (model 7700, Agilent 7700, Santa Clara, CA, USA) [26].

Determination of the PCs content

For extraction and derivatization of PCs, 100 mg of each sample was mixed with 300 µL 0.1% (v/v) trifluoroacetic acid (TFA) (Wako, Osaka, Japan) containing 6.3 mM diethylenetriamine-pentaacetic acid (DTPA) (Wako, Osaka, Japan). The solution was mixed on ice for 15 min, centrifuged at 14,000 g at 4°C for 15 min. 154 µL 200 mM 4-(2-hydroxyethyl)-piperazine-1-propane sulfonic acid (HEPPS) (Wako, Osaka, Japan) (6.3 mM DTPA, pH 8.2), 6.25 µL 20 mM tris (2-carboxyethy) phosphine hydrochloride (TCEP) (Wako, Osaka, Japan) (200 mM HEPPS, pH 8.2) and 2.5 ll of 0.5 mM internal standard N-acetyl-L-cysteine (NAC) (Wako, Osaka, Japan) (TFA 0.1%) were added to 62.5 µL of the supernatant, and incubated solution for 10 min at 45°C. Following the TCEP reduction of disulfide bonds to sulfhydryls, the monobromobimane (mBBr) (Wako, Osaka, Japan) derivatization was carried out for 30 min at 45°C by adding 5 µL of 50 mM mBBr (in HPLC-grade acetonitrile (ACN)). The the last step, 25 µL of 1 M methanesulfonic acid (MSA) (Wako, Osaka, Japan) was added to finish the reaction. Samples were filtered with the syringe filter (0.45 lm, MedStore, Canada) and were stored at -20°C [27].

The HPLC system (Shimadzu Corporation, Tokyo, Japan) consisted of a DGU–20A5 degassing unit, RF–10AXL fluorescence detector, and LC–20AD pump. PCs were separated on a Wakosil II5C18 analytical column (5 µm particle size, 250 mm 9 4.6 mm; Wako, Osaka, Japan). The column temperature was 40°C with a column oven (CTO–20AC). The emission wavelength was 470 nm and the excitation wavelength was 380 nm. Solvents A (99.9% ACN) and B (89.9% Water + 10% ACN) were used to separate the samples. Both solvents had a TFA of 0.1 % by volume. The flow rate was 1.2 $mL.min^{-1}$. 100% (B) was employed for the first 0.5 min. Further, a linear gradient for 11.2 min of mobile phase A from 0 to 10.6% was run. Then, the linear gradient of solvent A was increased from 10.6 to 21.1% in 13.6 min. Finally, the column was washed with 100% of mobile phase A for 10 min and a gradient of solvent B from 0 to 100% was

employed to re-equilibrate the column at the initial conditions. Solutions of each standard PC_2, PC_3, and PC_4 (AnaSpec, Fremont, CA, USA) were prepared by using deionized water in a concentration of 4.53, 3.17, and 2.34 µM, respectively. The injection volume was 20 µl. The unknown peaks were identified by comparing the retention time and response of the peak in the chromatogram of the standard mixture, with the sample chromatogram. Sample spiking was performed in cases of shift of retention time or where the known peaks co-eluted with other peaks. Sample spiking involved the addition of the standard mixture to a sample to determine the exact peak of PCs.

RESULTS

Morphological comparison between natural and introduced *Caulerpa lentillifera*

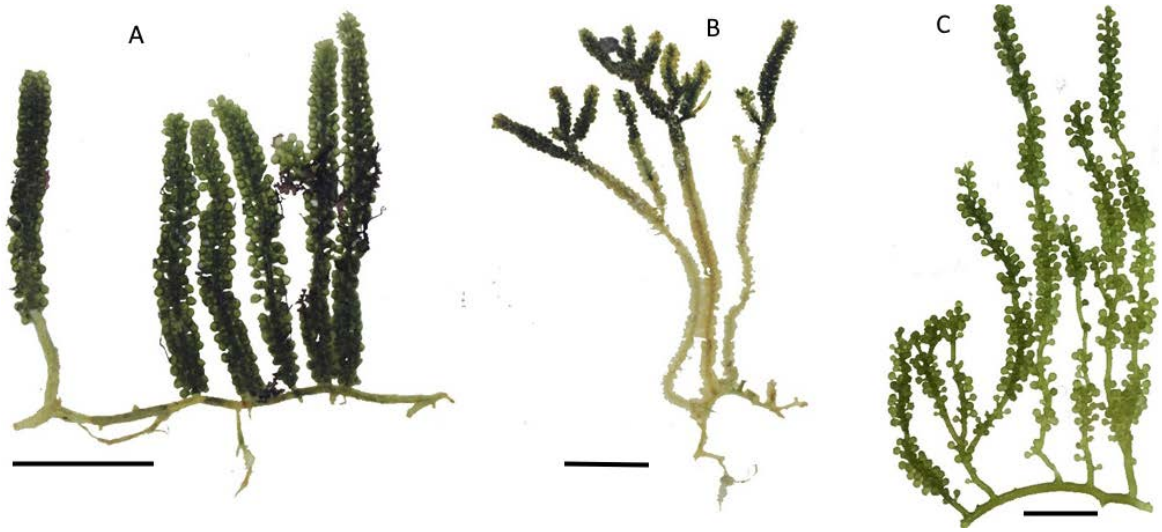

Figure 2. Samples collected at Ly Son, A: VMO.190345, B: VMO.190345 (Vietnamese clone), and herbarium voucher specimen which introduced seedling from Japan (Japanese clone) in 2003 (VMO.040316). Scale = 2 cm

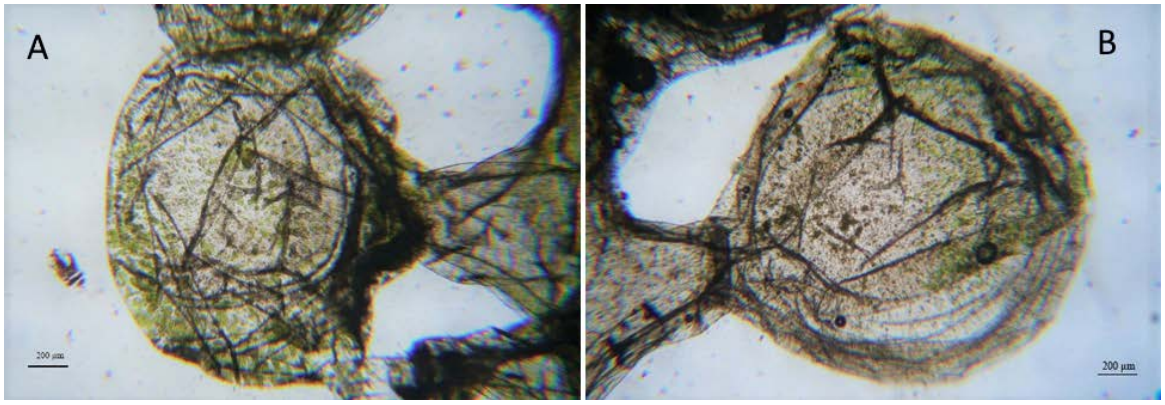

Figure 3. The ramuli of samples collected at Ly Son, A: VMO.190345 (Vietnamese clone), and introduced seedling from Japan in 2003 (VMO.040316). Scale = 200 µm

Morphological observation between the two clones showed that the average erect fronds of the Vietnamese clone (Figs. 2A, B) was 7.0 (±3.4) cm, shorter than the Japanese

clone (Fig. 2C) with 10.5 (±1.9) cm. There was a significant difference in erect fronds between the above two clones (F_{value} = 8.15, p value = 0.01 at α = 0.05). Comparing the ramuli diameter also indicated that the average ramuli diameter of samples collected in Ly Son was 1,470 (± 88) µm, smaller than the Japanese clone with values of 1,708 (± 97) µm (Fig. 3). There was a significant difference in ramuli diameter between the above two clones (F_{value} = 32.6, p value < 0.01 at α = 0.05). The average stalk between two clones was similar, 659 and 685 µm for Vietnamese and Japanese clone, respectively. There was no significant difference in stalk between the two above clones.

Phylogenetic analysis of *Caulerpa lentillifera*

The *tuf*A dataset for phylogenetic analysis consisted of 813 characters and 56 taxa. The alignment showed 544 bp (66.9%) of conserved sites, 269 bp (33.1%) of variable sites, 198 bp (24.4%) of parsimony informative characters, and 71 bp (8.7%) of singletons. Five samples of *Caulerpa lentillifera* collected from Vietnam grouped into the known clade: *Caulerpa lentillifera* and sister species with three species including *Caulerpa geminata* Harvey, *Caulerpa cactoides* (Turner) C.Agardh and *Caulerpa bartoniae* G.Murray with high support values (ML = 77%, BI = 1.0) (Fig. 4). No nucleotide difference exists between samples collected on offshore islands in Vietnam (Vietnamese clone), introduced in *Caulerpa lentillifera* (Japanese clone), and New Zealand.

HM concentration in algae collected from Van Phong Bay

The average concentration of 11 HMs on three farms was almost similar. Among them, the highest concentrations were Fe (117–167 µg.g^{-1}), Mn (42–69 µg.g^{-1}), Cd, and Hg, which showed lower concentrations than 0.15 µg.g^{-1}, while Pb was lower than 1 µg.g^{-1}. The order of HM concentration in *Caulerpa lentillifera* is Fe > Mn > Zn > As, Cu > Cr, Ni > Pb> Co > Hg, and Cd. Detail of each HM concentration was presented in Table 1.

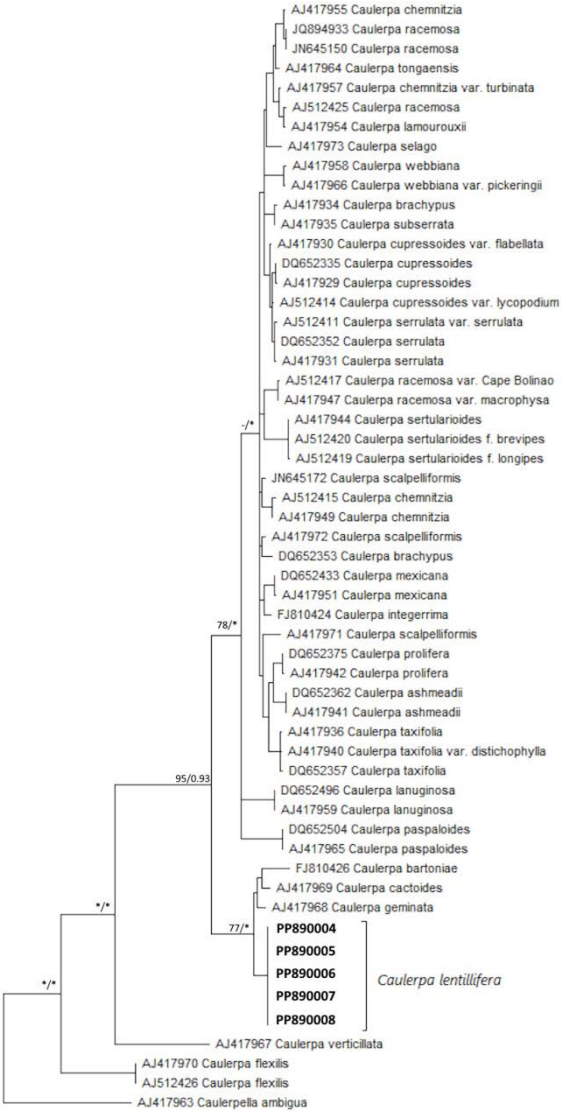

Figure 4. Caulerpa members' phylogeny inferred from maximum likelihood and Bayesian inference. The dataset is based on 813 bp of partial *tuf*A. Bootstrap values and posterior probability of each method are shown at each node: (left) maximum likelihood/(right) Bayesian inference; *: indicates full support (bootstrap value = 100%, posterior probability = 1.0); - indicates bootstrap value < 50%. Numbers before scientific names are GenBank accession numbers. In boldface, samples collected in Vietnam

Table 1. Average of HMs (µg.g^{-1}) in *Caulerpa lentillifera* collected in three different farms at Van Phong Bay and EU standard

	As	Cd	Co	Cr	Cu	Fe	Hg	Mn	Ni	Pb	Zn
Farm 1	1.58	0.09	0.10	1.42	2.03	117.19	0.12	42.12	0.94	0.56	7.85
Farm 2	2.05	0.09	0.17	0.44	1.94	117.20	0.10	69.50	0.92	0.60	6.40
Farm 3	2.22	0.12	0.18	0.80	1.94	167.37	0.13	69.39	0.93	0.58	5.74
Average	**1.95**	**0.10**	**0.15**	**0.89**	**1.97**	**133.92**	**0.12**	**60.34**	**0.93**	**0.58**	**6.66**
SD	*0.33*	*0.02*	*0.04*	*0.50*	*0.05*	*28.97*	*0.02*	*15.78*	*0.01*	*0.02*	*1.08*

Cu bioaccumulation of *Caulerpa lentillifera* cultivated in different levels of Cu

The concentrations of Cu in *Caulerpa lentillifera* cultivated in different levels of Cu are presented in Figure 5. In the control condition, the level of Cu was lower than 0.6 µg g^{-1} of dried tissue (DT). However, the seaweed cultivated with 2.5, 5.0, 7.5, and 10 µM of the metal showed an increasing Cu with 12.40, 15.27, 23.33, and 26.88 µg g^{-1} DT, respectively, on day 1. On days 3 and 6, the seaweed cultivated with 2.5, 7.5, and 10 µM of the metal showed 13.13, 17.07, 24.97 µg g^{-1} DT, and 15.39, 18.76, 29.02, respectively. Seaweed cultivated with 5.0 µM Cu on days 3 and 6. Cu in seaweed cultivated at 5.0 µM Cu L^{-1} was lower than cultivated at 2.5 µM Cu L^{-1}. In the following days (9 and 12), Cu in seaweed cultivated in different levels of Cu was still high, and Cu in seaweed cultivated in higher Cu in seawater showed higher than seaweed cultivated in lower Cu in seawater.

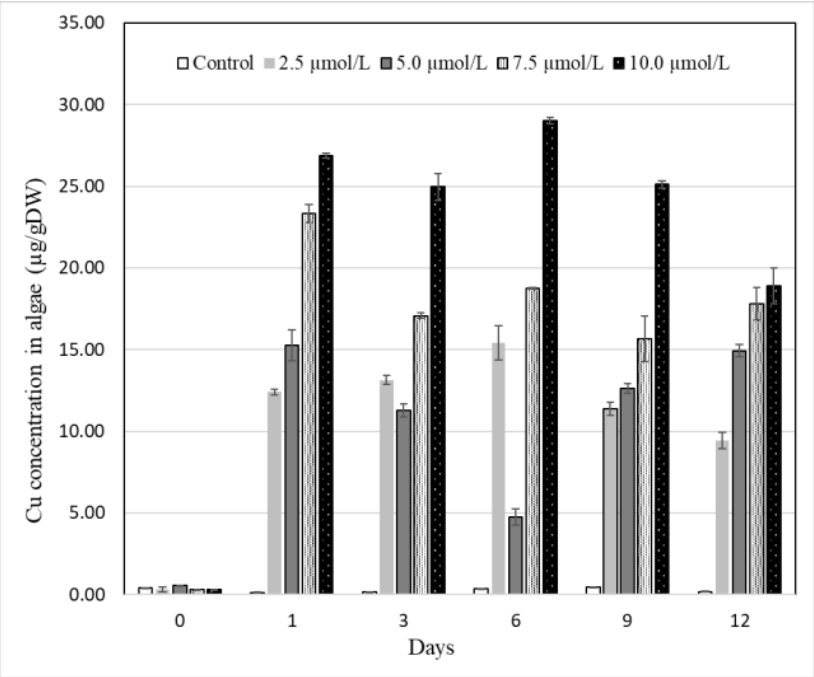

Figure 5. Cu bioaccumulation of *Caulerpa lentillifera* cultivated in seawater adding 2.5, 5.0, 7.5, and 10 µM L^{-1} for 0 to 12 days

Phytocheltins in algae

In this study, PCs were found in some samples. The chromatograms indicated NAC, Me4B, PC_2, PC_3, and PC_4 standards. The experiments were carried out three times, but the results on determining the PC content were the same.

DISCUSSION

In this study, two traits, including erect frond and ramuli diameter showed significant differences between two clones, and the Vietnamese clone seems smaller than the Japanese clone. So far, Su et al., [28] indicated that *C. lentillifera* was a kind of seaweed adapted to a low light intensity of 10 µmol photons $m^{-2}.s^{-1}$, and higher light intensity could cause photo-oxidation and damage its photosynthetic structure. The natural *C. lentillifera* growing at Ly Son Island occurred in rock or dead coral reefs, exposed in the air at the low time. In contrast, *C. lentillifera* in Van Phong Bay was cultivated in ponds under shadow mesh. Another study also showed that cultivation in shadow (around 50 µmol photons $m^{-2}.s^{-1}$) was encouraged due to higher biomass [29]. *Caulerpa* is a genus that showed morphological plasticity as a response to environmental changes. *Caulerpa lentillifera* and *C. racemosa* were misidentified in some cases [30]. This study also showed no genetic difference between natural and introduced *Caulerpa lentillifera* in Vietnam. The *tuf*A marker stands out as an excellent choice for molecular marker, and it was used in several families of Chlorophyta [31].

In this study, algae's HM concentrations of Cd, Cu, Hg, and Pb were low. Pb in *Caulerpa* spp. cultivated in ponds from Indonesia also showed 0.35 mg kg^{-1}, whereas Cd and Hg were below the detection limits of the method [32]. Cd and Pb in the commercialized *Caulerpa racemosa* var. *corynephora* from markets in Krabi, Thailand, were lower than 1.0 $mg.kg^{-1}$, however, Cu concentration was 17.4 $mg.kg^{-1}$ [33], much higher than this present study. The *Ulva rigida* showed lower Cd, Hg, and Pb than other green algae [5]. Al-Homaidan [34] reported that the green algae *Chaetomorpha aerea* (Dillwyn) Kutzing was significantly higher than in the other two green algae, including *Enteromorpha clathrata* (Roth) Greville and *Ulva lactuca* Linnaeus (Table 2). Unfortunately, the national standard (QCVN 8-2:2011/BYT) did not show standard regulatory limits for marine macro algae. Compared with International standard regulatory limits, the toxic heavy metal concentration in *C. lentillifera* found in this study were lower.

Table 2. Comparing Cd, Cu, Hg, and Pb concentrations in some green algae, and International standard regulatory limits. -: below the detection limits of the method; na: not available

Species	Cd	Cu	Hg	Pb	Sources
Caulerpa spp.	-	na	-	0.35	[32]
C. racemosa var. *corynephora*	0.89	17.4	na	0.97	[33]
C. lentillifera	**0.10**	**1.97**	**0.12**	**0.58**	**This study**
Chaetomorpha aerea	1.12	48.52	na	30.50	[34]
Enteromorpha clathrata	0.84	30.52	na	24.92	
Ulva lactuca	0.81	18.50	na	13.90	
Ulva rigida	0.03	3.15	0.02	0.05	[5]
European Commission Regulation (EC) No 488/2014	< 3.0	na	na	na	[35]
European Commission Regulation (EC) No 420/2011	na	na	na	< 3.0	[36]
Australia and New Zealand Food Authority	na	< 10	na	na	[37]

This present study indicated that increasing concentrations of Cu ranging from 2.5 to 10 $\mu M.L^{-1}$ induced a high Cu accumulation in the marine alga *C. lentillifera*, and the alga uptake Cu on the first day. It is a similar trend to other green algae. For example, in *Ulva compressa* cultivated in seawater with 10 $\mu M.L^{-1}$ Cd, the Cd concentration in algae reached 37.2 $\mu g.g^{-1}$ on the first day whereas the Cd concentration in algae was 2 $\mu g.g^{-1}$ in the control tank [24]. In green algae *Ulva prolifera* and *Ulva linza* cultivated seawater with 10 $\mu M.L^{-1}$ Cd, the Cd contents in algae were 78 and 26 times higher than the control after 7 days, respectively [38]. It is well-known that PCs were involved in transporting HMs from the cytosol into the

vacuole in plants [39]. In the study of Navarrete et al., [40] about cultivating marine macroalgae *Ulva compressa*, the increases in PCs were observed from day 1 at 7.5 and 10 µM Cu in seawater. Under high Zn treatment in seawater, *Caulerpa racemosa* showed gene expression for PCs, indicating that PCs induction occurred when the algae uptake Zn [26]. Unfortunately, PCs were not found in the study (Appendix 2). Therefore, the following study will use Zn or Cd as stressors, and the protocol of PC extraction should be modified.

CONCLUSION

There are significant differences in morphological traits of erect frond and ramuli diameter between Vietnamese natural and Japanese clones of *Caulerpa lentillifera*, but no genetic difference based on the *tuf*A marker. HM concentrations in *Caulerpa lentillifera* cultivated at Van Phong are lower than the International standard regulatory limit. Highly uptake Cu occurs on the first day, and higher Cu concentrations in algae were found in higher Cu concentrations in seawater.

Acknowledgements: We are deeply indebted to the VAST Key Lab on Food and Environmental Safety (Central Vietnam) for allowing us to use their equipment. This work was supported by the Vietnam Academy of Science and Technology, grant code TĐĐTMT.02/24–26.

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Appendix 1. GenBank accession and Herbarium voucher specimens of the sequences used in the alignment. Bold in face: samples collected in Vietnam; -/-: As above.

No.	Taxa	GenBank accession number/Herbarium voucher specimens	Sources
1	*Caulerpa ashmeadii*	DQ652362	Stam et al., 2006
2	*Caulerpa ashmeadii*	AJ417941	Patrizia Famà et al., 2001
3	*Caulerpa bartoniae*	FJ810426	Wynne et al., 2009
4	*Caulerpa brachypus*	DQ652353	Stam et al., 2006
5	*Caulerpa cactoides*	AJ417969	Patrizia Famà et al., 2001
6	*Caulerpa cupressoides*	DQ652335	Stam et al., 2006
7	*Caulerpa cupressoides*	AJ417929	Patrizia Famà et al., 2001
8	*Caulerpa cupressoides var. flabellata*	AJ417930	Patrizia Famà et al., 2001
9	*Caulerpa cupressoides var. lycopodium*	AJ512414	de Senerpont Domis et al., 2002
10	*Caulerpa distichophylla*	AJ417940	Patrizia Famà et al., 2001
11	*Caulerpa filiformis*	AJ417964	Patrizia Famà et al., 2001
12	*Caulerpa freycinetii var. integerrima*	FJ810424	
13	*Caulerpa flexilis*	AJ417970	Patrizia Famà et al., 2001
14	*Caulerpa flexilis*	AJ512426	Patrizia Famà et al., 2001
15	*Caulerpa geminata*	AJ417968	Patrizia Famà et al., 2001
16	*Caulerpa lanuginosa*	DQ652496	Stam et al., 2006
17	*Caulerpa lanuginosa*	AJ417959	Patrizia Famà et al., 2001
18	*Caulerpa lentillifera*	MZ855332	Woodhouse and Zuccarello 2023
19	-/-	VMO.190345[a] PP890004	This study
20	-/-	VMO.190346[b] PP890005	-/-
21	-/-	VMO.040316[c] PP890006	-/-
22	-/-	VMO.231102[d] PP890007	-/-
23	-/-	VMO.231103[e] PP890008	-/-
24	*Caulerpa mexicana*	DQ652433	
25	*Caulerpa mexicana*	AJ417951	Patrizia Famà et al., 2001
26	*Caulerpa paspaloides*	DQ652504	Stam et al., 2006
27	*Caulerpa paspaloides*	AJ417965	Patrizia Famà et al., 2001

No.	Taxa	GenBank accession number/Herbarium voucher specimens	Sources
28	*Caulerpa prolifera*	DQ652375	Stam et al., 2006
29	*Caulerpa prolifera*	AJ417942	Patrizia Famà et al., 2001
30	*Caulerpa racemosa*	JQ894933	Sauvage et al., 2013
31	*Caulerpa racemosa*	JN645150	Sauvage et al., 2011
32	*Caulerpa racemosa*	AJ512425	de Senerpont Domis et al., 2002
33	*Caulerpa racemosa var. occidentalis*	AJ417955	Patrizia Famà et al., 2003
34	*Caulerpa racemosa var. turbinata*	AJ417957	Patrizia Famà et al., 2001
35	*Caulerpa racemosa var. lamourouxii*	AJ417954	Patrizia Famà et al., 2001
36	*Caulerpa racemosa var. Cape Bolinao*	AJ512417	de Senerpont Domis et al., 2002
37	*Caulerpa racemosa var. macrophysa*	AJ417947	Patrizia Famà et al., 2001
38	*Caulerpa racemosa var. laetevirens*	AJ512415	de Senerpont Domis et al., 2002
39	*Caulerpa racemosa var. peltata*	AJ417949	Patrizia Famà et al., 2001
40	*Caulerpa scalpelliformis*	AJ417971	Patrizia Famà et al., 2001
41	*Caulerpa selago*	AJ417973	Patrizia Famà et al., 2001
42	*Caulerpa serrulata var. serrulata*	AJ512411	de Senerpont Domis et al., 2002
43	*Caulerpa serrulata*	DQ652352	Stam et al., 2006
44	*Caulerpa serrulata*	AJ417931	Patrizia Famà et al., 2001
45	*Caulerpa sertularioides*	AJ417944	Patrizia Famà et al., 2001
46	*Caulerpa sertularioides f. brevipes*	AJ512420	de Senerpont Domis et al., 2002
47	*Caulerpa sertularioides f. longipes*	AJ512419	de Senerpont Domis et al., 2002
48	*Caulerpa subserrata*	AJ417935	Patrizia Famà et al., 2001
49	*Caulerpa scalpelliformis*	AJ417972	Patrizia Famà et al., 2001
50	*Caulerpa taxifolia*	AJ417936	Patrizia Famà et al., 2001
51	*Caulerpa taxifolia*	DQ652357	Stam et al., 2006
52	*Caulerpa urvilleana*	JN645172	Sauvage et al., 2011
53	*Caulerpa verticillata*	AJ417967	Patrizia Famà et al., 2001
54	*Caulerpa webbiana*	AJ417958	Patrizia Famà et al., 2001
55	*Caulerpa webbiana var. pickeringii*	AJ417966	Patrizia Famà et al., 2001
56	*Caulerpella ambigua*	AJ417963	Patrizia Famà et al., 2001

Notes: [a]: place of collection: Ly Son Island (15.3177°; 109.1286°), collected and determined by: Nguyen Xuan Vy, date of collection: 15 February 2019, depth: 0.5 m; [b]: place of collection: Ly Son Island (15.3939°; 109.1159°), collected and determined by: Nguyen Xuan Vy, date of collection: 15 February 2019, depth: 0.5 m; [c]: place of cultivation: Nha Trang (12.2078°; 109.2158°); collected and determined by: Nguyen Huu Dai, date of collection: 20 March 2004, depth: na; [d]: place of collection: Van Phong Bay (12.5646°; 109.2082°); collected and determined by: Nguyen Xuan Vy, date of collection: 10 April 2023, depth: 1 m.

Appendix 2. Chromatograms indicating NAC, Me4B, PC_2, PC_3, and PC_4 in standard (A), but not found in samples (B, C, D, E)