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Herbicide Atrazine Alters the Microbiota of the Filamentous Green Alga *Cladophora* sp. Cultured from Thailand

(Herbisid Atrazin Mengubah Mikrobiota Alga Hijau Berfilamen *Cladophora* sp. yang Dikultur dari Thailand)

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ABSTRACT

The attached green alga Cladophora *known to harbor microbiota that play important roles in ecosystem, is one of the most common freshwater filamentous green algae in rivers globally, including those in the northern part of Thailand. These rivers mostly run through agricultural regions where herbicides are heavily used to improve crop quality and quantity. The extensively-used herbicide atrazine persists in soil sediments through transport by surface runoff to rivers. The effect of such herbicide contamination on* Cladophora *microbiota in Thailand have not been investigated. To acquire this information, 16S rDNA amplicons were used to compare microbiota of* Cladophora *sp. cultures treated with a spectrum of atrazine concentrations. The results showed that the* Cladophora *microbiome included at least 106 possible Operational taxonomic units (OTUs) representing twelve bacterial phyla which are Acidobacteria, Actinobacteria, Armatimonadetes, Chloroflexi, Cyanobacteria, Deinococcus-Thermus, Epsilonbacteraeota, Nitrospirae, Patescibacteria, Planctomycetes, Proteobacteria, and WPS-2, representing both core and local algal bacteria. The presence of atrazine was also correlated with changes in richness of bacterial taxa suggesting that these algal epibiotic bacteria were differently affected by atrazine treatments.*

Keywords: 16S rDNA amplicons; atrazine; Cladophora*; microbiomes*

ABSTRAK

Alga hijau Cladophora *telah diketahui melindungi mikrobiota yang memainkan peranan penting dalam ekosistem, ia adalah salah satu alga hijau filamen air tawar yang biasa dijumpai dalam sungai di seluruh dunia, termasuk di bahagian utara Thailand. Sungai ini kebanyakannya merentasi kawasan pertanian di mana herbisid banyak digunakan untuk meningkatkan kualiti dan kuantiti tanaman. Herbisid atrazin yang digunakan secara meluas kekal di dalam endapan tanah secara pengangkutan melalui larian permukaan ke sungai. Kesan pencemaran herbisid ke atas mikrobiota* Cladophora *di Thailand masih belum pernah dijalankan. Untuk memperoleh maklumat ini, amplikon 16S rDNA digunakan untuk membandingkan kultur mikrobiota* Cladophora *sp. yang dirawat dengan spektrum kepekatan atrazin. Hasil kajian menunjukkan bahawa mikrobiom* Cladophora *merangkumi sekurang-kurangnya 106 kemungkinan unit operasi taksonomi (OUT) yang mewakili dua belas filum bakteria seperti Acidobacteria, Actinobacteria, Armatimonadetes, Chloroflexi, Cyanobacteria, Deinococcus-Thermus, Epsilonbacteraeota, Nitrospirae, Patescibacteria, Planctomycetes, Proteobacteria dan WPS-2, yang mewakili kedua-dua bakteria alga teras dan tempatan. Kehadiran atrazin juga turut dikaitkan dengan perubahan kekayaan taksa bakteria yang mencadangkan bahawa bakteria epibiotik alga ini dipengaruhi secara berbeza oleh rawatan atrazin.*

Kata kunci: Amplikon 16S rDNA; atrazin; Cladophora*; mikrobiom*

Introduction

The green algal genus *Cladophora*, which is known to play important ecological roles, is common in freshwaters globally (Zulkifly et al. 2013). *Cladophora* has been documented to occur in the northern and the northeastern part of Thailand, where it forms conspicuous green masses or streamers 10 cm or longer along the shoreline of main rivers (Laungsuwon & Chulalaksananukul 2013; Peerapornpisal et al. 2006; Thiamdao et al. 2012). Resistant to grazers, *Cladophora* provides habitats for other

organisms, including biofilms of bacteria that may play important functional roles (Braus et al. 2017; Graham et al. 2015; Zulkifly et al. 2012).

To date, many studies of epiphytic microbes of *Cladophora* have focused on large lakes of the northern United States of America (U.S.A.) (Braus et al. 2017; Byappanahalli et al. 2009, 2007, 2003; Chun et al. 2013; Graham et al. 2015; Ishii et al. 2006; Olapade et al. 2006; Whitman et al. 2003; Zulkifly et al. 2012). These studies showed that *Cladophora* typically supports surface biofilms that include diverse bacterial and eukaryotic lineages, those of more restricted occurrence representing local taxa and those of broad occurrence representing a core microbiota that may provide key functions in algal growth. For example, *Cladophora* epibiotic bacteria have been hypothesized to provide many important functions such as nitrogen fixation and providing vitamin B12, which is required for *Cladophora* growth (Graham et al. 2015).

In Thailand, *Cladophora* is commonly present in rivers running through agricultural areas where herbicides are heavily used to improve crop quality and quantity. Atrazine (6-chloro-N2-ethyl-N4-isopropyl-1,3,5-triazine-2,4- diamine) is one of the top ten herbicides used in the country (Department of Agriculture 2019; EPA 2003). This herbicide is extensively used for pre-emergence and post-emergence weed controls in corn, sorghum, pineapple, sugarcane, and rice farming (EPA 2003). However, atrazine can persist long enough to contaminate soil sediments, and then enter surface waters in runoff, causing contaminations in various major rivers in Thailand (Kruawal et al. 2005; Phewnil et al. 2012, 2010; Sangchan et al. 2014).

The contamination by atrazine on these rivers can directly affect the survival of photosynthetic aquatic organisms, because atrazine interferes with the electron transport chain in photosystem II by binding to the reaction center of the quinone B protein, which obstructs electron flow (Shukla & Devine 2008; Trebst 2008). It is also known that atrazine can alter diversity of aquatic bacterioplankton communities by reducing presence of susceptible bacteria and increasing populations of atrazine degrading- and mineralizing-bacteria, which can use atrazine as a carbon and nitrogen source (Bohuss et al. 2005; Radosevich et al. 1995; Vargha et al. 2005). However, whether atrazine similarly impacts microbial communities associated with algal surfaces has been unknown. For this reason, 16S rDNA amplicon technology was used to compare the microbiota of *Cladophora* sp. treated with different atrazine concentrations (0, 12, 60, 300, and 1000 μg/L) to answer whether culturing of the host *Cladophora* affect its microbiota, and also to determine the effect of atrazine on the *Cladophora* microbiota.

Materials and Methods

Algal identification

A sample of a *Cladophora* was collected from Lumpini Park, Bangkok, Thailand (13.7314° N, 100.5414° E), and the alga was identified to genus level on distinctive microscopic features: Branching filaments, reticulate plastids, and relatively large cell size. A sample of the dried specimen has been deposited under barcode number BCU 5002 in the Kasin Suvatabandhu Herbarium, Department of Botany, Chulalongkorn University, Thailand (https://www.chula.ac.th/museum/763/). To perform the molecular identification, total DNA was extracted by using Thermo Scientific GeneJET Plant Genomic DNA Purification Kit (Thermo Scientific™, USA), before amplification of 23S ribosomal DNA (23S rDNA) was conducted using the methods described in Sherwood and Presting (2007). A PCR product of the expected size was sequenced using Sanger sequencing (Macrogen, South Korea).

The 23S rDNA sequence from the Bangkok *Cladophora* collection was deposited in Genbank under accession number MK863366. The phylogenetic analysis of this sequence and other *Cladophora* 23S rDNA sequences (Genbank accession numbers AJ544728.1, AJ544752.1, AJ544753.1, AJ544754.1, AJ544755.1, AJ544756.1, AJ544757.1, AJ544760.1, AJ544761.1, AJ544763.1, AJ544764.1, KX421223.1, KX421224.1, KX421225.1, KX421226.1, KX421227.1, KX421228.1, KX421229.1, KX421230.1, KX421231.1, KX421232.1, KX421233.1, KX421234.1, MG021092.1, MG021094.1) was performed using a partial 23S rDNA sequence from *Aegagropila linnaei* (MF683076.1) as an outgroup. All sequences were aligned using MAFFT alignment v 7.402 (Katoh et al. 2009) and tested for evolutionary model using jModelTest2 v 2.1.6 (Darriba et al. 2012), before performing Maximum-Likelihood analysis using RAxML v 8.2.12 (Stamatakis 2014) and Bayesian analysis using MBayes v 3.2.7a (Ronquist et al. 2012) available on the CIPRES XSEDE Portal (Miller et al. 2012) using a TrN+G substitution model. Four independent chains were run for 1,000,000 cycles and consensus topologies calculated after 25,000 burn-in cycles.

Bacterial microbiota of *Cladophora* sp.

The 16S rDNA amplicons were employed to infer the taxonomic diversity of bacterial microbiota of *Cladophora*

of the same morphological type for which 23S rDNA sequence had been obtained. Firstly, the algal biomass was well-washed with Bold's Basal Medium to remove debris. Then, total metagenomic DNA was extracted using Quick-DNA Fecal/Soil Microbe Kits (Zymo Research, Carolina Biological Supply, Burlington, NC, USA), from three samples to generate three technical replicates. DNA was sent to Omics Science and Bioinformatics Center, Faculty of Science, Chulalongkorn University for V3-V4 16S rDNA amplification and sequencing. Raw data were analyzed using QIIME2 v 2017.12.0 pipeline (Bolyen et al. 2019). The taxonomy of the assembled contigs was assigned using the SILVA 132 reference database (Quast et al. 2013) at 97% minimum similarity. To compare the presence of the taxa, the taxon was called 'present' if it was present in at least one replication of the data, otherwise, it was called 'absent'. Then, to find the core and environmental bacterial taxa, the bacterial taxa identified in this study was compared with those previously reported studies. The core bacterial taxa were bacteria co-presented in this study and previously reported studies from the north of U.S.A. meanwhile, the environmental bacterial taxa were bacteria present in this study but have not been reported anywhere else.

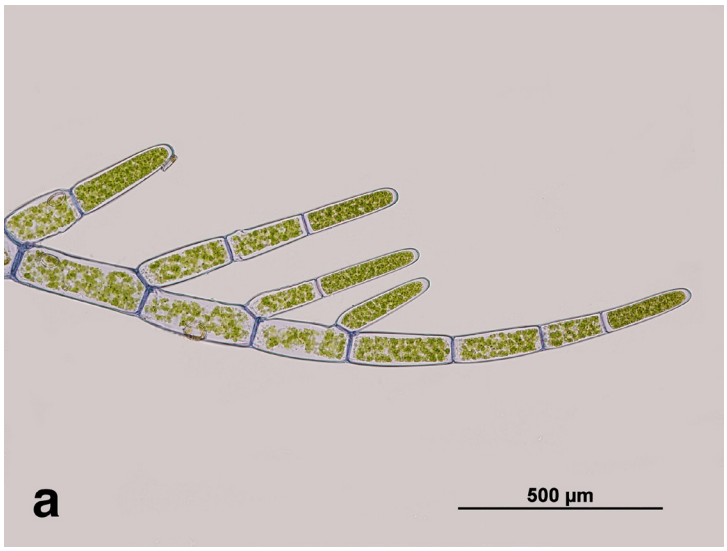
EFFECTS OF THE HERBICIDE ATRAZINE ON MICROBIOTA OF *Cladophora* sp. IN LABORATORY CULTURE

To investigate the effect of atrazine on microbiota of cultured *Cladophora* sp., the field-collected *Cladophora* was rinsed with Bold's Basal Medium until all debris was removed. Then, the alga was cultivated in Bold's Basal Medium for seven days before treating replicate algal cultures with five atrazine concentrations, including 0, 12, 60, 300, and 1,000 µg/L. The algal cultures were maintained with 16:8 daily light: dark cycle at room temperature. Total DNA was extracted on day 0 and day 14. Total metagenomic DNA was extracted by the same method described for the 23S rDNA-characterization of the *Cladophora*. Then, the level of taxonomic richness was compared between each experimental set using the non-parametric Kruskal-Wallis test with the Shannon index (H) as the dependent variable. The raw data for 16S rDNA amplicon analysis for field-collected and cultured *Cladophora* were deposited in the NCBI Short Read Archive accession number SAMN13351969.

RESULTS

ALGAL IDENTIFICATION

Morphological observation suggested that the alga belonged to genus *Cladophora* (Figure 1). Phylogenetic analysis of 23S rDNA analyzed using maximum likelihood and Bayesian frameworks (Figure 2) suggested that the *Cladophora* used in this study was closely related to an unclassified *Cladophora* isolate obtained from Northern California freshwater aquatic ecosystems (NCBI accession number MG021094).



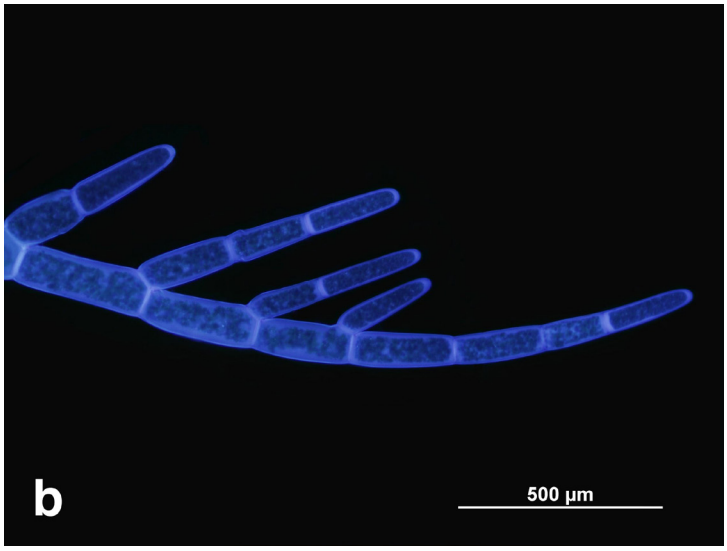


FIGURE 1. The morphology of (a) *Cladophora* sp. observed under light microscopy and (b) *Cladophora* sp. stained with Calcofluor White observed under fluorescence microscopy. The branching of the filament that was located close to the cross wall is the unique character of algae genus *Cladophora*

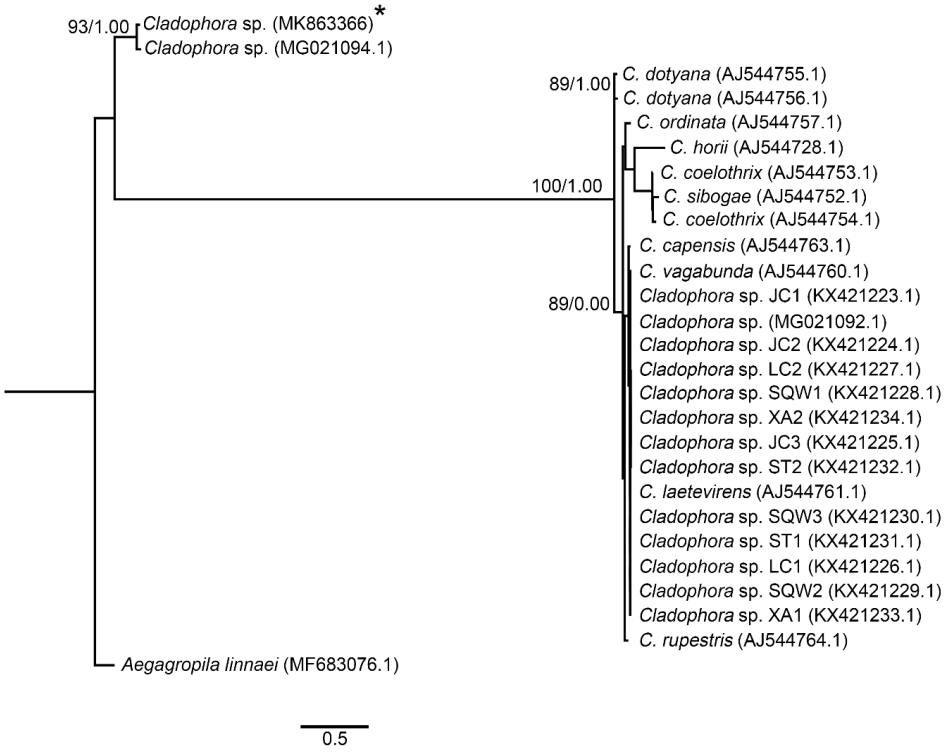


FIGURE 2. Maximum-Likelihood tree inferred from 23S rDNA of *Cladophora* species. The associated NCBI accession numbers were Maximum-Likelihood bootstrap values and Bayesian posterior probability values are shown at the respective nodes. The scale bar represents the estimated number of nucleotide substitutions per site. The closely related green alga *Aegagropila linnaei* was used as the outgroup

16S rDNA AMPLICON ANALYSIS

Our results from 16S rDNA amplicon analysis suggested that the microbiota included at least 106 possible OTUs representing 12 bacterial phyla which are *Acidobacteria*, *Actinobacteria*, *Armatimonadetes*, *Chloroflexi*, *Cyanobacteria*, *Deinococcus-Thermus*, *Epsilonbacteraeota*, *Nitrospirae*, *Patescibacteria*, *Planctomycetes*, *Proteobacteria*, WPS-2, and unknown bacterial phyla which consisted of 68 known bacterial genera (Figure 3).

The five most dominant bacterial phyla associated with the field-collected alga were *Proteobacteria* (36.77%), *Acidobacteria* (23.90%), *Cyanobacteria* (14.70%), Planctomycetes (6.67%), and *Chloroflexi* (6.35%). At the genus level, the five most dominant genera were *Acidobacteria aridibacter* (17.32%), uncultured *Proteobacteria* belonging to *Rhizobiaceae* (5.35%), uncultured *Chloroflexi* belonging to *Caldilineaceae* (3.96%), uncultured Proteobacteria belonging to *Rhizobiales incertae* Sedis (3.82%), and uncultured *Cyanobacteria* belonging to SepB-3 (3.72%).

To investigate the effect of laboratory culturing on the algal microbiota, we compared the microbiota of cultured *Cladophora* treated with different concentrations of atrazine. The results showed that after maintaining the algal culture in the laboratory for two weeks, the five most dominant phyla remained the same but with different taxonomic richness - *Cyanobacteria* (55.96%), *Proteobacteria* (20.59%), *Chloroflexi* (9.67%), *Planctomycetes* (5.21%), and *Acidobacteria* (4.40%). Hence, an increase of *Cyanobacteria* and *Armatimonadetes* and a decrease of *Proteobacteria*, *Acidobacteria*, *Planctomycetes*, *Chloroflexi*, *Actinobacteria*, *Patescibacteria*, *Deinococcus-Thermus*, *Epsilonbacteraeota*, WPS-2, and *Nitrospirae* were observed. Both WPS-2 and *Nitrospirae* were completely disappeared from the control (non-atrazine) treatment cultures.

The five most-dominant genera associated with non-atrazine cultured *Cladophora* were uncultured Cyanobacteria belonging to *Leptolyngbyaceae* (41.62%), uncultured *Chloroflexi* belonging to *Caldilineaceae* (6.88%), uncultured *Proteobacteria* belonging to A0839 (6.16%), uncultured *Planctomycetes* belonging to *Gemmataceae* (3.39%), and uncultured *Proteobacteria* belonging to *Reyranellaceae* (3.27%). Additionally, some bacterial genera were absent from the non-atrazine cultured *Cladophora*. These included *Actinobacteria gordonia*, *Planctomycetes planctomicrobium* and AKYG587, and *Proteobacteria azospirillum*, *Pseudaminobacter*, *Sphingoaurantiacus*, and *Tabrizicola*.

Then, we compared the *Cladophora* microbiota from each atrazine treatment. The results showed that after two weeks of treatment using different concentrations of atrazine, the five most dominant bacterial phyla remained almost the same, however, with different taxonomic richness. The five most dominant bacterial phyla in 12 µg/L atrazine were *Cyanobacteria* (34.75%), *Proteobacteria* (24.39%), *Acidobacteria* (16.71%), *Chloroflexi* (10.65%), and *Planctomycetes* (4.95%). The five most dominant bacterial phyla in 60 µg/L atrazine were *Acidobacteria (*39.95), *Proteobacteria* (28.30%), *Chloroflexi* (14.24%), *Cyanobacteria* (6.51%), and *Planctomycetes* (4.29%). The five most dominant bacterial phyla in 300 µg/L atrazine were *Chloroflexi* (22.06%), *Proteobacteria* (19.05%), *Acidobacteria* (16.26%), *Patescibacteria* (11.75%), and *Planctomycetes* (9.47%). The five most dominant bacterial phyla in 1000 µg/L atrazine were *Acidobacteria* (47.92%), *Proteobacteria* (21.51%), *Chloroflexi* (9.16%), *Planctomycetes* (6.30%), and *Actinobacteria* (5.36%).

A reduction of some bacterial genera was observed when the concentration of atrazine is increased. *Proteobacteria* (*Dongia* and *Amphiplicatus*) and *Cyanobacteria* (*Acaryochloris*, *Chamaesiphon*, and *Scytonema*) found in the control treatment were absent from the algal cultures treated with 60, 300, and 1000 µg/L atrazine. Meanwhile, *Cyanobacteria planoglabratella, Pleurocapsa*, *Schizothrix*, and *Xenococcus*, and *Acidobacteria aridibacter* were absent from the algal cultures treated with 300 and 1000 µg/L atrazine. From the algal cultures treated with 1000 µg/L atrazine, *Proteobacteria phreatobacter*, *Planctomycetes* and *Telmatocola*, *Acidobacteria blastocatella*, and *Cyanobacteria leptolyngbya* were absent. However, most of the bacterial genera present in the control were present in most of atrazine concentrations.

Statistical comparisons suggested that the diversity level present in the algal microbiota before the experimental atrazine treatment was the highest and the diversity level of the algal microbiota in the 12 µg/L atrazine treatment was the lowest. However, we did not observe a significant difference of the bacterial diversity level among the field-collected and the atrazine treated *Cladophora*.

Field Collected	0 µg atrazine L-1	12 µg atrazine L-1	60 µg atrazine L-1	300 µg atrazine L-1	1000 µg atrazine L-	Phylum	Genus
14.0	0.0	0.0	0.0	0.0	0.0	Proteobacteria	*Sphingoaurantiacus*
19.0	0.0	0.0	0.0	0.0	0.0	Proteobacteria	*Azospirillum*
20.3	0.0	0.0	0.0	0.0	0.0	Actinobacteria	*Gordonia*
32.7	0.0	0.0	0.0	0.0	0.0	Proteobacteria	*Tabrizicola*
84.3	0.0	0.0	0.0	0.0	0.0	Planctomycetes	*Planctomicrobium*
7.0	0.0	1.3	0.0	0.0	0.0	Planctomycetes	AKYG587
37.3	29.0	8.7	0.0	0.0	0.0	Proteobacteria	*Dongia*
1.7	2.3	9.3	0.0	0.0	0.0	Cyanobacteria	*Acaryochloris* MBIC11017
138.0	403.3	33.7	0.0	0.0	0.0	Proteobacteria	*Amphiplicatus*
69.0	265.3	71.7	0.0	0.0	0.0	Cyanobacteria	*Chamaesiphon* PCC-7430
16.3	25.3	87.7	0.0	0.0	0.0	Cyanobacteria	*Scytonema* UTEX 2349
5.3	0.0	117.3	0.0	0.0	0.0	Proteobacteria	*Novosphingobium*
40.3	97.0	88.7	2.7	0.0	0.0	Cyanobacteria	*Xenococcus* PCC-7305
346.0	41.7	30.3	4.3	0.0	0.0	Cyanobacteria	*Schizothrix* LEGE 07164
2201.0	76.3	70.7	4.3	0.0	0.0	Acidobacteria	*Aridibacter*
184.7	127.0	341.0	27.3	0.0	0.0	Cyanobacteria	*Pleurocapsa* PCC-7319
23.0	177.0	1657.3	430.3	0.0	0.0	Cyanobacteria	*Planoglabratella opercularis*
3.7	0.0	3.7	0.0	2.3	0.0	Actinobacteria	IMCC26207
95.3	153.3	127.7	63.7	4.0	0.0	Proteobacteria	*Phreatobacter*
3.0	0.0	0.0	0.7	6.0	0.0	Firmicutes	*Ethanoligenens*
22.3	162.7	104.7	21.7	10.7	0.0	Planctomycetes	*Telmatocola*
5.0	27.3	12.3	0.0	13.0	0.0	Proteobacteria	*Rhodovarius*
9.0	0.0	0.0	0.0	18.3	0.0	Proteobacteria	*Marivita*
109.0	455.7	556.7	62.3	36.7	0.0	Acidobacteria	*Blastocatella*
106.7	344.3	701.3	11.3	313.0	0.0	Cyanobacteria	*Leptolyngbya* ANT.L52.2
119.7	9.7	20.3	8.3	27.3	1.3	Planctomycetes	*Blastopirellula*
38.0	26.3	24.0	5.0	5.7	2.0	Cyanobacteria	*Synechococcus* PCC-7902
138.3	15.7	69.7	1.7	6.3	2.0	Acidobacteria	*Stenotrophobacter*
55.7	99.7	60.0	28.7	10.0	2.0	Deinococcus-Thermus	*Truepera*
28.0	139.0	3.7	4.7	18.3	2.3	Chloroflexi	OLB13
5.0	31.7	9.3	6.7	8.0	2.7	Armatimonadetes	*Chthonomonas*
5.7	0.0	0.0	0.0	6.3	4.3	Proteobacteria	*Silicimonas*
34.0	64.3	46.7	5.7	5.0	5.7	Proteobacteria	*Pedomicrobium*
3.7	0.0	0.0	0.0	10.7	6.0	Cyanobacteria	*Synechococcus* CC9902
11.7	11.0	11.7	0.7	12.3	6.0	Planctomycetes	CL500-3
3.0	6.3	22.0	13.3	20.3	6.0	Proteobacteria	*Pseudorhodoplanes*
64.3	52.7	102.7	22.0	9.7	6.7	Cyanobacteria	*Oedogonium cardiacum*
6.7	0.0	2.3	0.0	12.7	6.7	Cyanobacteria	*Cyanobium* PCC-6307
367.3	300.3	122.0	18.0	5.3	10.0	Cyanobacteria	HAVOmat113
44.3	519.0	289.0	120.0	4.3	11.7	Proteobacteria	*Bosea*
4.7	39.0	151.0	103.0	25.7	13.3	Proteobacteria	*Mesorhizobium*
362.0	153.3	265.3	268.7	12.3	13.7	Proteobacteria	*Rhodobacter*
13.0	0.0	0.0	5.3	20.7	13.7	Actinobacteria	*Pseudonocardia*
59.0	152.3	285.0	146.7	105.3	14.3	Acidobacteria	*Paludibaculum*
23.3	32.3	31.3	5.7	46.7	17.0	Planctomycetes	SM1A02
104.3	3.7	7.7	98.7	9.3	19.7	Proteobacteria	*Bdellovibrio*
6.0	11.7	17.7	70.0	20.0	20.0	Planctomycetes	*Gemmata*
5.3	21.3	27.3	9.3	7.0	26.7	Proteobacteria	AT-s3-44
50.3	134.0	393.0	37.7	279.3	32.0	Actinobacteria	*Ilumatobacter*
26.3	5.3	7.3	17.0	13.7	33.3	Actinobacteria	*Fodinicola*
22.7	26.7	26.3	3.0	118.3	34.3	Acidobacteria	*Chloracidobacterium*
75.3	193.3	244.0	191.0	61.7	52.3	Planctomycetes	*Fimbriglobus*
59.7	7.7	14.7	6.7	69.7	63.7	Epsilonbacteraeota	*Sulfurovum*
38.7	13.3	32.7	3.7	91.3	85.7	Proteobacteria	*Nordella*
25.3	180.7	77.7	25.7	28.3	95.3	Armatimonadetes	*Armatimonas*
303.3	304.3	265.0	140.0	102.0	109.3	Proteobacteria	*Hyphomicrobium*
185.7	35.7	126.3	173.3	57.3	122.7	Planctomycetes	SH-PL14
7.7	88.0	141.0	40.3	149.7	190.7	Actinobacteria	*Iamia*
23.7	0.0	0.0	0.0	44.3	226.0	Proteobacteria	*Pseudaminobacter*
20.0	30.7	122.3	228.7	348.7	255.7	Actinobacteria	CL500-29 marine group
22.7	167.7	345.7	655.0	248.3	294.0	Proteobacteria	*Reyranella*
102.7	174.0	465.0	85.3	214.7	312.3	Deinococcus-Thermus	*Meiothermus*
369.0	556.0	552.0	130.0	369.0	321.7	Proteobacteria	SWB02
122.3	133.0	206.7	69.0	249.7	353.7	Acidobacteria	*Bryobacter*
110.0	334.3	452.3	278.7	425.0	383.0	Acidobacteria	Elev-16S-1166
37.0	17.7	212.3	538.3	134.7	403.0	Proteobacteria	*Sphingopyxis*
61.3	247.7	104.3	28.3	182.7	455.7	Proteobacteria	*Hirschia*
60.0	76.0	401.0	201.3	230.0	474.0	Proteobacteria	*Candidatus Alysiosphaera*

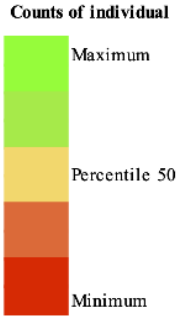


FIGURE 3. Known bacterial taxa present in field-collected *Cladophora*, cultured *Cladophora*, and atrazine-treated cultured *Cladophora*. The numbers and colors in the box represent the counts and percentile of the counts of individuals in each taxon

DISCUSSION

CORE MICROBIOME AND ENVIRONMENTAL OF FIELD-COLLECTED *Cladophora*

This is the first study of the microbiota of the green alga *Cladophora* in Thailand. We identified an isolate from northern Thailand and studied its microbiota before and after treatments of atrazine, an herbicide widely used in agriculture. Studies showed that specific phylogenetic groups of heterotrophic bacteria occur in close association with specific green algae as the algae provides organic exudates for bacteria. Therefore, it is hypothesized that these bacteria might play important roles in survival and dispersal of organisms.

Among the thirteen bacterial phyla identified in the field-collected *Cladophora* (*Acidobacteria*, *Actinobacteria*, *Armatimonadetes*, *Chloroflexi*, *Cyanobacteria*, *Deinococcus-Thermus*, *Epsilonbacteraeota*, *Nitrospirae*, *Patescibacteria*, *Planctomycetes*, *Proteobacteria*, and WPS-2), we found some bacterial phyla present in common with the *Cladophora* microbiomes previously reported from the north of U.S.A. (Braus et al. 2017; Graham et al. 2015; Zulkifly et al. 2012). These included *Acidobacteria bryobacter*, *Actinobacteria ilumatobacter*, *Armatimonadetes armatimonas*, *Cyanobacterial chamaesiphon*, *Deinococcus-Thermus truepera* and *Meiothermus*, *Planctomycetes gemmata* and *Proteobacteria rhodobacter*, *Bdellovibrio*, *Hyphomicrobium*, *Novosphingobium*, and *Sphingopyxis*.

These bacterial genera, i.e. *Acidobacteria bryobacter*, *Planctomycetes gemmata*, and *Proteobacteria novosphingobium* were also present in a close association with other green algae and early diverging land plants. For example, *Acidobacteria bryobacter* was reported as a closely associated taxon of a chlorophyte alga *Chlorella sorokiniana* (Lebrero et al. 2016), a streptophyte alga *Chara braunii* (Saltykova 2015), and a liverwort *Marchantia* sp. (Alcaraz et al. 2018). *Planctomycetes gemmata* was present in the microbiome of a chlorophyte alga *Ulva* sp. in Carreço, Portugal (Lage & Bondoso 2011; Faria et al. 2018) and a streptophyte alga *Chara braunii* in Japan (Saltykova 2015). *Novosphingobium* is known as Plant Growth Promoting Bacteria (PGPB) in a green alga *Nannochloris* sp. (Ramanan et al. 2015). However, their functions in the algal microbiomes have not yet been elucidated.

Actinobacteria Ilumatobacter, *Armatimonadetes Armatimonas*, *Cyanobacterial Chamaesiphon*, *Deinococcus-Thermus Truepera* and *Meiothermus*, and *Proteobacteria rhodobacter*, *Bdellovibrio*, *Hyphomicrobium*, and *Sphingopyxis* have not been reported from microbiome of green algae other than *Cladophora*. However, these bacterial genera were always present in the local environmental samples together with green algal species. For example, the *Actinobacteria ilumatobacter* was present in the co-occurrence networks among bacteria and microbial eukaryotes of Lake Baikal during a Spring phytoplankton bloom (Mikhailov et al. 2019) and in a eutrophic lake in South Norway (Parulekar et al. 2017). The presence of these bacteria suggested their essential functions for the survival of other photosynthetic phytoplankton in the system.

Some bacterial phyla previously reported in microbiota of temperate U.S.A. *Cladophora* were not present in our results. These included the bacterial phyla *Bacteroidetes*, *Verrucomicrobiae*, *Lentisphaerae*, *Nitrospirae*, and *Fusobacteria* (Braus et al. 2017; Graham et al. 2015; Zulkifly et al. 2012). This incongruence suggested that these bacteria might be environmentally specific and might not be required for growth and dispersal of *Cladophora* in tropical regions like Thailand. However, this does not mean that the functions performed by these bacteria were not essential. It could be that the functions provided by these bacteria were substituted by those performed by other local bacterial taxa observed in this study.

Additionally, some human pathogenic bacteria such as *Campylobacter*, *Escherichia*, *Salmonella*, and *Plesiomonas* previously reported from *Cladophora* in Lake Michigan (Byappanahalli et al. 2009, 2007, 2003; Chun et al. 2013; Ishii et al. 2006; Olapade et al. 2006; Whitman et al. 2003) were not present in the results from this study. This suggested that these pathogenic bacteria are not evolutionarily important for the growth of *Cladophora* and the presence of these pathogens in living and dead *Cladophora* from Lake Michigan were environmentally influenced.

EFFECTS OF LABORATORY CULTURING AND ATRAZINE ON THE MICROBIOTA OF *Cladophora*

Comparison of the bacterial genera from field-collected and cultured *Cladophora* not treated with atrazine revealed the absence of 13 bacterial genera in the cultured *Cladophora*. These included *Actinobacteria gordonia*, *Pseudonocardia*, and IMCC26207, *Cyanobacteria cyanobium* PCC-6307 and *Synechococcus* CC9902, *Planctomycetes planctomicrobium* and AKYG587, and *Proteobacteria azospirillum*, *Marivita*, *Novosphingobium*, *Pseudaminobacter*, *Sphingoaurantiacus*, and *Tabrizicola*. The absence of these bacterial genera suggested that they were not essential for the survival of *Cladophora* when the nutrients, light, and temperature were suitable for algal growth or these bacteria genera were out-competed in such conditions.

EFFECTS OF DIFFERENT ATRAZINE CONCENTRATIONS ON THE MICROBIOTA OF *Cladophora*

After two weeks of atrazine treatment, we observed that different atrazine concentrations differently affected the microbiota of *Cladophora*. By comparing the presence of bacterial taxa in different atrazine concentrations, these bacteria were categorized into three groups.

The first group consisted of bacteria that confers different degrees of susceptibility to atrazine. Certain bacterial genera - *Acidobacteria aridibacter* and *Blastocatella*, *Cyanobacteria acaryochloris*, *Chamaesiphon*, *Leptolyngbya*, *Planoglabratella*, *Pleurocapsa*, *Schizothrix*, *Scytonema*, and *Xenococcus*, *Proteobacteria amphiplicatus*, *Dongia*, *Phreatobacter*, and *Planctomycetes telmatocola* - were absent from the algal microbiota in certain concentrations of atrazine and in all other treatments with higher atrazine concentration. The absence of these bacteria was not phylum-specific, therefore, we hypothesized that the susceptibility of these bacteria might be taxa-dependent resulted from their intrinsic factor, for example, the presence of genes involved in atrazine metabolism.

Another group consisted of bacterial genera that were randomly present in several atrazine treatments. These included *Actinobacteria* IMCC26207 and *Pseudonocardia*, *Cyanobacteria cyanobium* PCC-6307, *Synechococcus* PCC-7902, and *Synechococcus* CC9902, *Planctomycetes blastopirellula*, *Proteobacteria rhodovarius*, *Marivita*, *Silicimonas*, and *Pseudaminobacter*. Lastly, most of the bacterial taxa were present in all conditions used in this study: Field-collected, cultured, and atrazine-treated cultured *Cladophora*. These included several bacterial taxa from phyla *Acidobacteria*, *Actinobacteria*, *Armatimonadetes*, *Chloroflexi*, *Cyanobacteria*, *Deinococcus-Thermus*, *Epsilonbacteraeota*, *Planctomycetes*, and *Proteobacteria*. Different bacterial taxa present under these conditions suggested that different degrees of adaptive ability were adopted by these bacteria to cope with the herbicide atrazine, as we observed both increases and decreases in their population when the concentration of atrazine was increased.

This increase and decrease of the bacterial taxonomic diversity (although not statistically different) and its change in richness suggested that these bacteria had the potential to adapt to the new environment when atrazine is present. In this study, the presence of atrazine did not involve only the addition of the chemical in the algal medium, but also changes that possibly occurred due to its effects. For example, the presence of this chemical caused a decline of highly atrazine-susceptible bacteria genera, which later decayed and became an organic resource for other surviving bacterial groups. Also, this chemical might alter the secretion of organic compounds of *Cladophora* or other associated organisms, which benefited certain bacterial groups.

Some of the bacterial taxa present in the atrazine-treated cultured *Cladophora* have previously been reported from atrazine contaminated environments. These included *Actinobacteria gordonia* (Drzyzga 2012) and *Pseudonocardia* (Desitti et al. 2017), *Cyanobacteria schizothrix* (Sugiura 2009) and *Synechococcus* (Weiner et al. 2007), *Deinococcus-Thermus truepera* (Fang et al. 2018), and *Proteobacteria azospirillum* (Gadkari 1991), *Bdellovibrio* (Liao et al. 2015), *Bosea* (Udiković-Kolić et al. 2012), *Dongia* (Wallace & May 2018), *Hyphomicrobium* (Liu et al. 2019), *Mesorhizobium* (Drouin et al. 2010), *Novosphingobium* (Sohn et al. 2004), *Pedomicrobium* (Satsuma 2009), *Phreatobacter* (Tóth et al. 2014), *Pseudaminobacter* (Topp et al. 2000), *Pseudorhodoplanes* (Esquirol et al. 2018), *Rhodobacter* (Zhang et al. 2012), and *Sphingopyxis* (Chen et al. 2015). The presence of these bacterial taxa in atrazine contaminated conditions might due to their intrinsic ability to cope with the chemical. For example, these bacteria present in atrazine-treated cultured might have acquired genes involved in atrazine metabolism through horizontal gene transfer. The atzABC genes which involved in atrazine metabolism were conserved, widely spread, and could be obtained by means of lateral gene transfer (De Souza et al. 1998; Devers et al. 2005; Marri et al. 2007; Ochman et al. 2000; Vos et al. 2015).

CONCLUSION

This is the first study that investigated the *Cladophora* microbiome in Thailand. We observed that different atrazine concentrations affected the algal microbiomes both in their taxonomic composition and the species richness. Comparisons between microbiomes of *Cladophora* present in Thailand and the U.S.A indicated that some bacteria, i.e. *Acidobacteria*, *Actinobacteria*, *Armatimonadetes*, *Chloroflexi*, *Cyanobacteria*, *Deinococcus-Thermus*, *Epsilonbacteraeota*, *Nitrospirae*, *Patescibacteria*, *Planctomycetes*, *Proteobacteria*, and WPS-2 were present in all *Cladophora* samples suggesting that these bacteria might be crucial for *Cladophora* growth and survival. They might represent the core *Cladophora* microbiome that could result in the algal successful worldwide distribution.

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