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

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

ANCHITTHA SATJARAK*, JITTRA PIAPUKIEW, WIKROM CHANTHAPATCHOT, KARNJANA RUEN-PHAM & ALISA S. VANGNAI

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

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 in 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; Varga 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 affects 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, 100.514° 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 MrBayes 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.

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.
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, Epsilonbacteriaeota, 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 Caldicilinaeae (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, Epsilonbacteriaeota, 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 Caldicilinaeae (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, Sphingoaaurantiacuis, 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 Scytomma) 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 blastocellata, 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.
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, Epsilonbacteria, Nitrospirae, Patescibacteria, Planctomycetes, Proteobacteria, and WPS-2), we found some bacterial phyla present in common with the *Cladophora* microbiome previously reported from the north of U.S.A. (Braus et al. 2017; Graham et al. 2015; Zulkifly et al. 2012). These included Acidobacteria, Actinobacteria, Armatimonadetes, Cyanobacteria, Deinococcus-Thermus, Epsilonbacteria, Nitrospirae, Planctomycetes, Proteobacteria, and WPS-2. Some bacterial phyla were not 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 IMCC cyanobium IMCC-6307 and Synechococcus CC9902, Planctomycetes planctomicrobium and AKYG587, and Proteobacteria azospirillum, Marivita, Novosphingobium, Pseudaminobacter, Sphingoaureantacius, 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, Planoglobratella, Pleurocapsa, Schizothrix, Scytomena, 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 (Töth 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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