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Abstract Monospecific microalgal dominance in nutrient-rich environments is usually attributed to exclusive resource competition or grazing selectivity. However, allelopathic interactions within microalgal communities may reinforce established dominance and limit species replacement. While many allelopathic interaction studies among autotrophic microorganisms focused on aquatic free-living species, scarce knowledge exists on microalgae aquatic biofilms, although chemical communication between species or communities is favored due to spatial proximity. We thus developed and applied a co-culture protocol using a disc diffusion method to evidence the inhibitory potential of biofilms of the marine diatom *Cylindrotheca closterium* growing in non-axenic conditions on different targeted microalgal strains. The biofilm exhibited an inhibitory effect on the individual growth of two microalgal strains, *Nannochloropsis oculata* and *Tetraselmis striata*, but did not affect the microalga *Porphyridium purpureum*. Bacteria isolated from the inhibition zone did not show any inhibitory activity on targeted microalgae. This suggests that the growth inhibition and cell deformation observed for the two sensitive microalgal strains may result from compounds produced during the growth of *C. closterium* biofilm or from associated bacteria identified but not isolated in the present study. Therefore, further investigations are required to identify the compounds and their microbial producers involved in the observed inhibition.

Keywords Microalgae, Allelopathic interactions, Biofilm, Bacteria, Microbial co-culture

Introduction

The dominance of microalgal species in a nutrient-rich environment may be explained by exclusive resource competition or grazing selectivity (Vallina et al. 2014). However, interactions within algal communities may also occur by allelopathy (Legrand et al. 2003), defined as a chemical communication involved in inter-species competition, where one species prevents other organisms from using available resources by producing effective molecules. Distinguishing competition from allelopathy under natural conditions remains difficult (Inderjit and del Moral 1997). If competition is mainly characterized by the species intrinsic ability for resource acquirement, the allelopathic effects depend on the sensitivity and physiological status of the targeted organism, the distance between the emitter and the target, and the concentration of produced molecules (Leflaive and Ten-Hage 2007). Therefore, allelopathic interactions are particularly expected in aquatic biofilms because organisms are physically close (Allen et al. 2016). When lipophilic, allelochemical compounds can readily diffuse through extracellular polymeric substances constituting biofilm matrix, or direct cell-to-cell contact, reducing their dilution. Their concentration can therefore be significant in the biofilm while being less detectable in the surrounding water (Allen et al. 2016, Uchida 2001). Allelopathic compounds may be produced to target planktonic organisms. In this case, these molecules must be sufficiently concentrated to affect targets over greater distances in a diluted aquatic environment (Gross 2003).

Diatoms are species known for the production of inhibitory compounds, such as polyunsaturated fatty acids (PUFAs), polyunsaturated aldehydes (PUAs), polyphenolic and halogenated compounds that have been proven to negatively impact other microalgae (Pichierri et al. 2017; Wenjing et al. 2019). The marine diatom *Cylindrotheca closterium*, living both as free-floating cells or in biofilm, displays allelopathy abilities when cultured in planktonic form (Wenjing et al. 2019). However, the allelopathy ability of *C. closterium* when growing in biofilm is unknown.

Most studies investigating microalgal *vs* microalgal allelopathy used non-axenic cultures and filtered growth medium dissolved fractions, without considering the ability of bacteria to produce inhibitory or algicidal compounds in the biofilm (Coyne et al. 2022), and some of the allelopathic compounds required to be continuously produced to be effective (Flores et al. 2012). The inhibitory activity of microalgal biofilms against potential competitors may be a valuable trait when cultivating commercially relevant microalgal species. Attached culture systems, an emerging technology replacing suspended culture systems, facilitate biomass growth as a biofilm on a support continually rotating between liquid and gaseous phases (Morales et al. 2020). Recent works highlighted the reduction of

environmental impact of such new production systems (Morales et al. 2020, Penaranda et al. 2023). The long-term growth of microalgae in attached culture systems showing very little microalgal contaminations during growing periods (F. Guiheneuf, personal communication), suggests potential allelopathy ability.

We therefore hypothesized that microalgae and associated bacteria established in biofilm under favorable growth conditions may have the ability to inhibit potential competitors such as allochthonous microalgae. To test this hypothesis, we examined the inhibitory potential of the diatom *Cylindrotheca closterium*‘s biofilm when cultivated using this attached culture system under laboratory conditions. Inhibition tests were performed using experimental *C. closterium* biofilm co-cultures from a modified version of the disc diffusion method (Hudzicki 2009) against three selected microalgae targets. Two of the studied targets are pelagic and marine microalgae, *Tetraselmis striata* and *Nannochloropsis oculata*, from the Chlorophyta and Ochrophyta family, respectively. The third studied target was a benthic microalga of marine origin, the red microalga, *Porphyridium purpureum* from the Rhodophyta family. These microalgae were selected for inhibition tests because they can grow on biofilm, and they belong to genera that are susceptible to be inhibited through allelochemicals such as *Tetraselmis* and *Nannochloropsis* (Khairy 2010, Chaïb et al. 2021) or less sensitive to allelochemicals such as *Porphyridium purpureum* (Hellio et al. 2002). In addition, those strains belong to different taxonomic divisions, promoting a broad representation of biological diversity, as well as a diversity of size, morphology, and pigments.

Materials and methods

Microalgae strains

The benthic diatom *Cylindrotheca closterium* AC170 was obtained from the Algobank Caen Collection (Université de Caen Basse-Normandie, France) and used as a potential emitter. Three marine non-axenic microalgal strains from three algal lineages were chosen as targets, for a broader representation of biological diversity, as well as diversity in size, morphology,

and physiology. The selected strains were: i) the planktonic chlorophyte *Tetraselmis striata* isolated and identified in 2018 from a high-rate algal pond located in Palavas-les-flots (Southern France, Pascon et al. 2021), ii) the planktonic ochrophyte *Nannochloropsis oculata* (CCAP 847/9) and iii) the benthic rhodophyte *Porphyridium purpureum* (CCAP 1380).

Culture conditions

Before experimentations, the four microalgal strains were cultured in planktonic mode using a modified f/2 medium (Guillard and Ryther 1962, Guillard 1975, Supl. Table 1), prepared with Instant Ocean (Aquarium Systems) adjusted to achieve a salinity of 40 $g.L^{-1}$. All cultures were incubated at 20 °C under continuous white light with a light intensity of 85 µmol photons $m^{-2}.s^{-1}$.

Cylindrotheca closterium was cultivated as a biofilm using a small rotating experimental culture system (Figure 1) under controlled laboratory conditions (controlled temperature of 20 °C and light of 80-90 µmol photons.$m^{-2}.s^{-1}$) with weekly sterile culture medium addition (modified f/2 medium with twice the concentration in nitrogen, phosphate and silicate, using synthetic sea salts Instant Ocean adjusted to reach a salinity of 40 $g.L^{-1}$). A plastic tank (L35 cm x W25 cm x H15 cm) was used and contained a roller with a length of 30 cm and a diameter of 10 cm, with a rotating bar allowing for tangential velocity ranging from 0.02 to 0.07 $m.s^{-1}$. A textile fabric was autoclaved and placed on the roller using zip ties. On March 10th 2023, the fabric was moistened on the roller with a sterile culture medium for one day and inoculated by evenly pouring onto the fabric the planktonic culture of *C. closterium* at 5.7 rotations per minute (rpm) under a light intensity of 90 µmol photons $m^{-2}.s^{-1}$. The plastic tank was then half-filled with 3.6 L of sterile culture medium, ensuring that ¾ of the roller was exposed to the air while ¼ was immerged in the culture medium. After 10 days and visual check of signs of biofilm establishment, the rotation speed was increased to 15 rpm and the light intensity was raised to 100 µmol photons $m^{-2}.s^{-1}$ in order to ensure higher access to light and nutrients without risking biofilm detachment. Once the biofilm onto the fabric reached maturity after three weeks (characterized by a dark brown colour, uniformity, and opacity, see Fig. 1A), it was collected by scraping the entire fabric. After the first harvest from the fabric, the rotation speed was increased to 37 rpm, equivalent to a linear speed of 0.2 m s-1 that is the optimal speed before inducing too much shearing and friction forces that will lead to cell detachment (F. Guiheneuf, personal communication). The entire biofilm was harvested every three weeks. The biofilm that developed at the bottom of the tank was

harvested every week before fresh culture medium renewal (Figure 1A). The harvested one-week biofilm at the bottom of the tank after 14 days of experiment (March 24th 2023, hereafter referred to as 14T) and after 131 days of experiment (July 19th 2023, 131T) and the harvested three-week biofilm from the fabric on the roller after 131 days of experiment (131R) were tested for potential inhibition on targeted microalgae strains.

Inhibition tests using *Cylindrotheca closterium* biofilm on targeted microalgae

Inhibition tests were developed based on the paper disc antibiotic sensitivity testing design, to visualize the formation of an inhibition halo against a microalgal target spread on a Petri dish, from a disc on which *C. closterium* biofilm was deposited. To get homogeneous targeted microalgal mats, and for each of the targeted microalga, a sterile cotton swab with 6 mm cotton wool tip was dipped into the planktonic microalgae culture in the exponential growth phase and streaked onto the 1.5% agar-containing modified f/2 medium by making tight streaks. For each targeted species, one such Petri dish was used as a control and three others for the inhibition tests. Sterile cellulosic discs were then arranged on the agar surface. One disc was inoculated with 20 µL of positive control (35% hydrogen peroxide), another disc with 20 µL of negative control (sterile modified f/2 medium), a third disc was inoculated with the *C. closterium* biofilm and two other discs were inoculated after centrifuging the *C. closterium* biofilm at 4500 g for 10 minutes at 20 °C. A volume of 20 µL was deposited on each disc with the respective pellet and raw supernatant of *C. closterium*. Centrifugation intended to separate the particulate from the dissolved fractions of the biofilm. Filtration at 0.2 µm using polycarbonate filter was also performed from *C. closterium* biofilm on roller fabric. The one-week biofilm of *C. closterium* that was developing at the bottom of the tank was sampled after 14 days and after 131 days of experiment, while the three-week *C. closterium* biofilm formed on the roller fabric was scraped after 131 days. After placing the two controls and biofilms onto the cellulosic discs, the Petri dishes were all incubated in duplicates for 15 days at 20 °C under continuous white light at an intensity of 85 µmol photons.m^{-2}.s^{-1}. After 15 days of incubation, when present, the diameters of the inhibition zones were measured and observed under the inverted microscope to evaluate the physiological condition of the cells. Statistical analyses comparing means were conducted using a Mann-Whitney test on cell dimension measurements, with a significance level set at a *p*-value of 0.05.

Bacteria isolates from inhibition zones

The potential role of bacteria in the inhibition activity was investigated. When inhibition zones around cellulosic discs were observed, samples were taken with an oese to isolate and identify the bacterial strains from these zones and to perform additional inhibition tests on targeted microalgae. Bacteria were isolated using serial dilutions (1/10, 1/100 and 1/1000), and then spread on Zobell agar medium to isolate colonies. The Petri dishes were incubated for eight days at 20 °C in the dark. Dilutions (1/10 or 1/100) that allowed the maximum number of identifiable bacterial colonies were preserved. Morphologically distinct colonies were isolated on new duplicated plates and incubated at 20°C in the dark until obtaining distinct and separate colonies (between 48 hours and 120 hours, depending on the bacterial strain). Individual colonies were then sub-cultured onto a new plate and incubated under the same conditions at least two more times to ensure strain purity.

The identification of isolated bacterial strains was carried out using a Sanger sequencing approach. A heat shock was performed on the bacterial cultures frozen at -20 °C, to a temperature of 95 °C for 15 minutes to access their DNA. Amplification of the 16S rRNA gene of each bacterial colony was then performed using universal primers: forward 8F (5'-AGAGTTTGATCCTGGCTCAG-3'; Edwards *et al.* 1989) and reverse 1492R (5'-CGGTTACCTTGTTACGACTT-3'; Stackebrandt and Liesack, 1993). A reaction mixture was prepared with: 4 µL of GoTaq buffer 1X, 0.4 µL dNTP at a final concentration of 0.2 mM each, 1 µL of each primer at a final concentration of 0.5 µM, 0.1 µL of GoTaq DNA polymerase 1.25 U (Promega, city), 12.5 µL of molecular-grade water, and 1 µL of bacterial DNA for a final volume of 20 µL per reaction. The PCR reaction was carried out according to the following program: initial denaturation for 2 minutes at 95 °C, 30 cycles of 30 seconds at 95 °C for denaturation, 45 seconds at 55 °C for hybridization, and 1 minute at 72 °C for extension, followed by a final extension step of 4 minutes at 72 °C. To validate the amplification of the 16S rRNA gene, an agarose gel of 0.9% in 1X Tris Acetate EDTA (TAE) buffer was prepared in 50 ml, and 2 µL of a DNA intercalating dye (Biotium, Gelred, 1/4 concentration) were added. The gel was placed in an electrophoresis chamber (Advance, Mupid-One) containing 1X TAE. Five microliters of each PCR product with 1 µL of 10X loading buffer (Invitrogen, Bluejuice) were loaded into the wells and allowed to migrate for 30 minutes at 100 volts. The gels were then observed under ultraviolet light in a gel reader. When a band around 1.5 Kb was visible, which corresponds to the expected size for the amplification of 16S rRNA gene, 2 µL of Exosap-IT Express were added to 5 µL of each PCR product to purify them and retain only the amplicons (removal of primers and excess dNTP). The mixture was incubated for 4 minutes at 37 °C to allow enzyme treatment,

followed by 1 minute at 80 °C to inactivate it. The DNA concentration of each purified PCR product was measured by spectrophotometry using a Nanodrop (ND-1000 spectrophotometer). A dilution was then performed to obtain a DNA concentration of 10 $ng.\mu L^{-1}$. Five microliters of the 8F primer at a concentration of 5 $pmol.\mu L^{-1}$ were added to the purified amplicons before sending them to Eurofins Genomics for sequencing of the first half of the 16S rDNA. Once the amplicons were sequenced, the quality of the obtained electropherograms was observed using the Bioedit software version (Hall, 1999). Sequences were compared to the GenBank database of the National Center for Biotechnology Information (NCBI) using the BLAST search program to establish a preliminary determination of their taxonomy. For each sequence, all hits with a sequence coverage greater than 99%, a sequence identity percentage greater than 97%, and an E-value of 0 were retained. The isolated strain *Alteromonas macleodii*, isolated from the inhibition zone obtained using centrifuged *T. striata* biofilm supernatant (see Table 2), was then tested at different cell concentrations (10^6, 10^7, 10^8 $cell.ml^{-1}$) against targeted microalgae strains to check for potential growth inhibition using the same diffusion test protocol as described in the previous paragraph.

Identification of the bacterial community in the biofilm

To identify the bacterial community from the biofilm of the experimental culture system, we further used a metabarcoding approach on the samples from the bottom of the tank on March 24th (14T) and July 19th 2023 (131T), and from the roller fabric on the same date (131R). DNA was extracted from the 3 biofilm samples using the DNeasy PowerBiofilm Kit (Qiagen). The V3-V4 variable region of the 16S rRNA gene was amplified using a primer set designed to avoid the amplification of chloroplastic 16S rRNA gene: S-D-Bact-0341-b-S-17: CCTACGGGNGGCWGCAG as forward and 799F_rc: CMGGGTATCTAATCCKGTT as reverse (Klindworth et al. 2013; Thomas et al. 2020). The PCR reaction mix was composed of 12,5µL of KAPA HiFi HotStart ReadyMix 2X (KapaBiosystems), 0.75 µL of each primer at a final concentration of 0.3 µM, 9 µL of molecular-grade water, and 2 µL of bacterial DNA (2ng uL-1) for a final volume of 25 µL per reaction. PCR were carried out using the following program: initial denaturation for 3 minutes at 95 °C, 10 cycles of [30 sec at 95 °C, 45 sec at 44 °C, and 90 sec at 68 °C], and 15 cycles of [20 sec at 94 °C, 45 sec at 50 °C, and 90 sec at 68 °C], followed by a final extension step of 4 minutes at 68 °C. Negative controls were used for the sampling, DNA extraction and PCR steps to control for potential contaminants. PCR were performed in triplicate and then pooled before metabarcoding.

Amplicons were then sent to the GeT-Biopuces platform for index addition, MiSeq Illumina sequencing (Run MiSeq 600 cycles V3) and sequence processing using the dada2 package (Callahan et al. 2016) in R (R Core Team, 2021). Sequence processing included removal of sequences with N, of low quality and of size below 100 bp, and filtration of the phiX sequences. It also included sequence dereplication, ASV formation and chimera removal. Taxonomic annotation was done using Silva version v138.1 (Quast et al. 2013) and GTDB r202 (Parks et al. 2022). Before further analyses, the ASV table was transformed by normalizing the number of sequences using median sequencing depth (Trefault et al. 2021).

Results

Microalgal inhibition from *Cylindrotheca closterium* biofilm

A zone of inhibition was observed from the one-week *C. closterium* biofilm collected at the bottom of the tank, on the microalgal targets *N. oculata* and *T. striata* (Figure 2). The percentages of inhibition relative to the positive control (hydrogen peroxide) for *N. oculata* and *T. striata* varied respectively between 65 to 158% and between 24 and 92%, according to the type of biofilm sample (i.e. pellet, supernatant, or whole) and days of experiment (Table 1). However, no zone of inhibition was observed for the target *P. purpureum* for the three types of biofilm sample. Zones of inhibition on the *N. oculata* and *T. striata* targets was also observed when *C. closterium* grown on fabric was used as emitter. The percentages of inhibition relative to the positive control for *N. oculata* and *T. striata* varied respectively between 16 to 39% and between 12 to 38%, according to the three types of biofilm sample (Table 1). These inhibition activities seemed to be lower than that measured using the bottom tank biofilm (Table 1). No inhibition was observed on these targeted algal strains when filtered (0.2 µm) centrifuged biofilm supernatant was used as an emitter.

Samples were taken from the diffusion tests in the inhibition zones and observed under an inverted microscope. Regarding the targeted microalgae *N. oculata*, we observed the presence of *C. closterium* cells in the samples taken from the inhibition zone of the paste, the pellet, and the supernatant. From the inhibition zones, different sizes of *N. oculata* cells were observed (Figure 3A) compared to *N. oculata* cultivated without any discs (control culture). In the inhibition zone, one population of *N. oculata* with cells significantly larger than the control culture (Mann Whitney, n=20, p-value < 0.05) was observed with an average size of 5.07 ± 0.09 µm, and a second population of *N. oculata* was also observed with cells

significantly smaller than the control culture (Mann Whitney, n=20, *p*-value < 0.05) with an average size of 1.76 ± 0.10 µm. In comparison, *N. oculata* population in the control culture had an average cell size of 2.90 ± 0.08 µm. In these inhibition zones, we did not observe a significant change (Mann Whitney, n=20, *p*-value > 0.05) in the length of *C. closterium* cells (50.9 ± 0.3 µm) in comparison to the *C. closterium* control, i.e. *C. Closterium* growing alone on Petri dish (51.6 ± 0.2 µm). However, we observed a significant decrease in the diameter of the *C. closterium* cells, with a diameter of 5.70 ± 0.10 µm for the control culture and 4.23 ± 0.11 µm for the samples taken from the inhibition zone (Mann Whitney, n=20, *p*-value < 0.05). Regarding the targeted microalgae *T. striata*, we also observed the presence of *C. closterium* in all the inhibition zones. Two types of *T. striata* cells were observed: one with an average size of 11.07 ± 0.16 µm, similar to the diameter of the control culture (10.90 ± 0.18 µm, Mann Whitney, n=20, *p*-value > 0.05), and a second type of cells with a significantly smaller average size of 4.60 ± 0.09 µm (Mann Whitney, n=20, *p*-value < 0.05). *T. striata* cells with a significantly smaller size than the control also showed membrane deformation (Figure 3B). As observed in the *N. oculata* inhibition zone, a change in the morphology of *C. closterium* cells in the *T. striata* inhibition zone was observed, with cells of diameter 3.80 ± 0.09 µm, significantly thinner than the control culture (Mann Whitney, n=20, *p*-value < 0.05). No change in cell size or number of *P. purpureum* was observed when growing in Petri dishes with or without *C. closterium* as emitter (Figure 3C)

Bacterial strains isolated from *Nannochloropsis oculata* and *Tetraselmis striata* inhibition zones

Six bacterial strains were identified using a Sanger approach, after isolation from the inhibition zones of *T. striata* and *N. oculata*, showing mainly small white colonies (Supplementary Table 1). In the inhibition zones of *N. oculata*, isolated bacteria were assigned to the genus *Alteromonas* sp. (sequence coverage = 100%, sequence identity = 98.96%), and to the species *Alteromonas macleodii* (s.c. = 100%, s.i. = 98.02%). In the inhibition zones of *T. striata*, isolated bacteria were identified as the species *Alteromonas macleodii* (s.c. = 98.09 and 96.86%, s.i. = 100 and 99% respectively), as well as the genus *Lacimonas* sp (s.c. = 96.97%, s.i. = 99%). The bacterial strain *A. macleodii* isolated from the inhibition zone of the centrifuged *T. striata* biofilm supernatant was tested as an allelopathic emitter against *N. oculata* and *T. striata*, but no inhibition zone was clearly observed.

The bacterial community associated with *C. closterium* biofilm

Sequences analysis in metabarcoding indicated that three samples were composed of the classes: *Alphaproteobacteria, Bacteroidia*, and *Gammaproteobacteria*. *Alphaproteobacteria* dominated all samples with 97.3%, 87.5%, and 69.9% sequences from the one-week biofilm collected at the bottom of the tank after 14 days (March 24th 2023, referenced as 14T) and 131 days of experiment (July 19th 2023, referenced as 131T), and from the three-week biofilm collected on the fabric of the roller after 131 days of experiment (July 19th 2023, referenced as 131R), respectively (Fig. 4, Suppl. Table 2). *Bacteroidia* and *Gammaproteobacteria* were similarly abundant in 14T with about 1% sequences. These two classes were also similarly abundant in 131T with about 6% sequences. *Gammaproteobacteria*, however, represented more sequences than *Bacteroidia* in 131R, with 26.1% and 3.9% sequences, respectively. Among *Alphaproteobacteria*, the genus *Roseinatronobacter* dominated the three samples with 80.7%, 56.4%, and 50.9% sequences for 14T, 131R and 131T. Other genera included *Marivita, Oceanicaulis, Salinarimonas,* and a *Rhodobacteraceae* ASV, with *Marivita* and *Oceanicaulis* similarly abundant in 131R with about 10% sequences, *Rhodobacteraceae* ASV more abundant than the two genera in 14T with 13.6% sequences and *Salinarimonas* less abundant with 0.9-2.6% sequences. In *Gammaproteobacteria, Marinobacter* dominated all samples with 1.3%, 4.5%, and 23.7% sequences in 14T, 131T, and 131R. Less abundant genera included *Congregibacter* and *Vibrio*, as well as *Alteromonas,* which was only present in 131T. Finally, within *Bacteroidia, Phaeodactylibacter* was the most abundant in the samples, with 0.8%, 4.6%, and 1.7% sequences respectively in 14T, 131T and 131R. Other *Bacteroidia* included *Muricauda* and the *Schleiferiaceae* UBA7878.

Discussion

Based on inhibition tests conducted using the biofilm of *Cylindrotheca closterium* as the emitter, the growth of the two targeted microalgae strains *Nannochloropsis oculata* and *Tetraselmis striata* was suppressed and resulted in change of cell size. This finding represents evidence of growth inhibition observed among microalgae cultivated in biofilm co-cultures. Xu et al. (2019) reported previously that the diatom *C. closterium* significantly reduced the growth of the dinoflagellate *Prorocentrum donghaiense* in planktonic cocultures. They showed that extracts derived from *C. closterium* culture filtrates affected the total chlorophyll content and therefore the efficiency of photosystem II of *P. donghaiense* cells. This led to the

inhibition of photosynthesis and to the reduction in growth rate of this dinoflagellate species. In the present study, we observed an effect of *C. closterium* biofilm on cell size of *N. oculata* as well as of *T. striata*, where cells exhibited altered morphology (cells with deformed membranes). Other studies have also reported morphological alterations due to an allelopathic effect. For example, Pichierri et al. (2017) observed *Ostreopsis* cf. *ovata* cells with abnormal morphologies when exposed to the individual acellular filtrate from two diatoms species (*Proschkinia complanatoides* and *Navicula* sp.), including membrane deformations and deleterious effects on the nucleus, resulting in chromatin dispersion in the cytoplasm. However, in the present study, no inhibition effect was observed from *C. closterium* the filtered biofilm supernatant at 0.2 µm, thus without bacteria or microalgae. This suggests that compounds leading to the inhibition or death and the cell deformation of targeted algal strains were highly labile or a physical contact is required between *C. closterium* biofilm and the targets. These compounds appeared to exhibit a limited bioactive duration, likely necessitating continuous production and release during diatom cell growth or direct contact with targeted cells to optimize their efficiency. The biofilm of *C. closterium* may have been able to produce metabolites, such as observed with other microalga (Zhao et al. 2019, Long et al. 2021) capable of altering chlorophyll pigment or cytoplasmic membranes, and thereby inhibiting photosystem II, which directly impacts photosynthesis functioning. This impact on photosynthesis would lead to modifications in the cell cycle, division, and growth of the targeted microalgae, and this would explain the appearance of various morphotypes. Furthermore, we also observed in our study a significant reduction of the diameter of *C. closterium* when growing in the inhibition areas, which was not observed in studies focused on this diatom. Nonetheless, we could hypothesize that the production of inhibitory molecules by the *C. closterium* biofilm may represent a substantial metabolic cost, leading to a reduction in the diatom's growth (Legrand et al. 2003, Allen et al. 2016).

In the present study, no inhibition was observed for *Porphyridium purpureum*, a benthic microalga. The discrimination of inhibitory compounds on the target could be related to the physiology of this microalgae that possesses sulfated polysaccharide cell wall exhibiting strong antioxidant activity (Tannin-Spitz et al. 2005). This ability may protect cells against the potential damaging effects of reactive oxygen species (ROS) triggering oxidative stress under the stimulation of allelochemicals. Indeed, antialgal substances from *Cylindrotheca closterium* have been suggested to provoke oxidative stress on the dinoflagellate *Prorocentrum donghaiense* (Xu et al. 2019).

The bacterial strain *Alteromonas macleodii* isolated from the inhibition zone of centrifuged *Tetraselmis striata* supernatant did not show any significant antialgal activity. As *Cylindrotheca*'s biofilm and the cultures from the targeted microalgae *Tetraselmis striata* were not axenic, the isolated bacterial strains could come from either of them. In the case where *A. macleodii* came from the biofilm, it was not involved in the antialgal activity while in the case where it came from *Tetraselmis striata* culture, it was likely living in association with the algae. Like several *Gammaproteobacteria*, bacteria belonging to the genus *Alteromonas* are often found living in association with microalgae (Grossart et al. 2005, Sapp et al. 2007), such as *N. oculata* (Chernikova et al. 2020) or as competitors of *T. suecica* for nutrients (Biondi et al. 2017). The bacterial community survey of the biofilm from the tank and the roller indicated that *Alteromonas* was present in only one sample and in low sequence numbers. *Alteromonas macleodii* may form short-lived 'blooms' of active cells that are missed using traditional single-point sampling (Wietz et al. 2022). Another hypothesis could be that the isolated strain came from one of the targeted cultures instead of the *C. closterium* biofilm. The bacterial community from the one-week biofilms of the tank and the three-week biofilm of the roller fabric also contained a large majority of *Alphaproteobacteria* with the genus *Roseinatronobacter* dominating in all samples. Bacteria from the genus *Roseinatronobacter* are facultatively phototrophic purple bacteria, found in environments characterized as extreme such as alkaliphilic hypersaline lakes and are highly tolerant to large environmental gradient variations (Boldareva et al. 2007, Wilson et al. 2012, Trutschel et al. 2023, Cubillos et al. 2024). Such large variations likely happen in our rotating system where bacteria are impacted in the biofilm by varying humidity conditions and salt concentrations due to evaporation. The other *Alphaproteobacteria* genera, *Marivita*, *Oceanicaulis*, and *Salinarimonas* have previously been observed associated with microalgal planktonic cultures or in algal biofilms and are believed to play a role in nutrient transformation and availability to the algae (Zhou et al. 2021, Lukwambe et al. 2023, Li et al. 2019, Cole et al. 2014, Strömpl et al. 2003). For instance, *Salinarimonas* has been shown to promote algal growth in the biofilm (Xiao et al. 2023). Within *Gammaproteobacteria*, *Marinobacter* has been identified in association with the phycosphere or the biofilm of microalgae, likely beneficiating from carbon source exudates and its cells have been shown to be chemically attracted by the diatom *Thalassiosira weissflogii* (Giraldo et al. 2019, Pinto et al. 2021, Sonnenschein et al. 2012). Within *Bacteroidia*, *Muricauda*, is a genus regularly found in biofilms and thought to be, as well as the genus *Marinobacter*, amongst the primary colonizers, and bacteria from this genus have been shown to play a role in cell aggregation of the microalgae *Nannochloropsis*

oceanica (Abed et al. 2019, Wang et al. 2012). *Phaeodactylibacter* bacteria are heterotrophic denitrifiers and salt tolerant, they are found in biofilms and associated with microalgae such as the diatom *Phaeodactylum tricornutum* (Chen et al. 2014, Li et al. 2021, Wang et al. 2020, Fan et al. 2023). Overall, the bacterial community associated with *C. closterium* biofilm contains genera that could play various roles such as making complex molecules available to the algae, cell aggregation or with various osmotic properties. Although the main bacterial strain isolated from the inhibition areas did not participate directly in the targeted microalgae inhibition, bacteria closely associated to *C. closterium* biofilm could have a role in the observed inhibition activity. Further analyses are needed to define the allelopathic capabilities of *C. closterium* and its microbial community in the biofilm. Additional experiments may include omics tools such as metabolomics or metatranscriptomics to identify allelochemical compounds and the metabolic pathways of the biofilm microbiota involved in algal growth inhibition. Additionally, a wider spectrum of algal targets may be tested to identify the potential allelopathic specificity of the *C. closterium* biofilm.

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Competing interests

The authors declare no conflict of interest.

Availability of data

Partial 16S rRNA gene sequences were deposited in GenBank under the Accession number PP551695-PP551700. Raw 16S rRNA gene sequences and sample metadata are available in the SRA under BioProject ID PRJNA1098477.

Authors' contributions

Conceptualization: Emilie Le Floc'h, Eric Fouilland; Methodology: Lucie Sanchez, Amandine Avouac, Emilie Le Floc'h, Christine Félix, Christophe Leboulanger, Angélique Gobet ; Formal analysis and investigation: Lucie Sanchez, Emilie Le Floc'h, Christine Félix, Angélique Gobet ; Validation: Lucie Sanchez, Emilie Le Floc'h, Christine Félix, Eric Fouilland ; Visualization : Lucie Sanchez, Emilie Le Floc'h, Angélique Gobet, Eric Fouilland ; Writing-original draft preparation: Lucie Sanchez, Eric Fouilland ; Writing-review and editing: Lucie Sanchez, Amandine Avouac, Emilie Le Floc'h, Christine Félix,

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Tables

Table 1: Results obtained of the inhibition test conducted after 15 days of incubation under continuous light with *Nannochloropsis oculata, Tetraselmis striata* and *Porphyridium purpureum* as the targeted microalgae strain and *Cyclindrotheca closterium* in biofilm (harvested from the bottom of the tank or from the fabric of the roller) acting as the emitter when (i) the whole biofilm, (ii) the centrifuged biofilm pellet, and (iii) the centrifuged biofilm supernatant filtered or not, incubated after 14 and 131 days of experiment using the rotoating system. Results are expressed as the percentage (%) of the inhibition zone measured around the diffusion discs with the emitter relative to the inhibition zone measured with hydrogen peroxide as the positive control. Mean and range from replicates (n=2) are reported.

		One-week old biofilm from the bottom of the tank			Three-week old biofilm from the roller fabric			
Days of Experiment	Target species	Whole biofilm	centrifuged biofilm pellet	centrifuged biofilm supernatant	Whole biofilm	centrifuged biofilm pellet	centrifuged biofilm supernatant	filtered centrifuged biofilm supernatant
14 days (March 24th 2023)	*N. oculata*	150 (±16)	91 (±60)	158 (±43)	not tested	not tested	not tested	not tested
	T. striata	33 (±5)	24 (±10)	33 (±2)	not tested	not tested	not tested	not tested
	P. purpureum	0	0	0	not tested	not tested	not tested	not tested
131 days (July 19th 2023)	*N. oculata*	65 (±21)	not tested	not tested	39 (±10)	16 (±4)	26 (±26)	0
	T. striata	92 (±19)	not tested	not tested	12 (±6)	18 (±18)	38 (±14)	0
	P. purpureum	0	not tested	not tested	0	0	0	0

Table 2. Identification of bacterial colonies isolated from inhibition zones of the targeted microalgae around the biofilm type, from the one-week biofilm growing at bottom of the tank collected on March 19th 2023. Details on sequence taxonomic identification are available in Supplementary Table 2.

Inhibition zone from the targeted microalga		Putative bacterial species	16S rDNA sequence length (bp)	Sequence coverage (%)	Sequence identity (%)	Accession number of the best hit
N. oculata	centrifuged biofilm supernant	*Alteromonas macleodii*	858	100%	98.02%	MT325885.1
	whole biofilm	*Alteromonas* sp.	1094	100%	98.17%	MT210817.1
	whole biofilm	*Alteromonas* sp.	772	100%	98.96%	MF599947.1
T. striata	centrifuged biofilm supernant	*Alteromonas macleodii*	941	100%	98.09%	MT325885.1
	whole biofilm	*Alteromonas macleodii*	834	99%	96.86%	HM584088.1
	whole biofilm	*Lacimonas* sp.	860	99%	96.97%	MK493541.1

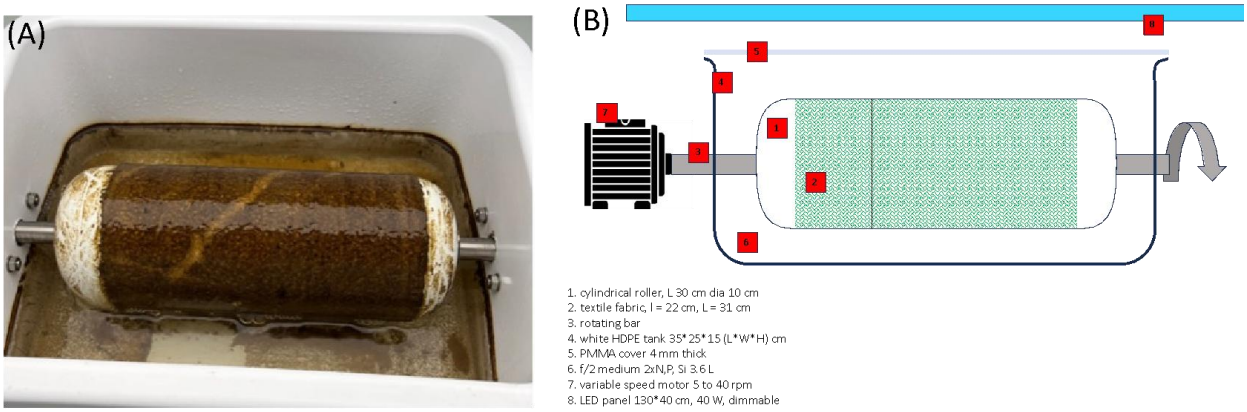

Figure 1: *Cylindrotheca closterium* growing as biofilm (A) using an experimental rotating culture system described in (B)

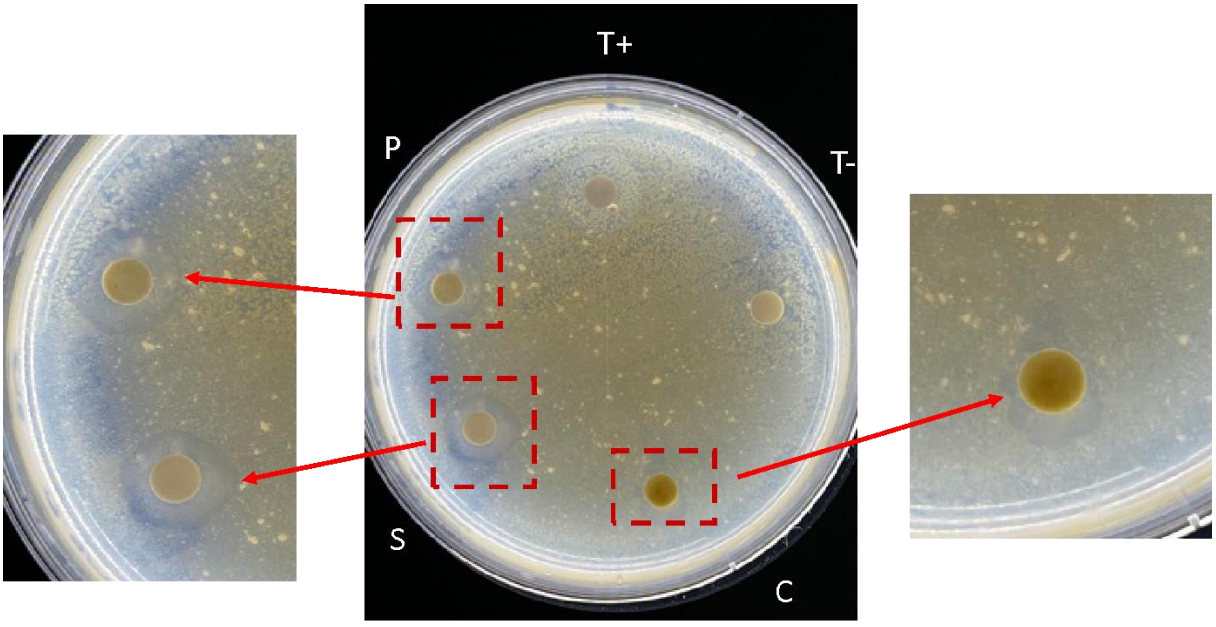

Figure 2: Result of one inhibition test using *Cylindrotheca closterium* one-week biofilm from the bottom of the tank (March 19th 2023) against the target *Nannochloropsis oculata*. T+: hydrogen peroxide, T-: sterile modified f/2 medium, P: paste from the one-week biofilm at the bottom of the tank, C: pellet obtained from centrifugation of the biofilm, and S: supernatant obtained from the centrifugation of the biofilm. Inhibition zones were observed after 15 days of incubation at 20 °C under continuous neutral light at an intensity of 80-90 µmol photons.$m^{-2}.s^{-1}$.

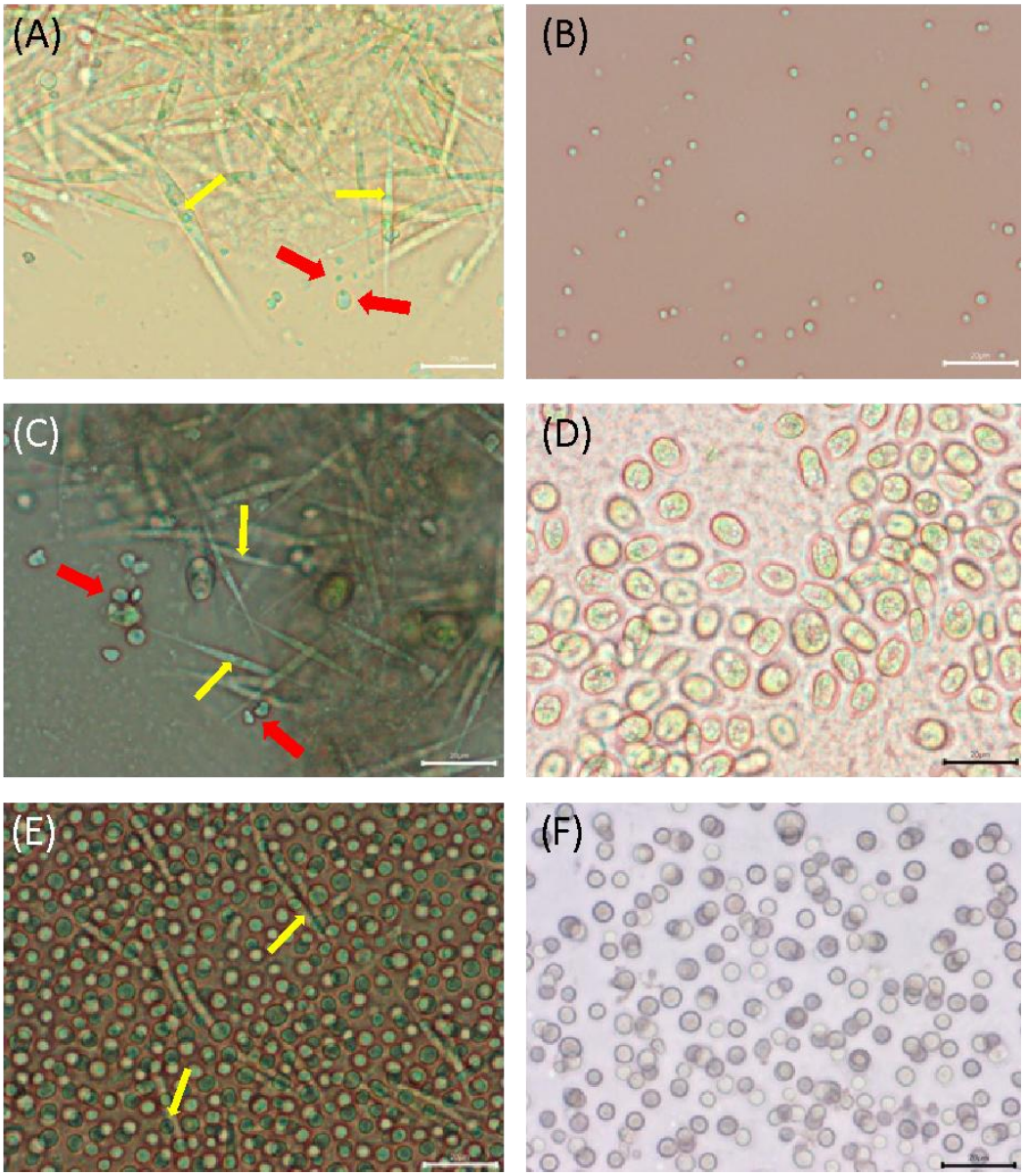

Figure 3: Observation under an inverted microscope of (A) *N. oculata* sampled from the inhibition zone with the one-week centrifuged *C. closterium* biofilm supernatant collected from the bottom of the tank (March 24th 2023) as the emitter, (B) *N. oculata* sampled from control cultures in Petri dish with no emitters, (C) *T. striata* sampled from the inhibition zone with the one-week centrifuged *C. closterium* biofilm supernatant collected from the bottom of the tank (March 24th 2023) as the emitter, (D) *T. striata* sampled from control cultures in Petri dish with no emitters, (E) *P. purpureum* sampled from the inhibition zone with the one-week centrifuged *C. closterium* biofilm supernatant collected from the bottom of the tank (March 24th 2023) as the emitter, (F) *P. purpureum* sampled from control culture in Petri dish with no emitters. Deformed cells are indicated by red arrows. *C. closterium* cells are indicated by yellow arrows.

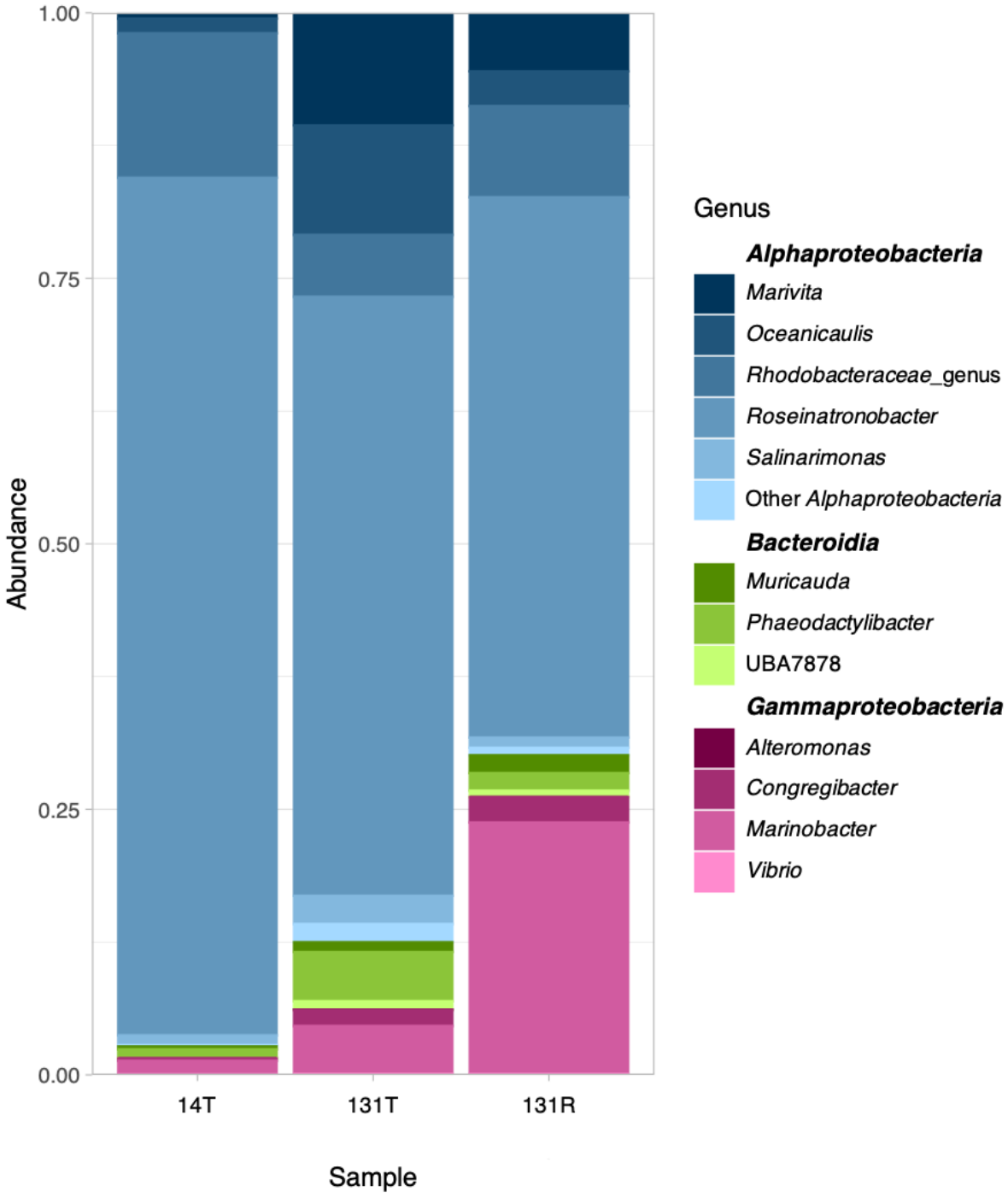

Figure 4 : Taxonomic composition of the bacterial community at the genus level, classified at the class level, in the 3 samples. Other *Alphaproteobacteria*, *Alphaproteobacteria* genera with less sequences than the five most abundant genera in the dataset. (14T: One-week biofilm collected from the bottom of the tank after 14 days of experiment (March 24th 2023), 131T: One-week biofilm collected from the bottom of the tank after 131 days of experiment (July 19th 2023), 131R: One-week biofilm collected from the fabric of the roller after 131 days of experiment (July 24th 2023). Details on sequence taxonomic identification are available in Supplementary Table 3.