TEP production under oxidative stress of the picocyanobacterium *Synechococcus*

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ABSTRACT

Transparent exopolymer particles (TEP) are mainly acidic polysaccharides directly or indirectly formed by phytoplankton and bacteria. These particles are often colonized by picoplankton and considered a hot spot for microbial activity. Recent studies suggested an important role of *Synechococcus* in TEP production found in lakes and prompted us to further investigate this issue using monoclonal xenic cultures of *Synechococcus*. We tested TEP production under oxidative stress in two treatments, one with hydrogen peroxide and another treated with ultraviolet radiation (UVR) and high photosynthetic active radiation (PAR), compared with an unstressed control. Our results showed a cell-normalized TEP production, ranging from 12 to 238 ng C cell^{-1} among strains, not only under stress but also in the control with non-limiting nutrients. Our data prove that freshwater communities of *Synechococcus* and their associated heterotrophic microflora, are capable of producing TEP even during growth phase. The oxidative stress induced extra production of TEP up to 400 ng C cell^{-1} in one of our phycocyanin-type (PC) strain. The phycoerythrin-type (PE) strains increased TEP production, particularly under UV-PAR stress, whereas the PC strains did it under H_2O_2 stress. This study provides new perspectives on the potential role of freshwater *Synechococcus* in TEP production.

INTRODUCTION

Transparent exopolymer particles (TEP) are predominantly composed of acidic polysaccharides forming sticky gel-particles, stainable with Alcian blue (Alldredge *et al.,* 1993; Passow *et al.,* 2001). TEP are present in oceans and inland waters and have an important role in the food webs, altering the size of the single cells by aggregating autotrophic and heterotrophic cells and increasing the sedimentation properties of the particle itself (Burd and Jackson, 2009; Deng *et al.,* 2015). Phytoplankton and picoplankton are able to release TEP-precursor or directly form TEP, mainly after a bloom in a period of senescence or decline of the population (Passow, 2002a; Grossart *et al.,* 2006; Berman-Frank *et al.,* 2007).

The mechanism of TEP production is complex: algae and picoplankton (autotrophic and heterotrophic) produce polysaccharide fibrils, which can be transformed into particles through abiotic processes like coagulation or gelation (Meng *et al.,* 2013, Jackson, 1995). TEP have a similar composition as extracellular polymeric substances (EPS), containing polysaccharides, proteins, lipids, and amino acids (Passow, 2002b). Nevertheless, TEP differ from EPS as they are present in the aquatic environment as discrete particles and not as cell-surface attached or dissolved molecules like EPS (Meng *et al.,* 2013).

TEP are considered a "hot spot" for microbial activity and are usually colonized by algae, bacteria and picocyanobacteria, that remain attached to these sticky particles (Azam and Malfatti, 2007). Many studies have been performed using filamentous cyanobacteria *Trichodesmium* spp. (Berman-Frank *et al.,* 2004), and marine diatoms (Rooney-Varga *et al.,* 2005; Grossart *et al.,* 2006; Gärdes *et al.,* 2011) to understand the importance of algae-bacteria interaction in TEP production and aggregation. Contrasting results were observed in different marine diatoms: the presence of bacteria in a *Thalassiosira rotula* culture promoted TEP production, conversely TEP substantially increased also in the axenic *Skeletonema costatum* culture (Grossart *et al.,* 2006). The different effects of attached or detached bacteria to *Thalassiosira weissflogii* were considered important to explain such contrasting results, which may depend on the changes in bacterial community composition (Gärdes *et al.,* 2011). Analogous to diatoms, *Microcystis aeruginosa* reacted

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differently in terms of TEP production if bacteria were or not present under high nitrogen concentrations (Pannard *et al.,* 2016). With unlimited nitrogen, TEP production by *M. aeruginosa* was higher in the presence of associated bacteria than in the axenic culture. This phenomenon only occurred in the exponential growth phase, but not in the stationary phase. These results indicate that the presence of associated bacteria may have a role in TEP production, but not under stressing conditions such as nitrogen limitation (Pannard *et al.,* 2016).

Only a few studies have considered the production of TEP by *Synechococcus* spp. which, contrary to the colonial cyanobacterium *Microcystis* spp., is present in nature in its single-cell form (Callieri *et al.,* 2012). The genus *Synechococcus* is composed by unicellular cyanobacteria that are abundant in diverse aquatic ecosystems and its plasticity is the base of its success on Earth (Callieri, 2017). It is a polyphyletic genus, *i.e.* composed by different clades without a common ancestor (Shih *et al.,* 2013) and only recently a reclassification scheme has been proposed (Walter *et al.,* 2017). This autotrophic prokaryote has accessory antenna pigments allowing the organism to adapt to different light conditions (Vörös *et al.,* 1998; Stomp *et al.,* 2007). They can be divided into two cell types differently sensitive to ultraviolet radiation (UVR) and high photosynthetic active radiation (PAR): phycocyanin-type (PC) and phycoerythrin-type (PE) with different light-harvesting pigments (Callieri, 2017).

Both marine and freshwater *Synechococcus* strains can potentially form microcolonies under stressing abiotic (UVR, high PAR, nutrient limitation) or biotic (grazing, viral attack) conditions (Passoni and Callieri, 2000; Callieri *et al.,* 2016; Huber *et al.,* 2017). Microcolony formation can be considered as a successful reaction to a stress event by *Synechococcus* spp. (Callieri *et al.,* 2012; Izaguirre *et al.,* 2015). The importance of UVR as stressing agent on a microorganism as *Synechococcus,* ubiquitous in the globe, is due to the increase of UVR in the ongoing climatic change. The formation of microcolonies by *Synechococcus* as induced by UVR was proven to vary in PE or PC strains depending on the different pigments they harboured (Callieri *et al.,* 2011). Whatever is the cause of microcolony formation, the mechanisms triggering the cell adhesion are related to the presence of TEP/EPS.

Deng *et al.* (2016) studied the aggregate formation and TEP production in an axenic culture of *Synechococcus* WH8102 of the 5.1A marine clade (Scanlan, 2012). They found that cell-normalized TEP concentration was higher in nitrogen-limited cultures and had an important function in aggregate formation. On the other hand, the relation between TEP formation and the effect of UVR and high PAR is not well known in *Synecchococcus* spp.. One of the effects of UVR is the induced production of reactive oxidative species (ROS) inside the cell, also at the phycobilisome level, when the electron transport exceeds the capacity to use the electron to fix CO_2 (Latifi *et al.,* 2008). One of the most common ROS, ubiquitous in natural waters at concentration <1 µM, is hydrogen peroxide (H_2O_2). Cell membranes are not so permeable to H_2O_2 as previously thought and its permeability depends on lipid composition and presence of enzymes such as catalase and glutathione peroxidase (Antunes and Cadenas, 2000). Therefore, the effect of H_2O_2 directly produced inside the cells as a reaction to harmful irradiance could be different from the effect of hydrogen peroxide directly added in the culture. A recent study showed that *Synecchococcus elongatus,* grown in batch cultures and exposed to different concentrations of hydrogen peroxide, undergoes a physiological stress that significantly enhances TEP production (Thornton and Chen, 2016).

Here, we planned a research to enlarge the experimentation on TEP production by using different *Synechococcus* strains. We tested the effect of UVR and PAR irradiation and the addition of H_2O_2 on TEP formation in 5 freshwater xenic *Synechococcus* monoclonal strains, four of which were already sequenced for their genome (Sanchez-Baracaldo *et al.,* 2019; Di Cesare *et al.,* 2018). The strains belong to different clades in the 16S rRNA phylogenetic tree (Callieri *et al.,* 2013) and in particular affiliate inside the sub-cluster 5.2 by phylogenomic analysis (Cabello-Yeves *et al.,* 2018; Di Cesare *et al.,* 2018; Sanchez-Baracaldo *et al.,* 2019). The strains have different accessory pigments (PE, PC), membrane composition and aggregation ability. The aim of this study was to verify if all these *Synechococcus* strains produced TEP under controlled conditions during their growth, and under oxidative stress induced directly by adding H_2O_2 in the cultures and indirectly by irradiating them with UVR and high PAR. We used xenic cultured *Synechococcus* strains and followed the dynamics of the associated microbiota that can be important in the *Synechococcus* response to different stress (Callieri *et al.,* 2017).

METHODS

Culture and experimental design

The *Synechococcus* strains used in this experiment were two phycocyanin-rich (PC) strains: BO8801 (BO) and MW101C3 (MW) and three phycoerythrin-rich (PE) strains: *Synechococcus* LL (LL) recently reclassified as *Vulcanococcus limneticus* (Di Cesare *et al.,* 2018), NH1G10 (NH) and ATX6A2 (ATX), with different spectral characteristics (Fig. S1) and all isolated from different lakes, and now belonging to the CNR-IRSA collection (Tabs. 1 and 2). The strains (except for ATX) were sequenced for their whole-genome (Di Cesare *et al.,* 2018;

Sanchez-Baracaldo *et al.,* 2019). All the strains are monoclonal and belong to different clades inside the sub-cluster 5.2 (Cabello-Yeves *et al.,* 2018; Sanchez-Baracaldo *et al.,* 2019). They also affiliate with different phylogenetic clusters in the 16S rRNA tree: LL=Group A *Cyanobium gracile*; NH=marine sub-cluster 5.2; MW=Group I near to the Antarctic group; BO=Group A near to *Microcystis elabens*. LL and NH have the new phycoerytrin Type IIB (Sanchez-Baracaldo *et al.,* 2019).

The batch cultures were grown in 1000 mL flasks with BG11 medium, at 20°C under a photon flux density of 30 µmol photons m^{-2} s^{-1} with a 12h:12h light:dark cycle in a walk-in culture chamber.

For each strain, the growth of three replicate cultures (1000 mL) was monitored for several days. Once the cultures were growing exponentially, each replicate was split into nine 100 mL subsamples. Three subsamples from each culture were exposed to 3 different treatments: i) UVR and PAR stress (150 µmol photons m^{-2} s^{-1} PAR and a pulse of 5 h per day of UVR, 70 mW m^{-2} UVB, 300 mW m^{-2} UVA); ii) oxidative stress with H_2O_2 (100 µM H_2O_2) and iii) control (no H_2O_2 and no UVR and PAR stress; 30 µmol photons m^{-2} s^{-1} PAR).

The cultures were sampled at T_0 (the time at which the treatments were set up) and every 24 h over the next 3 days (T_1, T_2, and T_3). Analyses in triplicates were done for the measurements of TEP, cell number, chlorophyll *a* concentration, and photosynthetic efficiency (F_v/F_m).

Soluble reactive phosphorus (SRP) and dissolved inorganic nitrogen (DIN) were analyzed at the end of the assay, to ensure nutrient availability throughout the experiment. All nutrient determinations were conducted by colorimetric standard protocols (Clesceri *et al.,* 1998). Details on the analytical methods and the quality controls adopted in the laboratory can be found at: http://www.idrolab.ise.cnr.it/.

Tab. 1. Characteristics of the lake of origin for the *Synechococcus* strains used in the experiments.

Strain	LL now *Vulcanococcus limneticus*	BO8801	MW101C3	1G10	ATX6A2
Lake of origin	Albano	Constance	Mondsee	Nahuel Huapi	Atexcac
Depth of origin (m)	0.5	-	-	70	2
Latitude	41°45' N	47°39' N	47°49' N	40°26'S	19°19' N
Longitude	12°40' E	9°18' E	13°24' E	71°33'W	97°27' W
Altitude (m asl)	291	395	481	764	2360
Area (km^2)	6.0	571	14.2	557	0.29
Maximum depth (m)	170	251	68	464	34
pH	8.5	7.5	7.9	7.5	8.9
Epilimnetic temp (°C)	7.5-14	6.5-20	6.5-20	7-17	16.5-21.5
Total P (µg L^{-1})	40	36	10.4	4.9	3.5-17
Total N (µg L^{-1})	400	900	600	67	19-30 DIN
Lake origin	Volcanic	Glacial	Glacial	Glacial	Volcanic

Tab. 2. Some genome characteristics of the *Synechococcus* strains used in the experiments.

Genome	Sequencing	Sequence length (bp)	GC conten (%)	Lake origin	GenBank number	References Accession
Synechococcus sp. 1G10	WGS	3340220	64.56	L. Nahuel Huapi (Argentina)	NQKW00000000	Sánchez-Baracaldo *et al.*, 2019
Synechococcus sp. BO8801	WGS	3271183	69.15	L. Constance (Germany)	NQKY00000000	Sánchez-Baracaldo *et al.*, 2019
Synechococcus sp. LL / *V. limneticus*	WGS	3548884	68.35	L. Albano (Italy)	NQLA00000000	Di Cesare *et al.*, 2018
Synechococcus sp. MW101C3	WGS	3029138	66.13	L. Mondsee (Austria)	NQKX00000000	Sánchez-Baracaldo *et al.*, 2019
Synechococcus sp. ATX6A2	-	-	-	L. Atexcac (Mexico)	-	-

Cell counts

For cell counting, samples of 1 mL were fixed with filtered formaldehyde (1% final concentration) and stored at 4°C in the dark. Counting was performed before and after sonication to avoid the underestimation due to aggregation or microcolonies presence.

A flow cytometer, Accuri C6 (Becton Dickinson, Oxford, UK) equipped with a 20 mW 488 nm Solid State Blue Laser and a 14.7 mW 640 nm Diode Red Laser was used to count both picocyanobacteria and heterotrophic bacteria. The light scattering signals (forward and side light scatter, FSC and SSC, respectively), green fluorescence (FL1 channel=533/30 nm), orange fluorescence (FL2 channel=585/40 nm), and red fluorescence (FL3 channel >670 nm and FL4 channel 675/25) were acquired and considered for cells identification and quantification (Callieri *et al.,* 2016).

The red autofluorescence signals, with threshold values set at 1000 for FL3-H channel and at 2000 for FL4-H channel, were used to identify *Synechococcus* cells owing to their pigment content. Density plots of FL2-H *vs* FL4-H or FL2-H *vs* FL3-H allowed optimal distinction and absolute counting of PE and PC. The associated bacteria living in cultures were counted by staining with SYBR Green I (1:10000 final concentration; Molecular Probes, Invitrogen). Density plots of FSC-H *vs* FL1-H allowed the distinction between the stained microbial cells and background noise, with a threshold value of 700 applied on the FL1-H channel and 1000 on FSC-H channel.

All data were acquired at a pre-set flow rate of 35 µL min^{-1}, in order to keep the number of total events below 1000 per second. The BD Accuri C6 software (v. 1.0.264.21) was used for data processing and a fixed template with the same gating strategy was applied to allow direct comparison between samples.

In addition, manual inspections with an epifluorescence microscope were conducted both in autofluorescence (blue: 450-490 nm; green: 510 nm) and in UVR (365 nm), after the addition of DAPI (5', 6-diamino-2-phenylindole, final concentration, 0.1 µg mL^{-1}). Black polycarbonate filters (Poretics, 0.2 µm pore size) were used and the cells observed by a Zeiss Axioplan microscope equipped with an HBO 100 W lamp, a Neofluar 100 x objective 1.25 x additional magnification and filter sets for UV (G365, FT395, LP420), blue (BP450-490, FT510, LP520) and green light excitation (LP510-560, FT580, LP590).

Transparent exopolymer particles

TEP concentrations were determined spectrophotometrically following the colorimetric method proposed by Passow and Alldredge (1995). For each treatment of the experiments, 6 to 10 mL volumes of the sample (depending on the clogging of the filter) were collected at each sampling time. Samples were filtered under low and constant pressure (30 mm Hg) on polycarbonate filters with 0.4 µm of pore size and of 25 mm of diameter. Particles on filters were subsequently stained with 500 µL of a 0.02% working solution of Alcian blue in 0.06% acetic acid (pH 2.5), (prefiltered through a 0.22 µm pore-size filter), and left in contact for 30 seconds, filtered again and rinsed with MilliQ water to eliminate the excess dye. The filters were then soaked in 6 mL of 80% sulfuric acid for 2 h, and the concentration of TEP was measured in a spectrophotometer (SAFAS), at 787 nm in a 1 cm cell against distilled water as reference. Empty stained filters were used as blanks.

Alcian blue absorption was calibrated using a Xanthan Gum (XG) solution (Gomma Xantano E415, Farmalabor, Assago, MI, Italy). The concentration of TEP was expressed as xanthan gum equivalent (µg XG eq L^{-1}), determined from the following equation (Passow and Alldredge, 1995):

$$\text{TEP } \mu\text{g XG eq } L^{-1} = [(\text{Abs} - \text{Abs Blank}) / (\text{Vol})] \times \text{fx}$$

where TEP is measured in XG eq; Abs is the absorbance of the sample at 787 nm; Abs Blank is the absorbance of the blank; Vol is the volume filtered in litres; and fx is the calibration factor, obtained by a regression line (3 points) of the calibration curve of absorbance *versus* dry weight of XG based on particles retained on the filters, performed each time in replicates. Every time we prepared a concentrated XG solution (C: 0.5 mg mL^{-1}) weighing 10 mg of XG and adding to 20 mL of MQ water. Carefully we dissolved the XG with the help of a glass stick. From this concentrated solution we prepared two replicates more diluted (20 µg mL^{-1}: 0.5 mL of C in 12.5 mL of MQ water). We filtered 100 µL, 250 µL and 500 µL to have the quantity of 2, 5 and 10 µg for the calibration curve, respectively.

From the TEP data (as XG eq in µg L^{-1}), we calculated TEP concentration as carbon (µg C L^{-1}) using the conversion factor of 0.63 (Engel and Passow, 2001; Engel, 2004) recently suggested by Berman-Frank *et al.,* (2016). These values must be considered with caution because they are an approximation of the actual TEP carbon (Callieri *et al.,* 2017). To quantify the effect of the treatments we normalized TEP concentration for cell number or for Chl-*a* concentration as the cultures had different cell densities.

Photosynthetic efficiency and Chlorophyll-*a* concentration

Photosynthetic efficiency, specifically the effective quantum yield of photosystem II (PSII), was used as an indicator of phytoplankton physiological status. It indicates the amount of light energy that is passing through PSII and is used to drive photosynthesis, rather than lost as heat or

fluorescence. We used a Phyto-PAM (Pulse Amplitude Modulated) fluorometer to assess the effective quantum yield of energy conversion at the reaction centre of PSII using saturation pulses (Schreiber *et al.,* 1986). In dark adapted samples, the fluorescence measured before the saturation pulse is F_0 and the maximal fluorescence measured after the pulse is F_m. The ratio of the maximum variable fluorescence ($F_v = F_m - F_0$) to the maximum yield (F_m) provides the measure of the maximal quantum conversion efficiency of PSII and is known as the ratio F_v / F_m (Genty *et al.*, 1989). We measured F_v / F_m for each treatment.

The concentration of chlorophyll-*a* (Chl-*a*) was measured by using the Phyto-PAM which works by using the reference spectra obtained from pure cultures of *Synechococcus* with different phycobiliproteins, and making a Chl-*a* calibration restitutes the total Chl-*a* concentration (µg L^{-1}).

Statistical analysis

Data were analysed using Statgraphics centurion 18 (Statgraphics Technologies, Inc., The Plains, VA, USA) and Sigma Stat (SigmaPlot 12.5, Systat software Inc., Bangalore, India). Analysis of variance (ANOVA) was conducted on raw data and residuals that met the assumptions of normality and equality of variance as suggested by Kozak and Piepho (2017). Multifactor ANOVA was designed to construct a statistical model describing the impact of two categorical factors Xj 1) treatments: UV-PAR, hydrogen peroxide *vs* the control and 2) time: T_0, T_1, T_2 and T_3 on a dependent variable Y (cell number, TEP production per cell, TEP production per Chl-*a*, F_v/F_m photosynthetic efficiency). For factors that showed significant P-Values in Multifactor ANOVA the analysis of Multiple Range Tests using the LSD method with 95 and 99 % confidence level was carried by pairwise comparison between treatments and control. Furthermore, the plot of main effects of means with LSD intervals was used to confirm the differences and verify the dependent variable responses in the two treatments against the control throughout the experimental time. The significance of the difference between treatments and control at each time we used all pairwise multiple comparison procedures (Holm-Šídák method).

RESULTS

Cell number

The total *Synechococcus* cells used for the estimates of TEP normalization for all strains were those counted after pulsed sonication for 1 minute (Fig. 1). The cell counts of *Synechococcus* PE at time zero (T_0) were 2.3 x 10^6 cell mL^{-1}, 4.9 x 10^6 cell mL^{-1} and 24 x 10^6 cell mL^{-1} for ATX, LL and NH respectively. The abundance of *Synechococcus* PC was 2.8 x 10^6 cell mL^{-1} and 3.6 x 10^6 cell mL^{-1} for BO and MW strains, respectively.

The cell number of the control increased throughout the experimental time (associated to the exponential growth phase) in all the strains with the exception of NH where we observed the highest initial *Synechococcus* concentration and a decrease from T_1 to the end of the experiment. The daily growth rate in the control of different strains was: LL= 0.36 d^{-1}, ATX= 0.31 d^{-1}, MW= 0.27 d^{-1}, BO= 0.22 d^{-1} and negative for NH= -0.01 d^{-1} (Tab. S1).

LL and ATX had the highest aggregation under UV-PAR treatment as shown in the cytograms of the flow cytometer (Fig. 2). A different state of aggregation was checked at the microscope for all the strains during the experiment and demonstrated also by the increase in cell number after sonication compared to the counting before sonication (Fig. S2). It is worth noting that ATX tend to aggregate under all the treatments (including the control), whereas LL formed microcolonies only under UVR-PAR (Fig. 2, Tab. S1).

The effect of the treatments differed in all the strains. LL, MW and BO abundances were negatively affected by the peroxide treatment and not by the UVR-PAR treatment (Fisher's LSD *post-hoc* test, $p<0.01$); ATX was affected by both treatments (Fisher's LSD *post-hoc* test, $P<0.01$) and NH only by UVR-PAR treatment (Fisher's LSD *post-hoc* test, $P<0.01$).

Synechococcus PC strains did not change greatly their morphology under treatments and control but the formation of large visible aggregates was observed, which in turn were not easily disaggregated with sonication.

TEP concentration

As a first observation, all *Synechococcus* strains produced TEP both in the control and treatments (Fig. S3). TEP was also visible at the microscope when stained with Alcian blue (Fig. S4). In general, *Synechococcus* produced up to a maximum of 1600 µg C L^{-1}, and it mainly increased under physical and chemical stress. In general, TEP production under UVR-PAR treatment was higher than in the peroxide treatment. We did not observe significant differences in both treatments for the final concentration of TEP in the ATX strain. In this strain (isolated from Lake Atexcac, Mexico) a continuous increment of TEP throughout time was observed in all treatments. In BO, a high production of TEP under UVR-PAR was also registered.

Normalized TEP production

TEP production per cell responded differently to physical and chemical stress in all strains. While in the control TEP production per cell remained constant (ranging 50-200 ng C $cell^{-1}$), in most of the treatments higher concen-

trations were yielded with time: with a maximum value ranging from 100 to 400 ng C cell^{-1} (Fig. 3). The PE strains showed a tendency to produce more TEP per cell in UVR-PAR treatment than in the control and peroxide treatment, with significant difference for LL and NH (Fisher's LSD *post-hoc* test, P<0.01). ATX showed a higher production of TEP per cell in UVR-PAR and peroxide treatments rather than in the control (Fisher's LSD *post-hoc* test, P<0.01) and there were not significant differences between both treatments. On the other hand, the

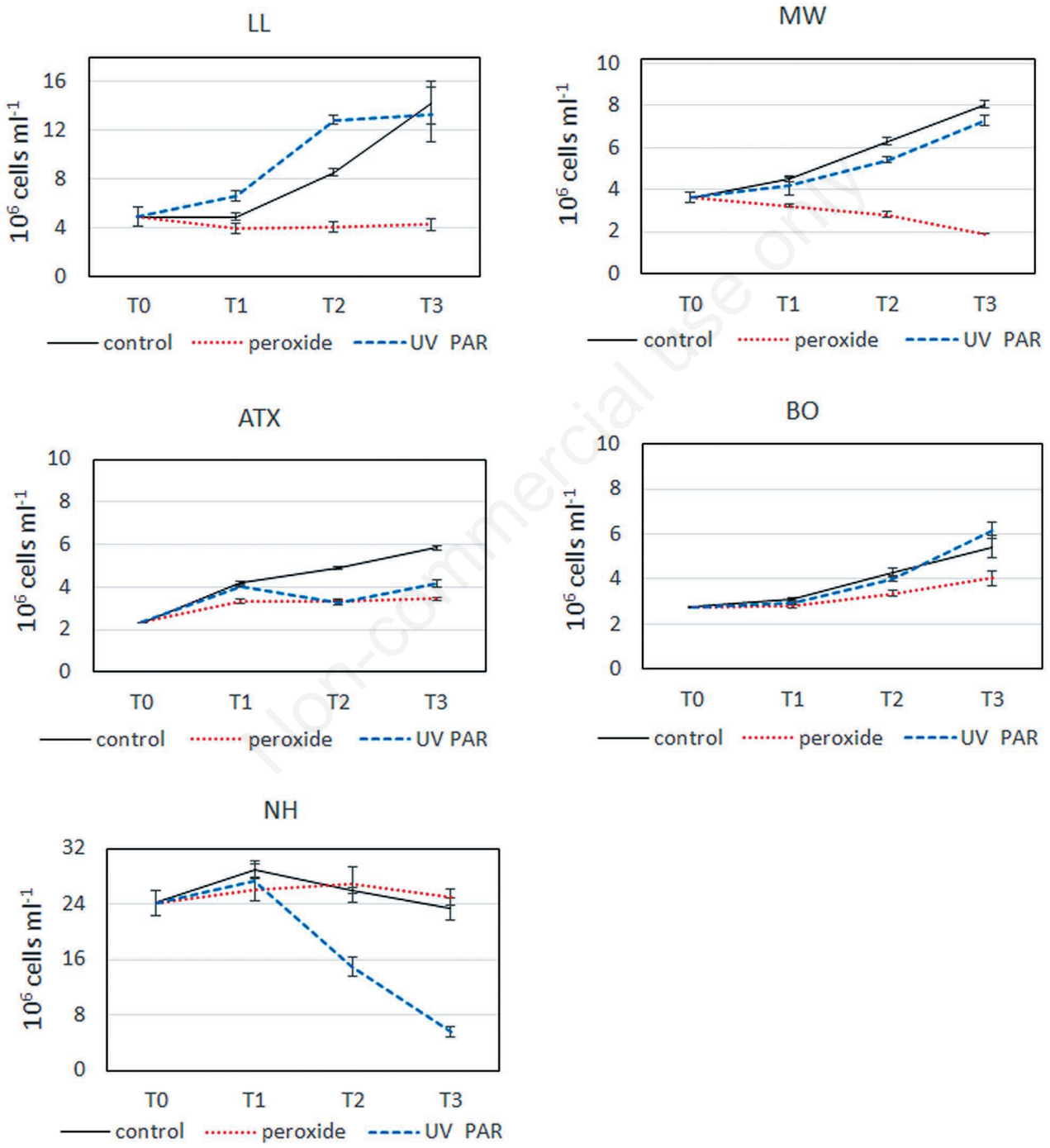

Fig. 1. Cell number of *Synechococcus* PE-rich strains (LL, ATX, NH) and PC-rich strains (MW, BO) sonicated, in the control and the two treatments, during the days of the experiment (T_0-T_3).

PC strains had a higher production of TEP per cell in the peroxide treatment against the control and UVR-PAR treatment (Fisher's LSD *post-hoc* test, P<0.01), observed after sonication. In fact, these strains had the highest production of TEP per cell in the peroxide treatment: BO 345 ng C cell^{-1} and MW 389 ng C cell^{-1}.

TEP production per Chl-*a* unit was higher in UV-PAR than in the peroxide treatment in PE strains, with significant differences in LL and ATX (Fisher's LSD *post-hoc* test, P<0.01). In NH there were not observable differences among treatments and control considering all the times, however TEP production per Chl-*a* unit was higher in UVR-PAR treatment than in the control, T_2 and T_3 (Holm-Šídák *post-hoc* test, P<0.01) (Fig. S5). In particular, ATX had the highest TEP production per Chl-*a* in UVR-PAR treatment with a maximum of 234, without

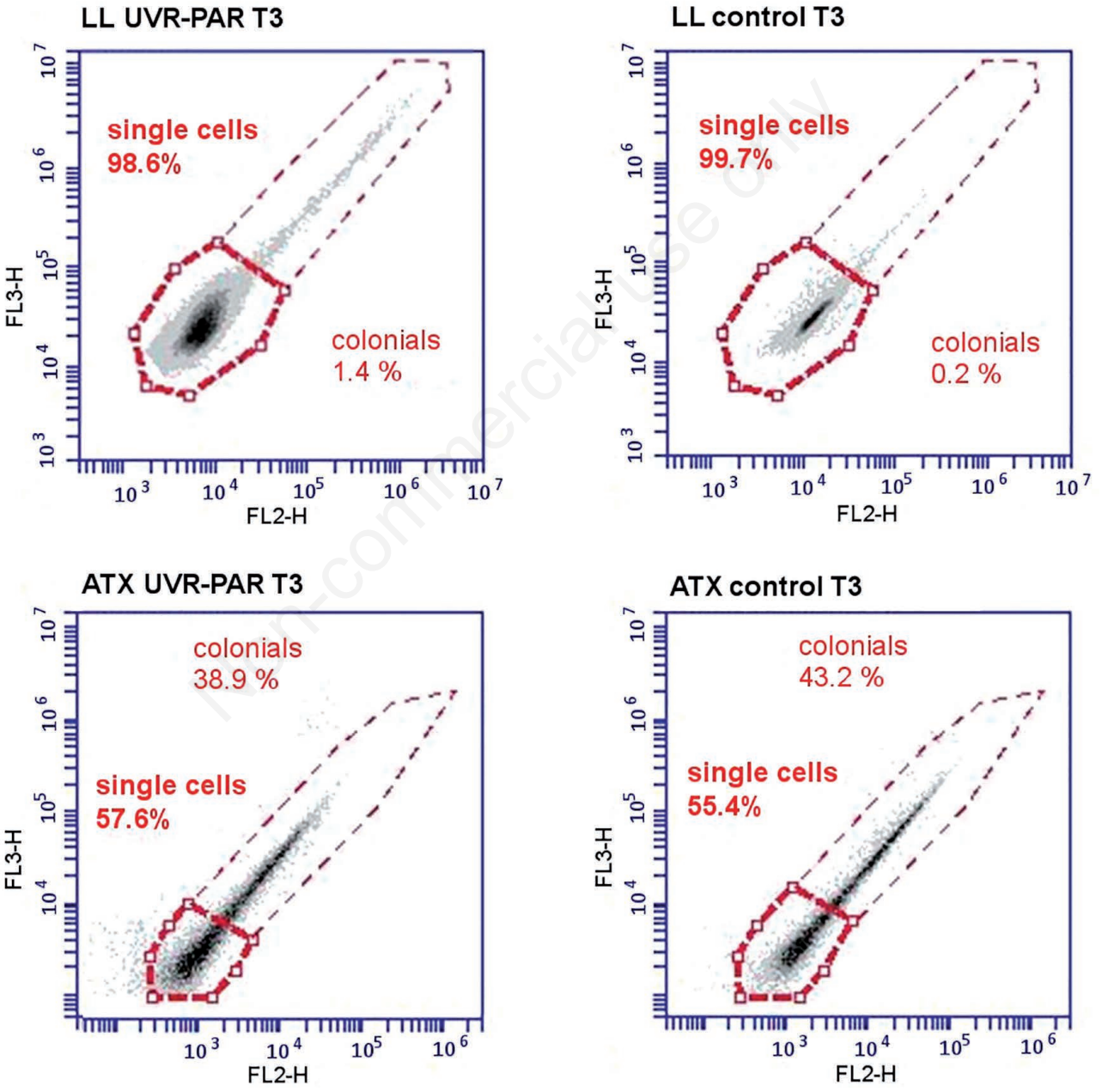

Fig. 2. Examples of the cytograms for *Synechococcus* strains cell counts showing the area of single cells and colonial events: LL (upper panels) and ATX (lower panels) in UVR-PAR treatment (left) and control (right) at T_3. FL2-H: orange fluorescence, phycoerythrin; FL3-H: red fluorescence, chlorophyll-*a*.

significant differences between peroxide treatment and control (Fisher's LSD *post-hoc* test, P<0.01). A similar result was observed in LL but with lower TEP production than ATX. MW showed a significantly higher TEP production per Chl-*a* in the peroxide treatment than in the UVR-PAR treatment (Fisher's LSD *post-hoc* test, P<0.01), while BO produced very low quantities of TEP per Chl-*a* unit in both treatments, but showed a significant difference against the control (Fisher's LSD *post-hoc* test, P<0.01).

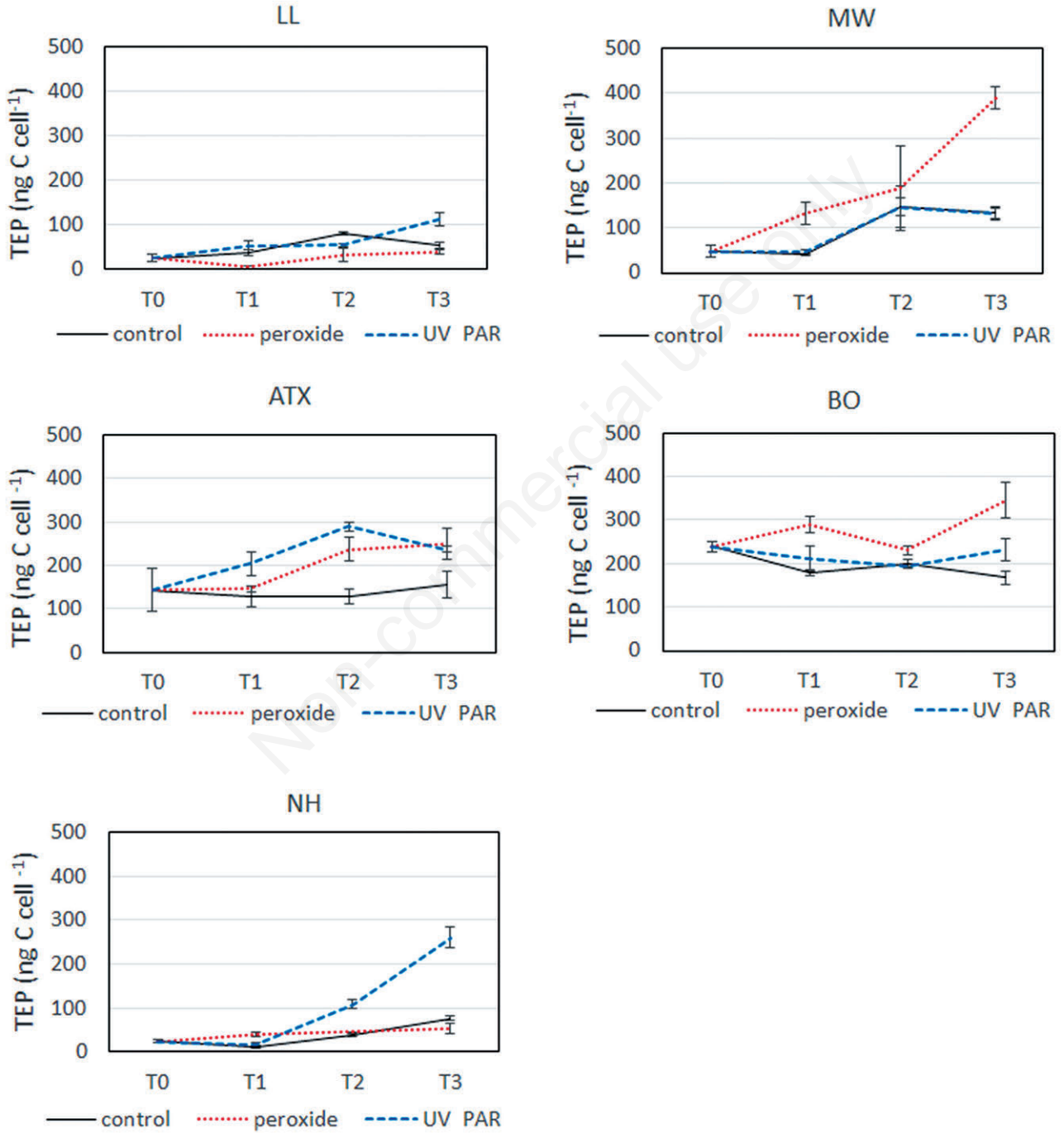

Fig. 3. TEP concentration normalized per cells of *Synechococcus* PE-rich strains (LL, ATX, NH) and PC-rich strains (MW, BO) in the control and the two treatments, during the days of the experiment (T_0-T_3).

Photosynthetic efficiency

The ratio F_v/F_m was quite constant in the control but showed variability among strains: LL showed 0.6, NH 0.5, BO 0.4 and MW and ATX ca. 0.2 (Fig. 4). Interestingly F_v/F_m in BO and ATX was not strongly influenced by the treatments, as conversely was observed for the other strains. In MW there was a significant decrease of F_v/F_m in peroxide and UV-PAR treatments *vs* control (Fisher's LSD *post-hoc* test, $P<0.01$).

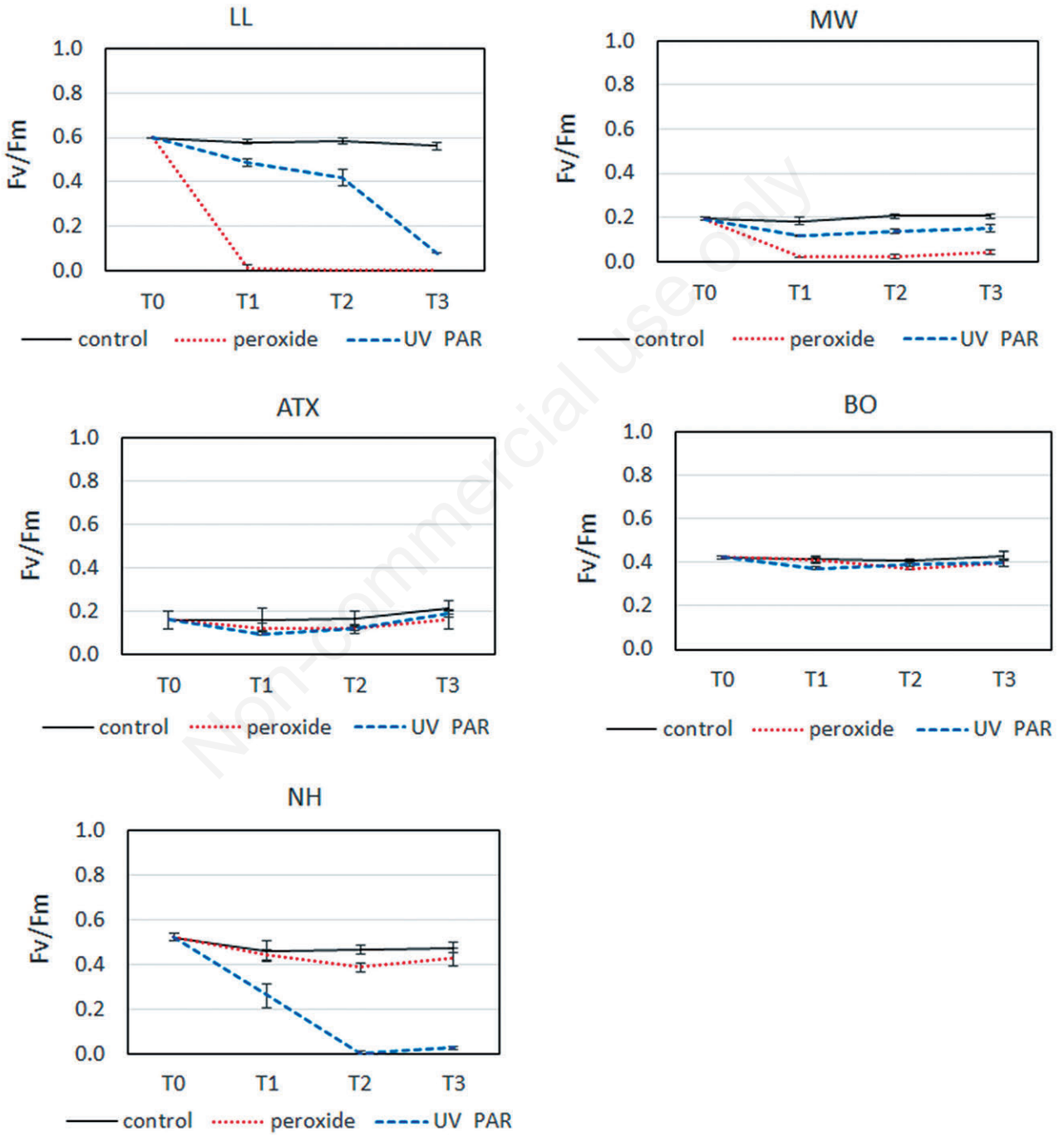

Fig. 4. F_v/F_m of *Synechococcus* PE-rich strains (LL, ATX, NH) and PC-rich strains (MW, BO) in the control and the two treatments, during the days of the experiment (T_0-T_3).

The decrease of photosynthetic efficiency was also statistically significant in LL in both treatments with respect to the control (Fisher's LSD *post-hoc* test, P<0.01). In NH the effect of UVR-PAR in F_v/F_m was stronger than in the peroxide treatment and control (Fisher's LSD *post-hoc* test, P<0.01). In BO strain, the ratio F_v/F_m was always very low but significantly different in the treatments against the control (Fisher's LSD *post-hoc* test, P<0.05).

Bacterial abundance

Bacterial abundance showed a different pattern throughout the experimental time. NH and LL strains showed a decrease in their abundance, NH had a stronger effect in the UVR-PAR treatment than in the peroxide and control treatments (Fisher's LSD *post-hoc* test, P<0.01), while LL had no significant differences between treatments and against the control (Fig. S6). Likewise, for ATX the bacterial abundance increased over time without showing significant differences between treatments. MW had the lower decrease of bacterial abundance with no significant difference between treatments and the control. Conversely, BO bacterial abundance increased in UVR-PAR treatment with significant difference between the peroxide treatment and the control (Fisher's LSD *post-hoc* test, P<0.01), however there was not significant difference between the two treatments at 99% confidence level.

DISCUSSION

Several studies on TEP production by marine autotrophic eukaryotes, mainly diatoms, have been published so far (Passow, 2002b; Fukao *et al.,* 2010). However, few experiments have been conducted on the production of TEP by the picocyanobacteria *Synechococcus* (Thorton and Chen, 2017; Deng *et al.,* 2016). In this work we added new evidence on TEP production in various *Synechococcus* strains isolated from different lakes around the world. Our data give a more realistic idea of the role of *Synechococcus* and its microbiome in the production of TEP in natural environments.

We observed a cell-normalized TEP production, ranging from 12 to 238 ng C $cell^{-1}$ in our strains, not only under stress but also in the control, in high nutrient concentrations. This could imply that *Synechococcus* and their associated heterotrophic community, can produce TEP even during growth. As has been observed in a previous work (Callieri *et al.,* 2017) in lakes TEP concentrations were significantly correlated to Chl-*a* and to picocyanobacteria abundance (mainly *Synechococcus*). Our experiments with a selected number of strains demonstrate that the oxidative stress induced extra production of TEP. In previous experimental designs using *Synechococcus elongatus* and *Synechococcus* sp. WH8102, the stress conditions were created by adding hydrogen peroxide (Thorthon and Chen, 2017) or by reducing nitrogen and phosphorus concentration in the culture media (Deng *et al.,* 2016). Here, we tested the difference in TEP production under UVR-PAR irradiation and exposure to hydrogen peroxide. Stressed or dying cells produced more TEP in all the strains, with differences mostly tied to pigment composition. The PC strains (MW and BO), like *S. elongatus*, were sensitive to the addition of 100 μM H_2O_2. There was a significant enhancement of TEP production together with a decrease in the photosynthetic activity and cell death. The autocatalytic cell death and caspase activity was observed in the cyanobacterium *Trichodesmium* (Berman-Frank *et al.,* 2004) and in the coccolithophore *Emiliania huxleyi* (Vardi *et al.,* 2012) under nutrient starvation, oxidative stress and high light intensity. Our experiments showed that in the PC strains, TEP production was significantly higher in the H_2O_2 treatment. The manual genomic inspection of our *Synechococcus* strains revealed that all of them have the genes involved in the caspase-like proteases, including the PC strains, so they could potentially increase the cell permeability, which triggers the programmed cell death like in *Trichodesmium* (Berman-Frank *et al.,* 2004). Nevertheless, only MW showed a significant decrease of cell number under H_2O_2. Conversely, the UVR-PAR treatment did not produce a significant increase of TEP production, comparable to the hydrogen peroxide treatment, in PC strains. One possible explanation of this result is that PC strains have protective pigments in the sheath around and inside the microcolonies, preserving them from the detrimental effects of radiation. The analysis of their genomes revealed that scytonemin related genes (similar to the core gene *scyA-F* observed in *Nostoc* spp.) were only present in BO, a PC strain that forms large aggregates also visible to the naked eye. These genes were only present in another two yet sequenced PC picocyanobacterial strains, *Cyanobium gracile* PCC 6307 and *Cyanobium* sp. CACIAM 14. This observation suggests that only some PC strains present scytonemin gene clusters. The remaining tested PC strain, MW and all the PE strains do not present these genes. We are not sure that the strains that contain the putative scytonemin gene clusters actually produce scytonemin. Having the genes does not mean that PC strains contain scytonemin, but surely the PE strains without the genes, cannot produce this pigment.

The absence of protective pigments like scytonemin in PE strains explains the results observed for these strains. PE strains were more sensitive to the UVR-PAR treatment, and formed weak microcolonies which disaggregated after sonication. The effect of UVR-PAR is to produce ROS directly inside the cell, thus affecting also the cells which are not permeable to H_2O_2. PE were not sensitive to H_2O_2 probably as they are not permeable to

this compound or have antioxidants like catalase (CAT) or peroxidase (APX) scavenging H_2O_2.

The study of the genomes showed that all our strains have (to a greater or lesser extent) CAT and APX genes (catalase/peroxidase HPI, *KatG* gene) and therefore the presence of antioxidants to explain the different reaction of PE and PC strains to TEP production under stress remains to be elucidated.

As PE strains responded differently to the UVR-PAR and H_2O_2 treatments we cannot robustly prove that TEP production is only dependent on the pigment type of the strains. All strains produced more cell-normalized TEP under UVR-PAR treatment, particularly on the second and third day of the experiment. We noticed that the formation of small microcolonies was a typical reaction of PE strains to UV and light irradiation (Callieri *et al.,* 2016). A soft sonication of the LL culture increased the number of cells counted by flow cytometer thus demonstrating that the microcolonies were neither too large nor strongly packed. A similar effect of aggregation was observed in ATX not only under UV-PAR but also under H_2O_2 treatment.

ATX produced higher TEP concentration under both stressing conditions. This strain originated from a high altitude tropical athassohaline Mexican lake, could survive at high PAR and UV irradiation. In fact, the ability to form microcolonies could be the survival strategy of ATX even if the TEP production indicates a bad health status of cells. ATX has a very low F_v/F_m ratio both in control and treatment conditions, hence the strategy of this strain could be to grow and photosynthesize at low photosynthetic efficiency, absorbing little energy for CO_2 assimilation or being able to dissipate excess energy using for example carotenoids (Latifi *et al.,* 2008). The peroxide affected the PSII of LL and MW very soon in the experiment (at T_1), while NH was affected later on (T_2) and only by UVR-PAR treatment, and BO and ATX had always a very low efficiency of the PSII. These results underline the wide variation of the response of the *Synechococcus* strains belonging to different phylogenetic clade.

NH produced more TEP under UVR-PAR and this is clearly due to cell death as demonstrated by the decrease in cell counts. We have no quantitative data on microcolony formation in NH (before and after sonication) but in general, this strain produces fewer microcolonies. This strain originates from a deep layer in a large North Patagonia lake, so it might be particularly sensitive to light. Nevertheless, it is well known that *Synechococcus* spp. can be very adaptable to different physiological conditions (Callieri, 2017; Huber *et al.,* 2017) and the characteristics of the lake origin are not necessarily the ones of the strain in culture. The rearrangement of accessory genes can give to *Synechococcus* its selective advantage to adapt to changing conditions.

The associated microflora in the cultures could potentially have an effect on the TEP production as observed previously in a marine *Synechococcus* strain (Deng *et al.,* 2016). Whatever the effect was, we considered important to work using xenic strains as the interaction bacteria-cyanobacteria could be the base of the physiological response of *Synechococcus* to any environmental stress. A different composition of the microbiome associated to *Synechococcus* ribotypes was found to influence the community response to grazing (Callieri *et al.,* 2017). The predominance of Gammaproteobacteria and Flavobacteria was found to be typical of MW strain while in LL Sphingobacteria and Alphaproteobacteria were found (Callieri *et al.,* 2017). In the present work, we only counted the bacteria, without analysing its composition, so we cannot confirm this community composition in our experimental conditions. Nonetheless, we observed that the ratio Bacteria/*Synechococcus* was in general low at T_0 (from 1.1 to 7) and only increased in ATX and BO strains. A microscopic analysis of the cultures showed that bacteria were mainly observed in aggregates and associated to the *Synechococcus* microcolonies (Fig. S7).

CONCLUSIONS

We found that *Synechococcus* and their associated microflora produced TEP actively under growth, but particularly under oxidative stress. The PE strains increased TEP production particularly under UVR-PAR stress whereas the PC strains under the H_2O_2 stress. PE strains produced TEP to contrast the UVR-PAR detrimental effect and formed microcolonies or larger aggregates from single cells. On the other hand, PC strains naturally present in larger colonies, seem to be less affected by UVR-PAR, possibly due to the presence of scytonemin in their colonies, but are more sensitive to the oxidative stress caused by the direct addition of ROS substances. Further studies on *Synechococcus* microcolony dynamics, composition and relation to TEP formation are necessary to better understand the role of these cyanobacteria in aquatic environments.

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