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Original Citation:

Shaping the phycosphere: Analysis of the EPS in diatom-bacterial co-cultures / Giulia Daly, F.D.. - In: JOURNAL OF PHYCOLOGY. - ISSN 1529-8817. - STAMPA. - 4:(2023), pp. 791-797. [10.1111/jpy.13361]

Availability:

The webpage https://hdl.handle.net/2158/1328811 of the repository was last updated on 2023-09-19T13:15:23Z

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DOI: 10.1111/jpy.13361

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(Article begins on next page)

17 October 2026

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Received: 9 June 2022 | Accepted: 5 June 2023

DOI: 10.1111/jpy.13361

NOTE

Shaping the phycosphere: Analysis of the EPS in diatom-bacterial co-cultures

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Funding information
Italian Ministry of University and Research, PNRR, Missione 4 Componente 2, "Dalla ricerca all'impresa", Investimento 1.4, Grant/Award Number: CN00000033

Editor: N. Poulsen

Abstract

The phycosphere is a unique niche that fosters complex interactions between microalgae and associated bacteria. The formation of this extracellular environment, and the associated bacterial biodiversity, is heavily influenced by the secretion of extracellular polymers, primarily driven by phototrophic organisms. The exopolysaccharides (EPS) represent the largest fraction of the microalgae-derived exudates, which can be specifically used by heterotrophic bacteria as substrates for metabolic processes. Furthermore, it has been proposed that bacteria and their extracellular factors play a role in both the release and composition of the EPS. In this study, two model microorganisms, the diatom *Phaeodactylum tricornutum* CCAP 1055/15 and the bacterium *Pseudoalteromonas haloplanktis* TAC125, were co-cultured in a dual system to assess how their interactions modify the phycosphere chemical composition by analyzing the EPS monosaccharide profile released in the culture media by the two partners. We demonstrate that microalgal–bacterial interactions in this simplified model significantly influenced the architecture of their extracellular environment. We observed that the composition of the exo-environment, as described by the EPS monosaccharide profiles, varied under different culture conditions and times of incubation. This study reports an initial characterization of the molecular modifications occurring in the extracellular environment surrounding two relevant representatives of marine systems.

KEYWORDS
dual-system co-culture, exopolysaccharides, extracellular environment, monosaccharide composition, phycosphere

The phycosphere is a metabolic hot spot surrounding the algal cell in which a variety of metabolites are involved in microalgae–bacteria interactions (Wichard & Beemelmanns, 2018). Nutrient trafficking and signaling molecules are central to these dynamic relationships (Daly et al., 2022), playing a vital role in these highly biodiverse aquatic ecosystems (Deng et al., 2022). The secretion of polymeric substances by these organisms plays a crucial role in physically defining the phycosphere, which serves as an adhesive that binds microbial cells and inorganic particulate matter together (Mühlenbruch et al., 2018; Rusanowska et al., 2019). Released substances from microalgal cells primarily consist of polysaccharides, proteins, lipids, and nucleic acids (Shi et al., 2017). Among these macromolecules, exopolysaccharides (EPS) are often the main

Abbreviations: Ara, arabinose; EPS, exopolysaccharides; Fru, fructose; Fuc, fucose; GalN, galactosamine; GlcN, glucosamine; Gal, galactose; Glc, glucose; GalA, galacturonic acid; GlcA, glucuronic acid; Man, mannose; Rha, rhamnose; Rib, ribose; Xyl, xylose.

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components, accounting for 45%–95% of the total polymers (Naveed et al., 2019). Polysaccharides play a fundamental role in the phycosphere, serving as valuable carbon sources for bacteria inhabiting this crowded microenvironment (Guerrini et al., 1998; Kamalanathan et al., 2019).

While phytoplankton are the main source of polysaccharides, bacterial EPS also represent a significant source of dissolved organic carbon in marine ecosystems (Thornton, 2014; Xiao & Zheng, 2016; Zhang et al., 2015). Bacterial EPS serve several functions, including ensuring the formation of a favorable microenvironment for attachment, maintaining exoenzyme activity, sequestering nutrients, and protecting against toxins (Decho, 1990). The composition of EPS varies considerably between microalgae and bacteria, potentially reflecting their fate in the extracellular environment (Bhaskar & Bhosle, 2005; Zhang et al., 2015). However, studying the surrounding extracellular environment of these complex microalgal–bacterial associations in real marine conditions is challenging. Indeed, enumerating metabolic exchanges in this crowded microenvironment becomes overwhelming, mainly because the exuded metabolites, such as the EPS, cannot be easily attributed to a particular microorganism or abiotic source (Ponomarova & Patil, 2015).

Accordingly, in this study, we chose to reconstruct the microenvironment surrounding the microalga, the phycosphere, using a simplified synthetic system composed of two model microorganisms: the heterotrophic bacterium *Pseudoalteromonas haloplanktis* TAC125 and the diatom *Phaeodactylum tricornutum* CCAP 1055/15. The focus of our investigation was on the monosaccharide components, which are readily taken up by the heterotrophic bacteria and are immediately available for their metabolism (Mühlenbruch et al., 2018). By employing this simplified model, we aimed to gain insights into the interactions and molecular modifications occurring in the extracellular environment of these relevant representatives of marine systems.

Our study investigated how the simultaneous presence of the diatom and the bacterium modifies their extracellular environment. Specifically, we focused on the EPS monosaccharide composition released by the two microorganisms. To analyze the chemical composition of the EPS in the lab-reconstructed exo-environment, we compared three different conditions: the diatom-bacterium co-culture, single cultures (diatom and bacterial controls), and the diatom grown within spent bacterial medium. (For the spent medium preparation and detailed culture conditions see Appendix S1 in the Supporting Information, which contains all supplementary tables and figures.)

The co-culture of *Phaeodactylum tricornutum*-*Pseudoalteromonas haloplanktis*, as well as the bacterial and diatom controls, were set up following the methods described in Daly et al. (2021) with some modifications. Briefly, the preculture of *Ps. haloplanktis* was grown for 2 d in a marine salt mix, called "Schatz Salts medium modified" (mSS; Daly et al., 2021), supplemented with L-glutamic acid (11 $g \cdot L^{-1}$) and used as inoculum for the co-cultivation experiments. For the co-culture, the bacterial preculture was washed once by centrifugation at $1254 \times g$ for 4 min and then added at a concentration of 10^5 cells $\cdot$ mL^{-1} to the fresh culture of *P. tricornutum* (10^4 cells $\cdot$ mL^{-1}) in mSS medium with no additional carbon source. In the case of the diatom grown within spent bacterial medium, *P. tricornutum* was inoculated at higher cell density (5.4×10^6 cells $\cdot$ mL^{-1}) to sustain its growth in this unusual culturing condition (spent mSS medium).

The diatom was inoculated from a growing stock culture of the axenic *Phaeodactylum tricornutum* in f/2 medium with vitamin B12. Control cultures were also set up: *P. tricornutum* alone in mSS medium as diatom control and *Pseudoalteromonas haloplanktis* alone in mSS medium without C source and *Ps. haloplanktis* alone in mSS medium containing additional L-glutamic acid, as negative and positive bacterial controls, respectively. The axenic diatom controls were checked for bacterial contamination at the beginning and at the end of the experiment by microscopy observation and plating aliquot on marine mgar (MA) plates (Condalab, Spain). All experimental cultures were performed in a working volume of 50 mL for 21 d, a duration calibrated to observe the cultures in a reciprocal stimulatory phase, before the instauration of possible competition for nutrients in the medium (Wang et al., 2014).

Samples were harvested at 7-d intervals over the entire cultivation period. The EPS released in the culture media were collected by centrifuging the culture at $1960 \times g$ for 15 min at room temperature. The EPS present in the supernatant were then precipitated using 70% ethanol and pelleted at $3500 \times g$ for 20 min. The pelleted EPS were hydrolyzed and subjected to analysis using a Dionex ICS-2500 ion exchange chromatographer (IEC) following the methods described in Zanolla et al. (2022). To assess the growth of both the diatom and the bacterium, measurements were taken every 7 d (See Appendix S1) in order to compare their growth rates and the EPS composition.

All of the analyses were conducted in experimental triplicates ($N = 3$). The significance of the data was evaluated using Student's *t*-test, one-way analysis of variance (ANOVA), or Kruskall–Wallis test at 95% significance. Statistical analysis was performed using GraphPad Prism version 6.00 (GraphPad software, USA), while principal component analysis (PCA) was performed on monosaccharides composition data (Rencher 1995) using OriginPro, Version 2022 (OriginLab Corporation, Northampton, MA, USA).

In our analysis of the EPS produced by the diatom and the bacterium cultures, we identified a total of 13 different monosaccharide moieties (Figure 1; nine sugars,

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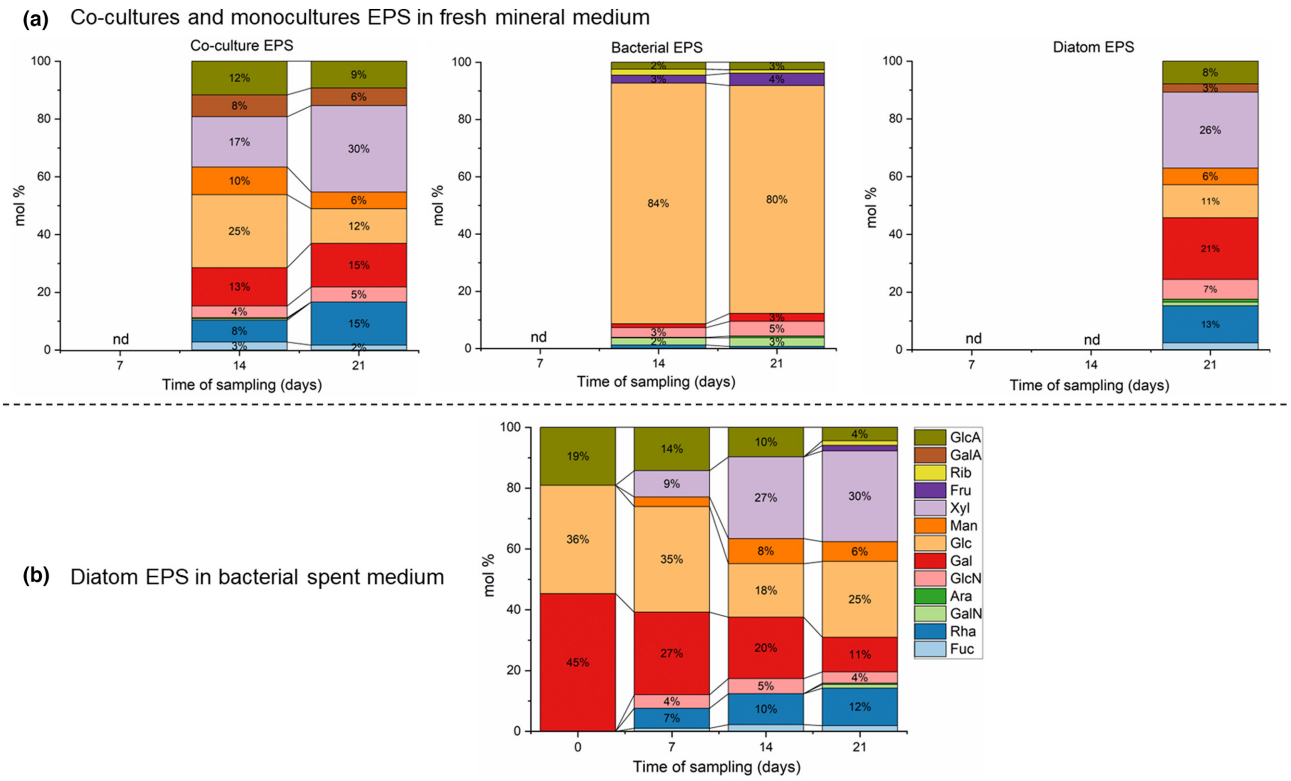

FIGURE 1 (a) Monosaccharide composition of EPS produced by diatom and bacterium as monocultures and by the co-culture at day 7, 14, and 21 in fresh mineral medium (mSS). (b) Monosaccharide composition of EPS produced by diatom grown in spent bacterial medium (diatom in SBacM) at four time points. Monosaccharide composition expressed as molar %. Abbreviations: Fuc, fucose; Rha, rhamnose, GalN, galactosamine; Ara, arabinose, GlcN, glucosamine; Gal, galactose; Glc, glucose; Man, mannose; Xyl, xylose; Fru, fructose; Rib, ribose; GalA, galacturonic acid; GlcA, glucuronic acid; nd, not detectable. Chemical characteristics of EPS: GlcN, Man, Xyl, Fru, Rib, Gal, Glc, GalA with hydrophilic character; Rha, Fuc, Ara, GlcA with hydrophobic character. [Color figure can be viewed at wileyonlinelibrary.com]

two uronic acids, and two amino sugars). Notably, the diatom EPS was composed of a relatively high number of monosaccharide units (Figure 1), indicating its heteropolysaccharide nature (Zhang et al., 2020). The EPS production consistently followed the growth curves (refer to Appendix S1 for detailed description of growth curves, Figure S1, and rates, Tables S1 and S2). At day 7, both the microorganisms grown in co-culture and the controls displayed low cell counts (Appendix S1: Figure S1a,b), and no monosaccharides were detected in the collected samples (Figure 1a). However, by day 21, there were increased diatom cell counts both in co-culture and in single culture that were accompanied by the detection of EPS.

After 14 days, it was possible to determine the monosaccharide composition for the co-culture and the positive bacterial control, but not for the diatom control due to low EPS concentration (Figure 1a). Our study has confirmed the exudation of polysaccharides by marine bacteria into the extracellular environment as previously observed in other research (Perera et al., 2022; Zhang et al., 2015). Specifically, the bacterium demonstrates the ability to release EPS when grown with glutamate as a substrate (bacterial positive control, bact+, Figure 1a). After 14 days, the EPS produced by the bacterium predominantly contained glucose, accounting for nearly 80% of the detected constituents. Additionally, small amounts of other sugars such as rhamnose, galactosamine, arabinose, glucosamine, galactose, fructose, ribose, and glucuronic acid were also present (Figure 1a). The monosaccharide profile of the bacterial EPS remained similar between day 14 and day 21.

After 21 days, the EPS in the exo-environment of *Phaeodactylum tricornutum* cultivated with *Pseudoalteromonas haloplanktis* was not influenced by the presence of the bacterium in terms of monosaccharide composition (Figure 1a). However, the presence of the bacterial cells enhanced the release of EPS by the diatom as demonstrated by the detectable amounts of EPS already after 14 days for the co-culture, while the diatom cultivated axenically released detectable amounts only after 21 days. This effect has been previously observed in various diatoms cultivated with different natural bacterial isolates (Bruckner et al., 2011), demonstrating that the model organisms chosen to reconstruct the phycosphere in this study can be used to represent the dynamics occurring in natural systems.

Overall, the monosaccharide profile of the EPS produced by the co-culture was very similar to that of the diatom in single culture but significantly different from the bacterial control (Figure 1a; Appendix S1: Table S3). As expected, these outcomes indicate that

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the EPS in co-culture were predominantly released by *Phaeodactylum tricornutum*. This is consistent with observations from epilithic diatom/bacteria co-cultures, in which diatoms were found to produce most of the carbohydrates, whereas the carbohydrate fraction secreted by bacteria was negligible (Bruckner et al., 2008). Moreover, bacterial growth in co-culture was low (Appendix S1: Figure S1a), resulting in no significant release of EPS into the medium. For detailed comparisons of the monosaccharide composition between the different conditions at different time points, refer to Table S3 in Appendix S1.

Microalgal-derived EPS are known to play a critical role in the attraction, recruitment, and retention of heterotrophic bacteria within the phycosphere (Smriga et al., 2016) as the excretion of EPS by diatoms provides a food source for bacteria (De Brouwer et al., 2002; Underwood et al., 2004). However, bioavailability of carbohydrates in the phycosphere depends on the ability of bacteria to hydrolyse algal polymers since EPS is less efficient for microbial growth compared to the monomers (Zhang et al., 2015). In our experiments, bacterial cell growth was sustained by the presence of the diatom in co-culture, where no additional C-source was provided. However, the growth rate was moderate when compared to the positive control, wherein the C source was freely available, highlighting the effort required for the bacterial cultures to efficiently feed on diatom EPS (Appendix S1, Figure S1a).

Interestingly, *Phaeodactylum tricornutum* showed enhanced growth in the presence of the bacterial spent medium compared to the control in mineral medium. This was evident from both cell count (Appendix S1: Figure S1b) and chlorophyll *a* content (Appendix S1: Figure S1c). This stimulating effect, previously reported by Bruckner et al. (2011) and Daly et al. (2021), is possibly due to the presence of bacterial exudates, which serve as substrates to support *P. tricornutum* under mixotrophic growth (Villanova & Spetea, 2021). Previous studies revealed that glucose and fructose exerted significant enhancement on the growth of *P. tricornutum* under mixotrophic conditions (Cerón-García et al., 2013; Villanova et al., 2017). Moreover, the diatom cultured in the spent bacterial medium showed the highest growth rate during the first phase (0–7 d), followed by a rapid decrease in the second phase, suggesting a rapid utilization of bacterial exudates (Appendix S1: Table S2).

The composition of microalgal EPS is influenced by several factors, including the species, strain, nutrient availability, cultural conditions (e.g., temperature, pH, light, salinity), physiology, and age of the culture (Kumar et al., 2018; Xiao & Zheng, 2016). Different parameters have been shown to induce changes in EPS

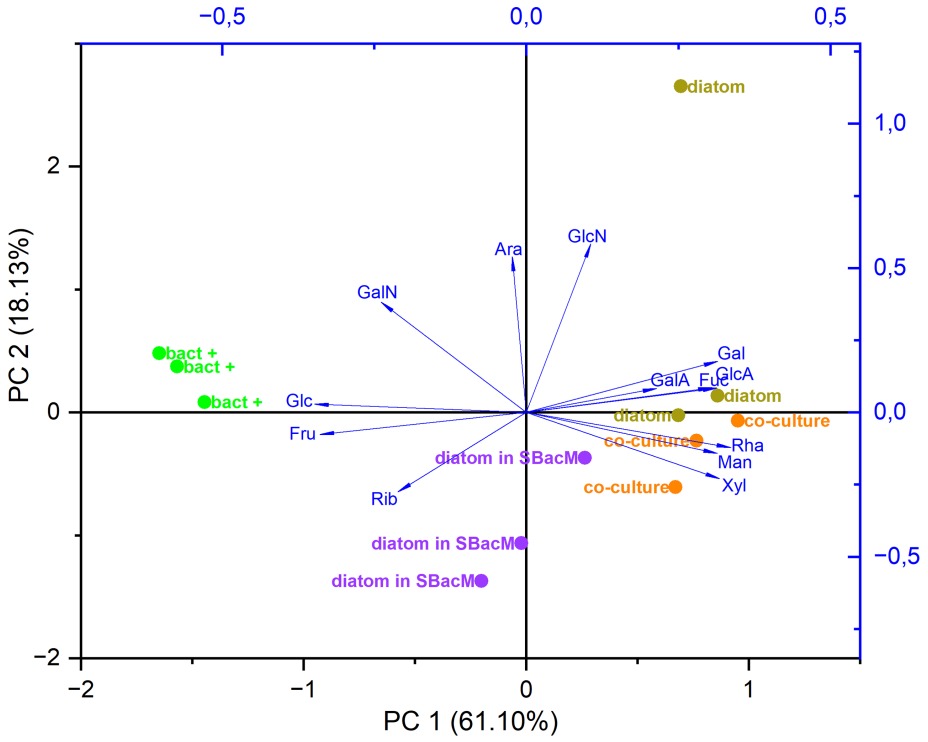

FIGURE 2 PCA biplot of PC1 vs. PC2 showing the distribution of samples according to the monosaccharide composition of the EPS at day 21. Samples: diatom (diatom) and bacterium (bact +: bacterium cultivated with glutamate as C source) as monoculture, the co-culture (co-culture), and the diatom cultivated in spent bacterial medium (diatom in SBacM). Abbreviations: Fuc, fucose; Rha, rhamnose, GalN, galactosamine; Ara, arabinose, GlcN, glucosamine; Gal, galactose; Glc, glucose; Man, mannose; Xyl, xylose; Fru, fructose; Rib, ribose; GalA, galacturonic acid; GlcA, glucuronic acid. [Color figure can be viewed at wileyonlinelibrary.com]

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monosaccharide composition and synthesis in different microalgae. For example, light intensity affects EPS composition in *Arthrospira platensis* (Phélippé et al., 2019), while different salinity and temperature conditions impact the EPS composition in the diatom *Fragilariopsis cylindrus* (Aslam et al., 2018). Additionally, the growth phase influences EPS composition for marine diatoms (Penna et al., 1999) and the red microalga *Porphyridium purpureum* (Li et al., 2020), highlighting the flexibility of the EPS-related pathways (Aslam et al., 2018). In the case of *Phaeodactylum tricornutum*, the EPS monosaccharide composition can change depending on the physiological state of the diatom (Willis et al., 2013) and growth phase (Bellinger et al., 2005; Underwood et al., 2004). Our results suggest that *P. tricornutum* modulates its polysaccharide biosynthesis machinery to adapt to the different cultural conditions, thereby influencing its bioactivity. The monosaccharide composition of the EPS produced by the diatom grown in spent bacterial medium at day 21 showed a marked difference in sugar profiles compared to the diatom control (at the same sampling point and the same stationary phase; Figure 1b) grown in the mineral medium mSS. Not only did the relative composition of specific moieties differ, but we could also detect the presence of unique monosaccharides (as fructose and ribose) that were absent in diatom control and the initial spent bacterial medium (Appendix S1: Figure S2).

Regarding the chemical changes occurring in the reconstructed phycosphere, we observed that the EPS profiles of the conditions analyzed changed over time (Figure 1). This effect was particularly pronounced in the diatom grown in the spent bacterial medium, in which significant changes in the EPS profiles were detected at the different sampling times (Figure 1b). The relative content of galactose decreased significantly from day 0 to day 21; rhamnose and xylose showed a significant increase from day 7 to day 14, while glucose exhibited a decrease at day 14 followed by an increase at day 21 (Appendix S1: Table S4).

The initial bacterial EPS (initial medium, t0) was dominated by hydrophilic and negatively charged moieties, primarily galactose, glucose, and galacturonic acid. The diatom growth within the spent bacterial medium gradually modified its phycosphere toward a more amphiphilic and heterogeneous environment: Indeed, the resulting EPS was composed of increasing relative amounts of hydrophobic moieties as deoxy sugars (rhamnose and fucose), while a wider diversity of hydrophilic moieties were produced (glucosamine, mannose, xylose, fructose, and ribose). The amphiphilic nature of the diatom-produced EPS is particularly significant, as hydrophobic interactions play a crucial role in the physical attachment (Dang & Lovell, 2016), facilitating bacterial colonization of the phycosphere.

PCA performed on EPS monosaccharide composition profiles collected after 21 days showed that the first and the second components (rhamnose and fucose) explained 61.10% and 18.13% of the variance, respectively (Appendix S1: Table S5). The monosaccharide composition expressed as a molar %, was used for all the variables. The biplot for PC1 × PC2 is shown in Figure 2, where the first component allowed discrimination of the bacterial samples from all the other conditions. The PCA indicated that the bacterial clustering was driven by glucose, which represented the main sugar component in the bacterial EPS, while the grouping of diatom and the co-culture were driving mainly by xylose, mannose, galactose, fucose, rhamnose, and galacturonic and glucuronic acid. The PCA biplot (Figure 2) seems to confirm the similarity between the diatom and co-culture EPS monosaccharides composition. It is worth noting that the outlier diatom sample in the biplot did not significantly affect the grouping (data not shown).

This study provides an initial description of the molecular modifications occurring within the polysaccharide matrix formed by these two model microorganisms in the phycosphere, highlighting how the microalgal–bacterial interactions can alter and shape the surrounding environment. The presence of bacterium stimulated the release of EPS by the diatom, mimicking natural environments, and showed that bacterial exudates could serve as substrates for diatom growth. Moreover, it was shown that diatom growth significantly modified the extracellular environment, which is ultimately dominated by the presence of diatom exudates, and that diatom and bacterial EPS composition varied over time and growth phase. Overall, the model-based phycosphere showed that these two microorganisms constitute representative models for the natural phycosphere, reaffirming the importance of analyzing simplified systems to unravel the complexity of the interactions occurring in diverse natural ecosystems.

AUTHOR CONTRIBUTIONS

Giulia Daly: Conceptualization (supporting); data curation (lead); formal analysis (lead); writing – original draft (lead). **Francesca Decorosi:** Methodology (supporting); writing – original draft (equal); writing – review and editing (equal). **Carlo Viti:** Conceptualization (supporting); supervision (equal); writing – review and editing (supporting). **Alessandra Adessi:** Conceptualization (lead); data curation (supporting); supervision (equal); writing – review and editing (lead).

ACKNOWLEDGEMENTS

The authors acknowledge the support of NBFC to the University of Florence-DAGRI, funded by the Italian Ministry of University and Research, PNRR, Missione 4 Componente 2, "Dalla ricerca all'impresa," Investimento 1.4, Project CN00000033.

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DATA AVAILABILITY STATEMENT

The data used to support the findings of this study are included within the article and its Supplementary Material.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Appendix S1 Supplementary methods, figures and tables supporting the main document.

How to cite this article: Daly, G., Decorosi, F., Viti, C., & Adessi, A. (2023). Shaping the phycosphere: Analysis of the EPS in diatom-bacterial co-cultures. *Journal of Phycology*, *59*, 791–797. https://doi.org/10.1111/jpy.13361

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