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1 **Investigating the role of extracellular polymeric substances produced by *Parachlorella***
2 ***kessleri* in Zn(II) bioremediation using atomic force microscopy**

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23 **Abstract**

24 Microalgae, such as *Parachlorella kessleri*, have significant potential for environmental remediation,
25 especially in removing heavy metals like zinc from water. This study investigates how *P. kessleri*,
26 isolated from a polluted river in Argentina, can remediate zinc. Using atomic force microscopy (AFM),
27 the research examined the interactions between Zn particles and cells grown with different nitrogen
28 sources—nitrate or ammonium. The results showed that cells grown with nitrate produced
29 extracellular polymeric substances (EPS), while those grown with ammonium did not. Raman
30 spectroscopy revealed distinct metabolic responses based on the nitrogen source, with nitrate-grown
31 cells showing altered profiles after zinc exposure. Zinc exposure also changed the surface roughness
32 and nanomechanical properties of the cells, particularly in those producing EPS. AFM force
33 spectroscopy experiments then confirmed strong Zn binding to EPS in nitrate-grown cells, while
34 interactions were weaker in ammonium-grown cells that lacked EPS. Overall, our results elucidate
35 the critical role of EPS in Zn removal by *P. kessleri* cells and show that Zn remediation by *P. kessleri* is
36 mediated by EPS adsorption. This study underscores the significance of regulating nitrogen sources
37 to stimulate EPS production, offering insights that are essential for subsequent bioremediation
38 applications.

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56 **Keywords**

57 *Parachlorella kessleri*, bioremediation, heavy metal, extracellular polymeric substances, atomic force
58 microscopy

59

60 1. Introduction

61 Rivers are the main source of clean water in many parts of the world. Their contamination
62 represents a problem not only for the populations living nearby, but also for the ecosystems linked to
63 the streams. The Reconquista River, located in the province of Buenos Aires, Argentina, is an example
64 of a heavily polluted river, as it receives tributaries from both domestic and industrial sources with
65 varying levels of contamination. Nutrients, such as nitrate and ammonium, and heavy metals are
66 among the pollutants found in the water and sediments (Basílico et al., 2022; Tufo et al., 2018).
67 Contamination levels vary between the upper, middle, and lower basins, and different biological
68 community compositions can be found along the river. In particular, microalgae are found in water
69 and sediment, even in the middle and lower Reconquista basins where contamination levels are
70 higher due to the discharge of industrial and domestic effluents. These microalgae are adapted to
71 live in extreme conditions, making them suitable for a variety of biotechnological applications
72 (Castañé et al., 1998; Ferraro et al., 2021; Grimolizzi, 2019).

73 Microalgae are highly diverse phototrophic organisms that play an important role in the
74 global carbon and nutrient cycles. They also have the capacity to produce commercially viable
75 metabolites for pharmaceutical, nutraceutical and industrial applications (Sreenikethanam et al.,
76 2022). Among their many interests, in recent years, they have attracted attention for their
77 remediation potential (Areco et al., 2021; Dayana Priyadharshini et al., 2021; Rude et al., 2022; Li and
78 Meng, 2023). Indeed, their presence in an aquatic environment can reduce high concentrations of
79 phosphate, nitrate and ammonium that generally cause eutrophication in rivers and lakes (Kakade et
80 al., 2021). Also, many studies demonstrate that some microalgae can interact with heavy metals and
81 reduce their concentrations in solution (Areco et al., 2018, 2022; Spain et al., 2021). Other studies
82 highlight the benefits of this bioremediation approach, noting that it can generate biomass for
83 producing commercially valuable byproducts as part of a biorefinery strategy (Satya et al., 2023;
84 Tripathi et al., 2023). It has been found that the main mechanisms involved in heavy metal
85 remediation by microalgae are metal precipitation due to pH changes linked to their nitrogen
86 metabolism (Areco et al., 2022) and passive adsorption, or biosorption, owing to their large surface
87 area and high binding affinity, which varies among species.

88 Biosorption is a surface phenomenon that occurs independently of microbial metabolism.
89 The size, shape, and cell wall composition of different algal species can affect their metal binding
90 capabilities. The process mainly involves the interaction between positively charged metal ions and
91 negatively charged cell surfaces (Gupta and Diwan, 2017). Even though many papers describe heavy
92 metal biosorption process (He and Chen, 2014; Volesky, 2007), for the moment, the molecular basis
93 of the interactions between the microalgae cell surface and heavy metals, and therefore the
94 mechanisms underlying their adsorption, are not entirely clear. For instance, an important question
95 that still requires clarification is the role played by extracellular polymeric substances (EPS) attached
96 to the cells in the adsorption of the heavy metals. EPS are a diverse group of macromolecules that
97 are secreted in relative high amounts by several genera of eukaryotic red and green microalgae, such
98 as *Parachlorella kessleri*, and some prokaryotic microalgae (Xiao and Zheng, 2016; Sendra et al.,
99 2017). Different factors, such as culture conditions, can potentially influence the generation and
100 structure of EPS, even those derived from the same species (Sasaki et al., 2020; Ye et al., 2022). They
101 can include polysaccharides, proteins, lipids, and nucleic acids, and can vary in composition and
102 structure depending on the microalgae species (Wang et al., 2018). EPS may provide many beneficial

103 functions for the microalgae, including energy storage, cell-to-cell communication, and protection
104 from environment stressors, among others. In addition, EPS can also play a role in shaping the
105 microalgae environment, by contributing to the formation of biofilms and aggregates and by
106 mediating the interaction with other organisms and/or abiotic components, such as dissolved
107 pollutants (Ciempiel et al., 2022). In this sense, they could also play a key role in the interaction with
108 heavy metals and in the further bioremediation capacities of cells, given that EPS have demonstrated
109 a remarkable affinity for heavy metals (Li et al., 2022).

110 Using microalgae adapted to live in polluted environments is crucial when studying the
111 possibility to apply bioremediation to contaminated waters (Afonso et al., 2024). In this sense,
112 several species have been isolated from the Reconquista River in order to study their remediation
113 potential. Among them is *Parachlorella kessleri*, a green microalgae species that generates EPS. *P.*
114 *kessleri* has been studied for its potential to grow with different nitrogen sources (O'Rourke et al.,
115 2015; Lv et al., 2019). Notably, in these studies, it was showed that depending on the nitrogen
116 source, nitrate or ammonium, the capacity of *P. kessleri* cells to remove pollutants from water was
117 different and was better when cells were in the presence of nitrate. Another study showed that *P.*
118 *kessleri* cells were able to remove heavy metals from water, in particular cadmium and chromium,
119 and could thus be considered as a bioremediation agent for this type of pollutants (Bauenova et al.,
120 2021). Furthermore, our team has obtained preliminary results indicating that the remediation of
121 Zn(II) by *P. kessleri* varies when cultivated in different nitrogen sources. Thus, in this study, we chose
122 to evaluate the interactions of Zn(II) and *P. kessleri* when cultivated in different nitrogen sources,
123 ammonium and nitrate. Our aim is to understand cell-Zn interactions when microalgae are cultured
124 with different nitrogen sources and elucidate the potential role of the EPS they produce in the
125 remediation. To conduct such study, we chose to work with atomic force microscopy (AFM) as a main
126 tool. Since its initial development in 1986 (Binnig et al., 1986), AFM has emerged as a powerful tool
127 for nanoscale surface characterization. Beyond its capabilities for high-resolution imaging down to
128 the nanometer scale, AFM is also a sensitive force machine capable of capturing forces at the
129 piconewton level (Dufrêne et al., 2017). This characteristic enables the exploration of
130 nanomechanical and adhesive properties of samples, as well as their interactions with their
131 environment. In the specific field of microalgae studies, AFM has been instrumental in unraveling the
132 morphology, nanostructure, nanomechanics, and adhesive behavior of cells (Formosa-Dague et al.,
133 2018; Demir-Yilmaz et al., 2021, 2023c). Notably, it has provided insights into their interactions with
134 particles or molecules present in their environment (Demir et al., 2020; Demir-Yilmaz et al., 2022)
135 and on the properties of the EPS they can produce (Vergnes et al., 2019).

136 In this work, we will first evaluate the effect of different nitrogen sources, ammonium and
137 nitrate, as well as Zn(II) on the metabolism of *P. kessleri* by determining the spectral fingerprints of
138 the cell metabolites, such as chlorophyll a, beta-carotene, nucleic acids and lipids, through Raman
139 spectroscopy. Then AFM will be used to understand the effect of the nitrogen source on the EPS
140 production, to evaluate the effects of the presence of Zn(II) on the cells and their EPS, and finally to
141 probe the interactions between Zn particles and cells at the molecular scale. This approach will
142 enable to assess the possibility of using *P. kessleri* cells to decontaminate heavy metals from water,
143 by providing a complete understanding of the mechanisms involved in the interactions between cells
144 and the Zn(II) present in the medium. Such findings could have great impacts on environmental
145 management and to develop efficient bioremediation strategies.

146 **2. Experimental Section**

147 **2.1. *Microalgae strain and culture***

148 *P. kessleri* was obtained from the culture collection of the Environmental Research and
149 Engineering Institute (IIIA CONICET-UNSAM). This strain was previously isolated from the sediments
150 of the middle basin of the Reconquista River, at San Francisco Park (S 34°34'14.8" W 58°39'46.4").
151 Cells were cultivated in sterile conditions in two different nutritive condition. The culture medium
152 used was Bold Basal Medium (BBM) containing either NaNO₃ (BBM-NO₃⁻, nitrate condition) or
153 (NH₄)₂SO₄ (BBM-NH₄⁺, ammonium condition) as a nitrogen source, with the same initial nitrogen
154 concentration (≈ 2.9 mM). Initial pH comprised between 6 and 6.5. The cultures were maintained at
155 21°C, under 120 rpm of orbital agitation, in an incubator (Panasonic, Japan) equipped with white
156 neon light tubes providing an illumination of approximately 40 μmol photons m⁻² s⁻¹, with a
157 photoperiod of 18 h light: 6h dark. All experiments were performed with 7 days exponential phase
158 batch cultures. For cultures in the presence of Zn(II), the same procedure was conducted, but an
159 aliquot of a concentrated solution of ZnSO₄·7H₂O (30 mM of Zn(II)) was added to each culture (BBM-
160 NO₃⁻ and BBM-NH₄⁺), resulting in a final concentration of 0.25 mM of Zn(II). The initial pH was
161 adjusted to 5.5 to avoid metal precipitation.

162 **2.2. *Cellular metabolites characterization using RAMAN spectrometry***

163 The Raman spectra were obtained from freeze-dried pellets of *P. kessleri* using a Raman
164 microspectrometer Bruker Optics, (Senterra, Germany) driven by Opus software (Bruker Optics,
165 v7.2). This device was equipped with a CCD camera cooled at - 60°C and a BX51 Olympus microscope.
166 Spectral data were collected using a 532 nm excitation laser with a power output of 2mW at the
167 sample. Each spectrum was obtained by averaging five sequential acquisitions, each with an
168 integration time of 10 seconds (Lieutaud et al., 2019). Raman spectra were processed using MATLAB
169 software (version R2019b, MathWorks Inc, Natick, MA, USA). The raw data, covering a spectral range
170 of 170–4000 cm⁻¹, was first cut in the spectral zone of 170–1600 cm⁻¹. All spectra were subsequently
171 baseline corrected employing an elastic concave method (64° and ten iterations), smoothed using
172 the Savitzky-Golay algorithm, and finally normalized via min–max normalization. The treated spectra
173 were subjected to multivariate data analysis, specifically Principal Component Analysis (PCA). The
174 optimal number of principal components (PCs) was determined using a scree plot. Then, PC scores
175 (specifically PC1 scores) were subjected to the Kruskal Wallis (KW) test, a non-parametric method for
176 comparing samples from three or more groups of independent observations (Kruskal and Wallis,
177 1952). Both PCA and KW were computed using the SAISIR Package (Cordella and Bertrand, 2014). All
178 statistical analysis was performed using MATLAB software (version R2019b, MathWorks Inc., Natick,
179 MA, USA).

180 **2.3. *Cell immobilization for AFM experiments***

181 Before AFM experiments, 1 mL of cells from 7-days cultures were diluted into 5 mL of either
182 BBM-NO₃⁻ or BBM-NH₄⁺. After that, cells were immobilized on polyoctyl-chitosan (PO-chitosan)
183 coated glass slides. For that, briefly, a 1.0 g L⁻¹ solution of PO-chitosan synthesized in a previous study
184 was spin-coated on plasma cleaned glass surfaces at 1000 rpm for 3 min (Demir-Yilmaz et al., 2023a).
185 The glass slides were then dried in an incubator at 37°C overnight before use. Then, 1 mL of cell
186 suspension was deposited on the surface, incubated during 30 min, and rinsed using either BBM-NO₃⁻
187 or BBM-NH₄⁺ before performing the AFM experiments.

188 **2.4. *AFM imaging and roughness analysis***

189 Height images of the whole cells were recorded using the Quantitative Imaging mode
 190 available on the Nanowizard IV XP AFM (Bruker, USA), with MLCT cantilevers (Bruker, nominal spring
 191 constant of 0.01 N m^{-1}). Images were recorded with a resolution of 150×150 pixels using an applied
 192 force comprised between 0.8 and 1.5 nN and a z-length comprised between 1 and 6 μm . In all cases,
 193 the cantilevers spring constants were determined using the thermal noise method prior to imaging
 194 (Hutter and Bechhoefer, 1993). To obtain high-resolution height images of the cell surface for
 195 roughness analysis, small areas of $0.5 \times 0.5 \mu\text{m}$ on top of cells were recorded using the contact mode
 196 available on the Nanowizard IV XP AFM (Bruker, USA), with MLCT cantilevers (Bruker, nominal spring
 197 constant of 0.01 N/m), at a resolution of 512 lines, using an applied force $< 1 \text{ nN}$. The height images
 198 obtained were then analyzed using the data processing software (Bruker, USA) to determine the
 199 arithmetic average roughness (Ra). In each condition, 10 different images were recorded on 10
 200 different cells coming from at least two independent cultures. To assess the significance of the
 201 differences observed between the different conditions tested, non-parametric statistical Mann and
 202 Whitney test was used. The differences were considered significant at p-value < 0.05 .

203 **2.5. AFM nanomechanical analysis**

204 For nanoindentation experiments, the AFM was used in force spectroscopy mode using an
 205 applied force comprised between 0.5 and 5 nN depending on the condition, with MLCT cantilevers
 206 (Bruker, nominal spring constant of 0.01 N m^{-1}). Young's moduli were then calculated from 70 nm
 207 indentation curves in the case of cells grown in BBM-NH_4^+ , and 500 nm indentation curves in the case
 208 of cells grown in BBM-NO_3^- using the Hertz model (Hertz, 1881) in which the force F , indentation (δ),
 209 and Young's modulus (Y_m) follow Equation 1, where α is the tip opening angle (17.5°), and ν the
 210 Poisson ratio (arbitrarily assumed to be 0.5). The cantilevers spring constants were determined by
 211 the thermal noise method (Hutter and Bechhoefer, 1993):

$$F = \frac{2 \times Y_m \times \tan \alpha}{\pi \times (1 - \nu^2) \times \delta^2} \quad (1)$$

212 In each condition, 10 different cells coming from at least 2 independent cultures were probed. To
 213 assess the significance of the differences observed between the different conditions tested,
 214 parametric statistical student t-test was used. The differences were considered significant at p-value
 215 < 0.05 .

216 **2.6. AFM dynamic mechanical analysis (DMA)**

217 For these experiments, colloidal probes were used. These were prepared as previously
 218 described in (Beaussart et al., 2014). In brief, a single silica microsphere (5 μm of diameter, Bangs
 219 Laboratories) was attached to triangular tipless NP-O10 cantilevers (Bruker, USA) using a thin layer of
 220 UV-curable glue (NOA 63, Norland Edmund Optics). Then, measurements were performed by
 221 applying a determined force of 1 nN (initial indentation), before applying sinusoidal excitation signals
 222 at frequencies of 1, 10, 20, 50 and 100 Hz. During these measurement, in-phase and out-phase
 223 signals are detected, and further used to determine the shear modulus G^* using the following
 224 Equation 2, where G' is the storage modulus, G'' is the loss modulus, i is the complex unit, ν the
 225 Poisson ratio (assumed to be 0.5), δ_0 the initial indentation, R the spherical colloidal probe radius
 226 (here of 2.5 μm), δ^* the sinusoidal indentation, and F^* the sinusoidal force.

$$G^* = G' + iG'' = \frac{1 - \nu}{4\sqrt{R\delta_0}} \frac{F^*}{\delta^*} \quad (2)$$

227 From these measurements, the G' and G'' values were extracted from 70 curves recorded on 23 cells
228 for each condition, and $\tan(\delta)$ was calculated, which corresponds to the ratio of G'' over G' .

229

230 **2.7. AFM cantilevers functionalization with Zn particles and force spectroscopy experiments**

231 To prepare functionalized AFM cantilevers with Zn particles, the edge of NP-O10 tipless
232 cantilevers (Bruker, USA) was first dipped into a thin layer of UV-curable glue (NOA63, Norland
233 Edmund Optics). Then the cantilever was approached towards a metallic Zn particle deposited on a
234 glass surface to attach it at the edge of it. The functionalized cantilevers were then put under UV-
235 light for 10 min to allow the glue to cure. These functionalized cantilevers were used in force
236 spectroscopy experiments to probe the interactions with cells grown either in BBM-NO_3^- or in BBM-
237 NH_4^+ for 7 days. The interactions were recorded with an applied force of 0.25 nN, at a speed of $1 \mu\text{m}$
238 s^{-1} , using a contact time of 1 s. In each condition, 10 different cells coming from at least 2
239 independent cultures were probed using 5 different probes. To assess the significance of the
240 differences observed between the different conditions tested, parametric statistical student t-test
241 was used. The differences were considered significant at p-value < 0.05.

242 **2.8. Scanning electron microscopy imaging of AFM cantilevers**

243 AFM cantilevers with tips functionalized or not were imaged using a Jeol 6400 electron
244 microscope (Jeol, Tokyo, Japan) equipped with an EDS Bruker SDD detector, at an acceleration
245 voltage of 20 kV.

246

247 **3. Results and Discussion**

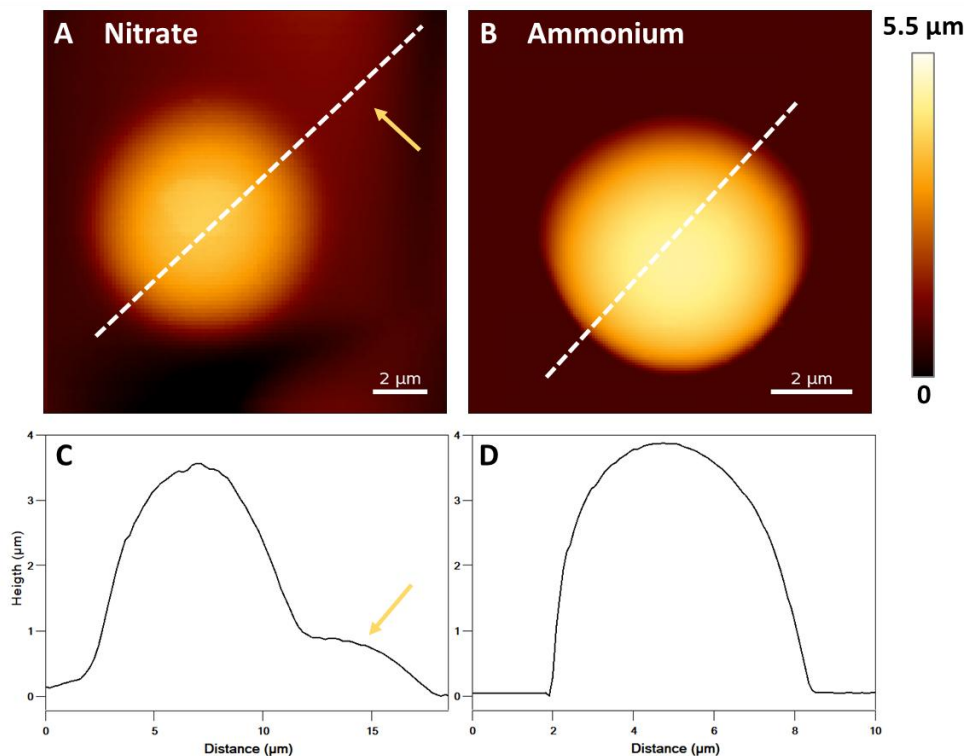
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249 **3.1. The nitrogen source influences the production of EPS by cells**

250 It is well known that nutritive conditions can affect the production of EPS by microalgae cells.
251 This has already been showed for different microalgae species (Wang et al., 2014; Babiak and
252 Krzemińska, 2021), and in the case of *P. kessleri*, one study has showed that a deprivation in nitrogen
253 could lead to an overproduction of EPS by the cells (Sasaki et al., 2020). In addition, a study
254 performed in 1994 showed that in the case of *Botryococcus braunii*, the amount of EPS produced was
255 different depending on the nitrogen source. In particular, it was showed that the highest production
256 of EPS was detected in the presence of nitrate and that the lowest yield was obtained in the presence
257 of ammonium (Lupi et al., 1994). To verify if this was also the case for the *P. kessleri* cells used in this
258 study, we first used AFM to image cells and detect the presence of EPS in the two different nutritive
259 conditions. The results obtained are presented in Fig. 1. In the case of cells grown in the presence of
260 nitrate (Fig. 1A), cells present a spherical shape, but the edges of the cells are not well-resolved,
261 indicating that the tip could not get into contact with them during the acquisition. Moreover, as
262 indicated by the arrow on Fig. 1A, some material seems to surround the cell. This is confirmed by the
263 cross-section taken along the white dashed line (Fig. 1C) that shows that indeed, the cell is
264 surrounded by a layer of approximately 1 μm in height, that could correspond to EPS. When cells are
265 grown with ammonium (Fig. 1B), the AFM image obtained shows also a spherical cell but this time,
266 the edges are well-resolved, which tends to indicate that no layer of material could prevent the tip to
267 get into direct contact with the cell. This is also confirmed by the cross-section (Fig. 1D) that shows
268 that no material surrounds the cell. Thus, these imaging results indicate that cells grown with nitrate
269 produce a layer of material, most likely EPS, that is not detected when they grow with ammonium.
270 This was further confirmed by SEM imaging experiments, (Fig. S1) which also demonstrated that EPS
271 are present in a greater extent when cells are grown with nitrate than when they are grown with
272 ammonium. It is important to note that the EPS observed on ammonium-grown cells in SEM images
273 might be soluble EPS, made visible due to the sample preparation process. These EPS most likely are
274 not bound to the cell surface, explaining why they are not detected under the physiological
275 conditions in which the AFM experiments were conducted.

276

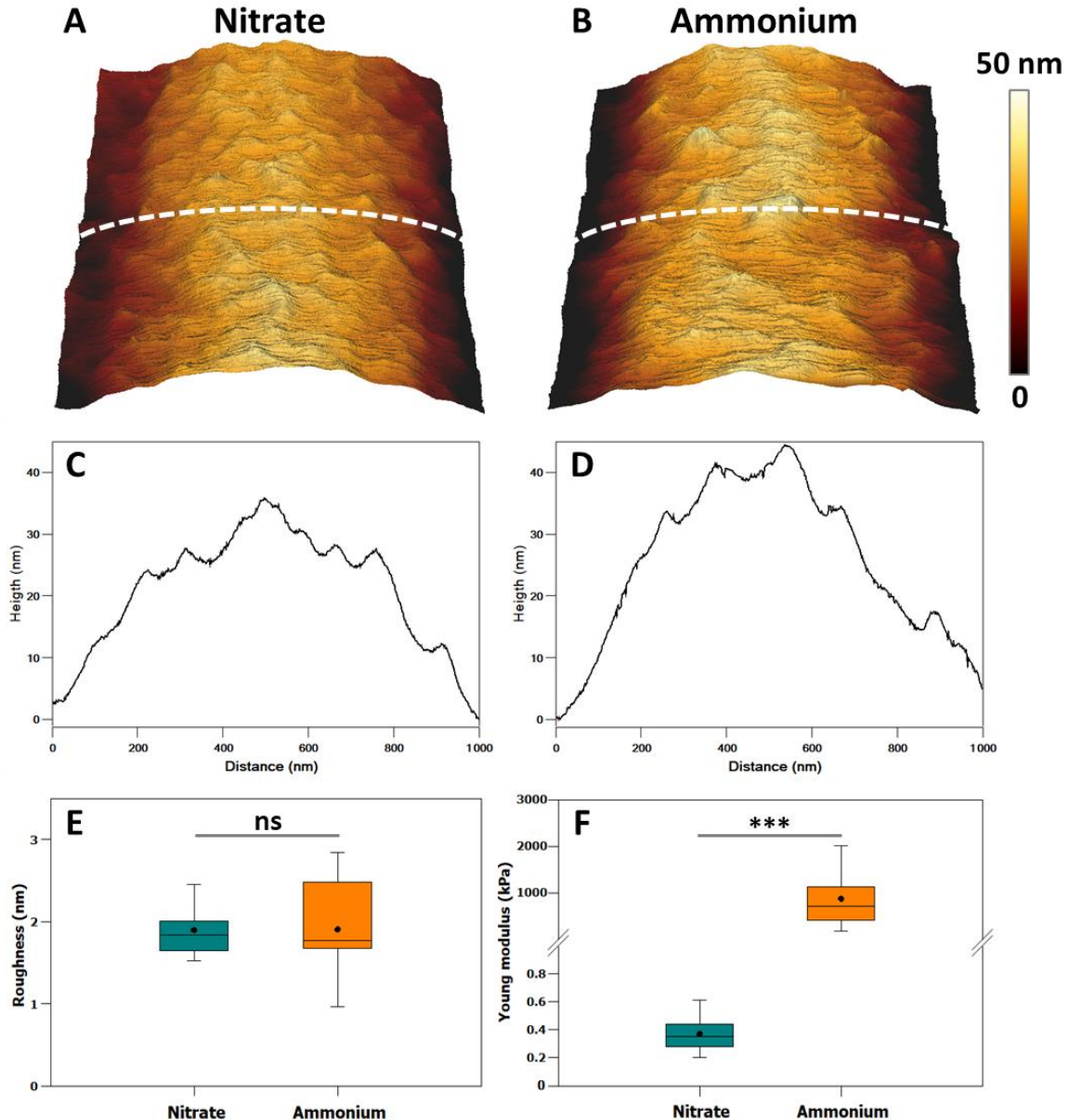
277



278 **Fig. 1. AFM imaging of *P. kessleri* in different nutritive conditions.** A) AFM height image of a *P. kessleri* cell
 279 grown with nitrate and B) grown with ammonium. The yellow arrow in A) indicates the presence of a layer of
 280 material surrounding the cell. C) and D) Cross-section taken along the white dashed line in A) and B)
 281 respectively. The yellow arrow in C) corresponds to the layer of material observed in A).

282 We then further characterized the EPS layer surrounding the cells by performing two
 283 different types of experiments: roughness measurements and nano-indentations experiments. First,
 284 to perform roughness measurements, images of small areas of $0.5 \times 0.5 \mu\text{m}$ were recorded on top of
 285 the cells using AFM in contact mode. This recording mode enables the acquisition of images with
 286 nanoscale resolution, offering the capability to unveil the ultrastructure of the cell surface. The
 287 images obtained for both cells grown with nitrate or with ammonium are showed in Fig. 2A and B.
 288 They both show a rather rough surface, featuring structures which are several nm in height, as
 289 indicated by the cross-section taken along the white lines in both images (Fig. 2C and D). Quantitative
 290 analysis was then performed using images recorded on 10 different cells in each condition to
 291 determine the average roughness, which is of $1.9 \pm 0.3 \text{ nm}$ for cells grown with nitrate, and of $1.9 \pm$
 292 0.7 nm for cells grown with ammonium (Fig. 2E). Cells in both conditions thus exhibit a similar surface
 293 structure, which is surprising. Indeed, in the case of cells grown with nitrate, the cells are covered by
 294 an EPS layer, and this is the EPS layer that should be imaged. While for cells grown in the presence of
 295 ammonium, there is no EPS layer on top of cells thus the images show directly the cell surface. There
 296 are two options; either both the EPS and the cell surface have a similar ultrastructure, or one
 297 possibility is that the EPS layer on top of cells is too soft, so that the tip goes through it directly and
 298 touches the cell surface. While EPS of microalgae cells have been imaged using AFM when deposited
 299 on surfaces (Cybulska et al., 2016) or in dried conditions (Demir-Yilmaz et al., 2023b), no images of
 300 EPS imaged directly on top of cells in liquid conditions have been reported. However, one previous
 301 AFM study conducted in our team showed that EPS produced by a cyanobacteria formed a gel-like
 302 material that was extremely soft (approximately 20 kPa). If this is also the case for *P. kessleri* cells, it
 303 could elucidate why the force applied by the tip in contact imaging, even when kept at a low level,

304 might be enough to penetrate the EPS layer. To verify this point, we then performed nano-
305 indentation experiments to determine the nanomechanical properties of cells grown in both nutritive
306 conditions. In this type of experiment, the tip, with known mechanical properties, is pressed against
307 the surface at a known applied force and retracted. This gives access to nano-indentation curves,
308 that can then be fitted by a model to extract the Young modulus (Y_m) value, that corresponds to the
309 rigidity of the cell surface, *i.e.* its resistance to compression. The lower the Y_m value, the softer the
310 material. The results obtained by probing 10 different cells in each condition show an important
311 difference between the two nutritive conditions. Indeed, cells cultured with nitrate exhibit a
312 remarkably low average Young's modulus (Y_m) value of 0.4 ± 0.1 kPa. In contrast, for cells cultivated
313 with ammonium, the average Y_m value is higher at 880.1 ± 727.4 kPa (Fig. 2F). The value recorded for
314 ammonium cells is in line with measurements performed on the cell wall of other microalgae species
315 (Demir et al., 2020; Demir-Yilmaz et al., 2023c), and reflects the rigidity of the cell wall. However, the
316 value obtained for cells grown with nitrate is very low, and most likely corresponds to the
317 nanomechanical properties of the EPS present on top of the cells, that form, like it was previously
318 showed, a gel-like structure (Vergnes et al., 2019). Given this, it is then most likely that the contact
319 image recorded (Fig. 2A) corresponds to the cell wall and not to the EPS layer. Thus, it can be
320 concluded that the different nitrogen source does not have an effect on the cell wall ultrastructure,
321 even though the metabolism of cells may be different in these two conditions.



322 **Fig. 2. AFM characterization of *P. kessleri* cell surface.** A) AFM height image of an area of $0.5 \times 0.5 \mu\text{m}$ on top
 323 of a cell grown in the presence of nitrate or B) in the presence of ammonium. C) and D) Cross-sections taken
 324 along the white dashed lines in A) and B) respectively. E) Boxplot showing the distribution of roughness values
 325 obtained on 10 different cells coming from at least two independent cultures for both nutritive conditions. F)
 326 Boxplot showing the distribution of Y_m values recorded on 10 different cells coming from at least two
 327 independent cultures for both nutritive conditions.

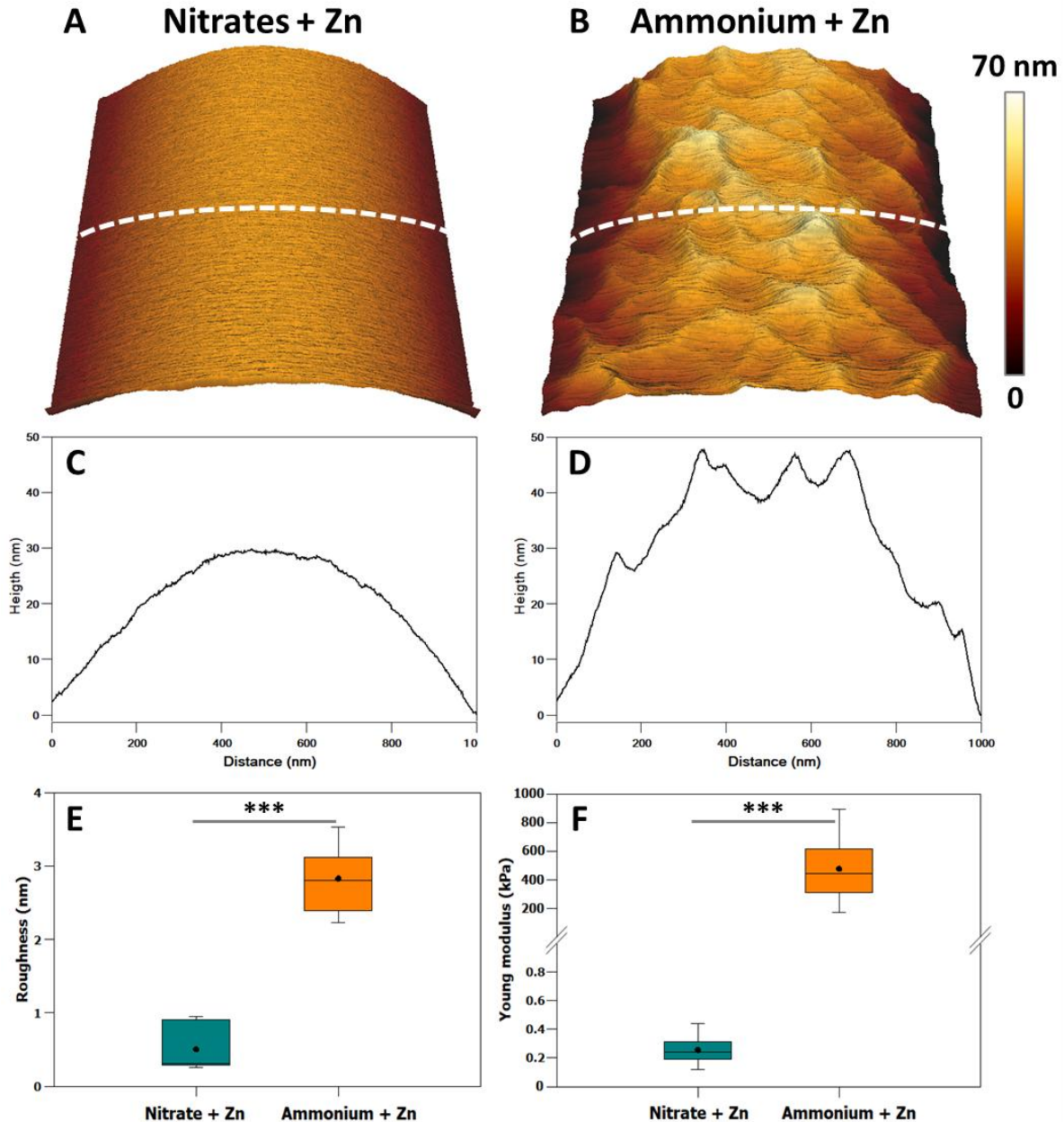
328

329 **3.2. Exposure of cells to Zn affects their surface properties**

330 In a next step, we then repeated these experiments with cells exposed during culture to a
 331 Zn(II) concentration of 0.25 mM in both nutritive conditions. The first aspect was to check if the
 332 exposure to Zn could have an impact on the EPS production, as in some cases, it has been showed
 333 that the exposure of cells to a stress could result in the overproduction of EPS by the cells (Pletikapić
 334 et al., 2012). For that, AFM images of the whole cells were recorded, and showed no differences with
 335 the ones obtained with cells that had not been exposed to Zn(II) (Fig. S2). Thus it does not seem that
 336 Zn(II) exposure has an effect on the EPS production. We then took a closer look at the surface of cells

337 and measured their roughness and nanomechanical properties. The results obtained are presented in
338 Fig. 3. The ultrastructure observed for cells without Zn(II) (Fig. 2A and B) seems to be conserved only
339 in the case of cells grown with ammonium. Indeed, cells grown with nitrate present a smooth surface
340 in the presence of Zn(II) with no structures emerging from the surface as indicated by the cross-
341 section in Fig. 3C. For cells grown with ammonium, the cross-section indicate the presence of
342 structures at the surface of cells that seem to have a height increased compared to the condition
343 without Zn(II). To confirm these observations, the quantitative analysis on the images recorded
344 showed that the average roughness in the case of cells grown with nitrate is of 0.5 ± 0.3 nm, and of
345 2.8 ± 0.5 nm for cells grown with ammonium (Fig. 3E). Thus, the response of the cells to the presence
346 of the pollutant in the culture medium is different depending on the nitrogen source used. For cells
347 grown with nitrate, the cell wall becomes smooth, while for cells grown with ammonium, it becomes
348 rougher, featuring higher structures on its surface. Regarding the nanomechanical properties, there
349 are also important changes compared to the conditions without Zn(II). For instance, for cells grown
350 with nitrate, the average Y_m value decreases from 0.4 ± 0.1 kPa to 0.2 ± 0.1 kPa with Zn(II) and for
351 cells grown with ammonium, it decreases from 880.1 ± 727.4 kPa to 478.3 ± 225.1 kPa in the
352 presence of Zn(II) (the differences in both cases are significant at a p-value of 0.001, student t-test).

353 A few AFM studies of the microalgae cell surface changes induced by an exposure to heavy
354 metal pollutant are available in the literature. In one of this study, the effects of cadmium were
355 evaluated on a green microalgae species, *Dunaliella tertiolecta* (Ivošević DeNardis et al., 2019). Their
356 results showed that cells in the presence of cadmium had a cell wall 80% more rigid compared to
357 control cells. While our results are opposite in the sense that cells get softer in the presence of Zn(II),
358 they are still in line with the fact that heavy metal exposure has an impact on the cell's
359 nanomechanical properties. Moreover other stress conditions have been studied using AFM, such as
360 salinity changes (Demir-Yilmaz et al., 2023b), N-deprivation (Yap et al., 2016), or pH changes
361 (Formosa-Dague et al., 2018; Pillet et al., 2019). All these different stresses resulted in changes in the
362 nanomechanical properties of cells. These alterations are frequently linked to modifications either in
363 the biochemical composition of the cell wall, which can shift as cells adjust their metabolism in
364 response to stress, or to a restructuring of the cell wall architecture. This restructuring implies that
365 the various components of the cell wall can be reorganized to adapt to the stress (Demir-Yilmaz et
366 al., 2023c). Regarding cell surface roughness, this parameter has also been showed to be impacted by
367 stress condition in microalgae. For instance, in the case of *C. vulgaris*, our team has previously
368 showed that both a salinity change in the culture medium or a change in the pH had for consequence
369 to increase the cell wall roughness (Demir et al., 2020; Demir-Yilmaz et al., 2023c). In these
370 investigations, it was proposed that this might be attributed to a rearrangement of molecules at the
371 cell surface, protruding more prominently under stress conditions, consequently causing the
372 observed increased roughness.



373
 374 **Fig. 3. AFM characterization of *P. kessleri* cell surface upon exposure to Zn(II).** A) AFM height image of an area
 375 of $0.5 \times 0.5 \mu\text{m}$ on top of a cell grown in the presence of nitrate and Zn(II) at a concentration of 0.25 mM or B) in
 376 the presence of ammonium and Zn(II) at a concentration of 0.25 mM. C) and D) Cross-sections taken along the
 377 white dashed lines in A) and B) respectively. E) Boxplot showing the distribution of roughness values obtained
 378 on 10 different cells coming from at least two independent cultures for both nutritive conditions in the presence
 379 of Zn(II). F) Boxplot showing the distribution of Ym values recorded on 10 different cells coming from at least
 380 two independent cultures for both nutritive conditions in the presence of Zn(II).

381

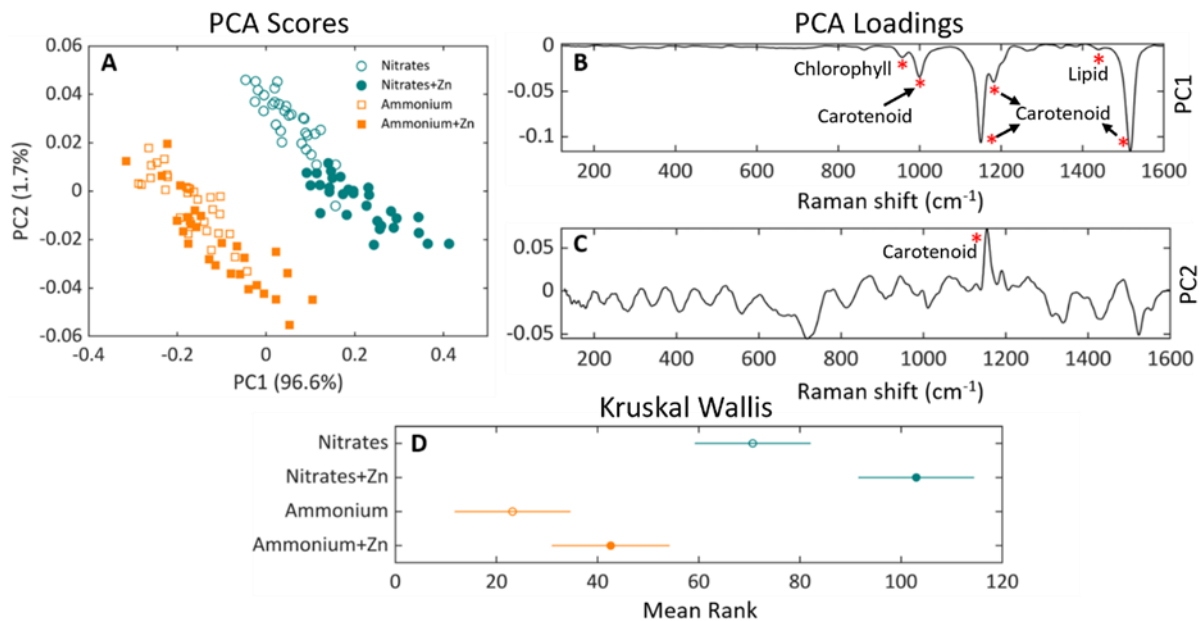
382 **3.3. The metabolism of *P. kessleri* is affected both by the nitrogen source and the exposure to**
 383 **Zn(II)**

384 In a next step, Raman spectroscopy was employed to investigate the impact of different
 385 culture media and Zn(II) exposure on the metabolism of *P. kessleri* cells. The Raman spectrum,
 386 detailed in the supplementary data (Fig. S3), highlights key peaks or molecule of interest detected

387 within the cells when they are exposed to the Raman laser. Microalgae, in general, exhibit common
388 bands or Raman shifts at 957 $\delta(\text{C-H}_3)$, 1182 $\delta(\text{C-H}_3)$, 997 $\nu(\text{C-C})$, 1149 $\nu(\text{C-C})$, 1182 and 1571 $\nu(\text{C-C})$ cm^{-1}
389 ¹, which are associated with chlorophylls and carotenoids. Additionally, a shift at 1436 cm^{-1} $\sigma(\text{C-H}_2)$
390 indicates the presence of lipids (Wieser et al., 2023). To assess the effect of the different nitrogen
391 sources (nitrate and ammonium) and of Zn(II) exposure on *P. kessleri* cells, a PCA multivariate
392 statistical analysis was applied to the Raman spectra obtained in the different conditions. Fig. 4A
393 shows that the first principal component PC1 accounts for the majority of spectral variances,
394 explaining about 96.6 % of the variance, while the second principal component PC2 explains only
395 1.7%. All other principal components contained less than 1% of the variance between samples. The
396 PCA plot demonstrates a clear separation between cells cultured with ammonium, indicated by
397 negative score values on PC1, and those cultured with nitrate, indicated by positive sign on PC1. It
398 seems that all bands with a negative sign on loading 1 (Fig.4B) are associated with cells cultured in
399 ammonium as they have a negative sign on PC1 scores. This indicates that *P. kessleri* cells grown in
400 culture medium with ammonium are strongly associated with higher level of chlorophyll, carotenoids
401 and lipids as indicated by the negative loadings on PC1. In contrast, cells cultured with nitrate did not
402 show the same high level of association with these biomolecules. This is further confirmed by the
403 Kruskal Wallis test performed on PC1 scores, which shows that cells cultured with ammonium are
404 significantly different ($p < 0.05$) from those grown in nitrate (Fig 4D). These results thus show that the
405 nitrogen source has an impact on the metabolism of cells, ammonium inducing the production in
406 cells of more pigments (chlorophyll and carotenoids) and more lipids compared to nitrate. While
407 Raman spectroscopy has already been used to evaluate the impact of nitrogen concentration in the
408 culture medium (Zhang et al., 2017; He et al., 2018; Li et al., 2019), this is the first time that the
409 impact of a different nitrogen source is evaluated. What is interesting to note is that in both cases,
410 pigments and lipid contents were affected either by a changing nitrogen concentration, or in our case
411 by a different nitrogen source. Our results are thus in line with existing literature on this topic.

412 Regarding the effect of adding Zn(II) to both cultures conditions, cells cultured with
413 ammonium did not exhibit any significant changes as confirmed by PC1 results and Kruskal Wallis (no
414 significant difference, $p > 0.05$). However, for cells cultured with nitrate as the nitrogen source, the
415 addition of Zn(II) had a negative impact. This is indicated by loading 2 (Fig 4C), where carotenoids
416 were more associated with cells cultured with nitrate and without Zn(II). The Kruskal Wallis test also
417 indicated a significant difference between the latter two groups (Fig 4D). It is interesting to compare
418 and correlate these results with the ones obtained using AFM. Indeed, in the case of cells grown with
419 nitrate, AFM experiments showed that both the roughness and the mechanical properties of the EPS
420 layer around the cells were impacted by the exposure to Zn(II). The Raman results obtained here
421 show that these changes on the “outside” of the cell, are also associated with changes in the cell’s
422 metabolism as they present a lower level of carotenoids than cells that were not exposed to Zn(II).
423 For cells grown with ammonium, while the cell’s surface is also modified by the presence of Zn(II) in
424 the medium (roughness and mechanical properties changed), cells do not have a modified
425 metabolism. These observations suggest the following hypothesis: although both types of cells are in
426 contact with the Zn(II) present in the culture medium, as indicated by the surface changes, only the
427 cells grown with nitrate, which produce EPS, are affected by Zn(II), generating stress, as evidenced by
428 metabolic changes observed here with Raman spectroscopy. The production of EPS by microalgae
429 can have varying effects on the absorption of metals like Zn(II), that can be influenced by the
430 microalgal species, environmental conditions, and the properties of the EPS. Indeed, EPS can either

431 hinder metal absorption by creating a protective layer around the cells, thus limiting direct metal
 432 uptake, or promote metal adsorption by forming complexes with the metal ions, which enhances the
 433 microalgae's ability to absorb these ions from the environment (Babiak and Krzemińska, 2021;
 434 Tripathi and Poluri, 2021). Furthermore, the solubility of EPS-metal complexes can influence the
 435 bioavailability of metals to microalgae (Hasani Zadeh et al., 2023). The results here obtained would
 436 suggest that EPS favors the absorption of the Zn present in the medium. To confirm this hypothesis,
 437 further investigation into the interactions between Zn(II) and the cells is necessary. Specifically, it
 438 should be verified whether the interaction of Zn(II) with the EPS produced by cells grown with nitrate



439 is indeed favored, thereby confirming the role of EPS in Zn(II) absorption into cells.
 440 **Fig. 4. PCA analysis of Raman spectra from Parachlorella kessleri cultivated using BBM with different**
 441 **nitrogenous compounds.** (A) The PC1 scores of *P. kessleri* cultivated with ammonium or ammonium + Zn are
 442 clearly distinguished from the two other groups. (B) The loading plot highlights the impacted molecules
 443 (represented by red asterisks) responsible for differentiating *P. kessleri* cultivated with ammonium or
 444 ammonium + Zn from the other groups on PC1. (C) The loading plot highlights the impacted molecules
 445 responsible for differentiating *P. kessleri* cultivated with nitrates from those cultivated with nitrates + Zn based
 446 on PC2. (D) Kruskal-Wallis on PC1 scores identify the groups that are significantly different from each other ($p <$
 447 0.05).

448 3.4. The EPS produced by cells interact and absorb zinc

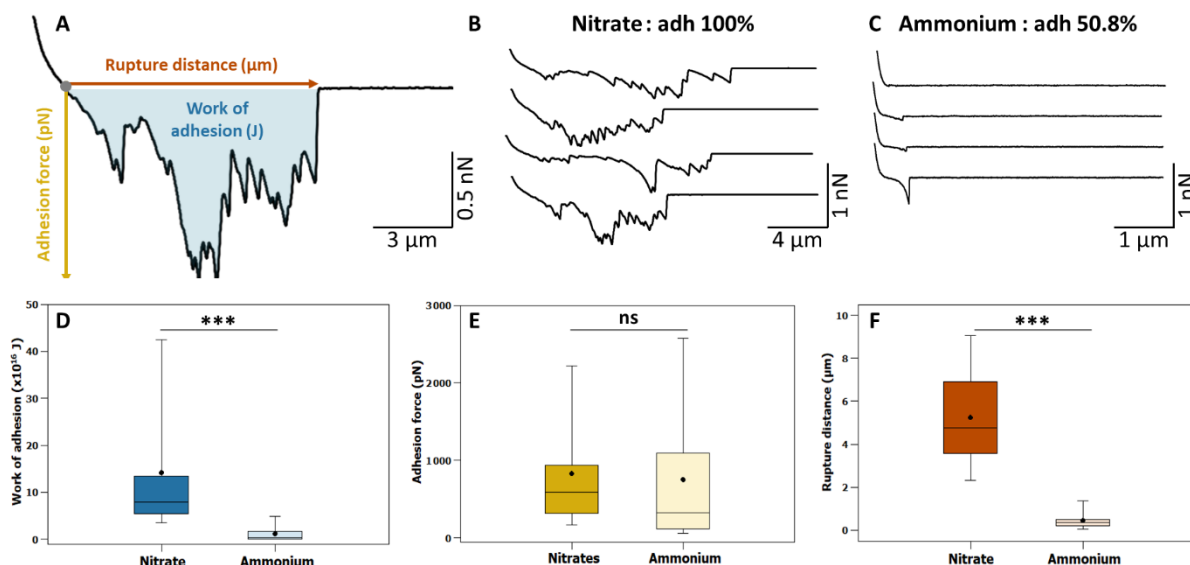
449 To investigate the potential role of EPS in the interactions with Zn, we then conducted force
 450 spectroscopy experiments to probe the direct interactions between metallic Zn particles
 451 functionalized on cantilevers, and the surface of cells grown in both nutritive conditions. To
 452 functionalize the cantilever, a protocol that was previously developed in our team was used
 453 (Formosa-Dague et al., 2018), which allowed to produce cantilevers with a single particle attached at
 454 the edge of it, as it can be seen on the SEM images presented in Fig. S4. While AFM has already
 455 been used to characterize the effects of pollutants on microalgae cells, this is the first time the direct
 456 interactions between a pollutant and the surface of cells are probed. The results of the force
 457 spectroscopy experiments obtained are presented in Fig. 5. In these experiments, the functionalized
 458 cantilever is first approached towards the surface of the cells, stays in contact with it during 1 s, and
 459 is then retracted. This gives access to force curves, an example of which is presented in Fig. 5A. From

460 these force curves, a number of information on the interactions measured can be extracted. First, if
461 an interaction takes place between the cantilever and the cell surface, the curves will present retract
462 adhesions, that are materialized by negative peaks on the curve. By measuring the maximum height
463 of these retract adhesions, it is possible to access to the adhesion force, *i.e.* the maximum force that
464 was needed to break the interaction upon retraction (in yellow in Fig. 5A). Then, the area under the
465 curve can be measured, which gives access to the work of adhesion, that represents the total
466 quantity of energy that was needed to break the interaction (in blue on Fig. 5A). For both adhesion
467 force and work of adhesion, higher values indicate a stronger interaction between the cantilever and
468 the sample. Finally, the rupture distance can be determined, which corresponds to the distance
469 between the contact point (when the cantilever touches the surface) and the last adhesion peak on
470 the curve (in red on Fig. 5A). This parameter is a function of the length of the molecules that are
471 unfolded from the sample surface upon retraction. These three parameters allow describing the
472 interactions recorded.

473 In a first step we examined the force curves obtained (Fig. 5B and C). When probing the
474 interactions with cells grown with nitrate (Fig. 5B), the force curves all presented multiple retract
475 adhesions on long distances, without a defined pattern. These force curves indicate the elongation of
476 extended molecules from the cell wall interacting with Zn (Formosa et al., 2012; Feuillie et al., 2017).
477 In the case of cells grown with ammonium, only 50.8% of the force curves presented adhesion peaks,
478 meaning that the adhesion probability between Zn and cells in this condition is much lower. Then the
479 curves that presented retract adhesions presented a small, single peak, close to the contact point.
480 These first observations already show a clear tendency of Zn to interact with EPS but not with the cell
481 wall. To quantify these differences, both the work of adhesion and the maximum adhesion force was
482 measured (Fig. 5D and E). The adhesion forces recorded showed no significant differences between
483 the two conditions: the average values are of 832.1 ± 1063.9 pN for cells grown with nitrate as the
484 nitrogen source and of 764.9 ± 960.0 pN for cells grown with ammonium (Fig. 5E). Note the high
485 heterogeneity of the measurements, which can be attributed to the natural heterogeneity of living
486 cells and the potential differences in the size of the Zn particles used in the experiments. However,
487 when measuring the work of adhesion, a significant difference can be observed between the two
488 conditions, as the average value is of $14.2 \times 10^6 \pm 26.8 \times 10^6$ J for cells grown with nitrate and of $1.2 \times$
489 $10^6 \pm 2.0 \times 10^6$ J for cells grown with ammonium (Fig. 5D). Thus, while the force of the interaction is
490 the same between the two conditions, which could mean that the same molecules are involved in
491 the interaction, the amount of energy needed to break the interaction, and thus the number of
492 molecules involved in the interactions is much larger in the case of cells grown with nitrate. Finally,
493 regarding the rupture distances measured, they are of 5.2 ± 2.1 μm for cells grown with nitrate and
494 of 0.4 ± 0.5 μm for cells grown with ammonium. These results mean that for cells grown with nitrate,
495 upon retraction of the functionalized cantilever, long and most likely several molecules are unfolded
496 from the surface, while this is not the case for cells grown with ammonium. To make sure that the
497 interactions recorded correspond to interactions between Zn and cells, these experiments were then
498 repeated in the same conditions (same applied force and contact time) with bare AFM tips. The
499 results showed that in the case of cells grown with nitrate, 86.3% of the force curves obtained
500 presented no adhesions, and that the adhesions recorded were of 40.5 ± 40.3 pN (Fig. S5A). For cells
501 grown with ammonium, a similar % of force curves interact with the cantilever (54.0%) with a level of
502 force significantly lower, of 365.3 ± 412.8 pN (Fig. S5B). These control experiments thus prove that
503 the interactions recorded with Zn particles are indeed due to the presence of the Zn particles on the

504 cantilevers. Altogether these results allow us to draw two conclusions: the first one is that Zn
 505 particles can interact with the cell wall of *P. kessleri* cells, but these interactions do not occur often
 506 (50.8% of adhesion) and involve a few molecules from the surface (single peaks and low distance
 507 ruptures). But this could explain the results obtained before regarding the biophysical properties of
 508 ammonium cells exposed to Zn (Fig. 3): while there is no strong attachment of the Zn particles on the
 509 surface of cells, the particles can still interact with it and could be a cause of the changes observed in
 510 terms of roughness and nanomechanical properties. The second conclusion is that the EPS produced
 511 by cells grown in nitrate conditions can interact with Zn particles *via* multiple bindings with long
 512 molecules. This results in the high work of adhesion recorded, and it highlights the key role probably
 513 played by the EPS in the remediation of Zn from the medium. Indeed, because of these strong
 514 adhesions, the Zn in the medium can penetrate into the EPS layer on the cells, from where it can
 515 then maybe diffuse into the cell and change its metabolism, and this way removing it from the
 516 culture medium.

517 While the role of EPS in the adsorption of pollutants is now a commonly accepted
 518 mechanism (Xiong et al., 2021; Nguyen et al., 2021), their role in the bioremediation of heavy metals
 519 is so far poorly documented. It is known that many microalgae species can be used to remediate
 520 heavy metals (Manikandan et al., 2022), however, in most studies, the role of EPS is not investigated.
 521 One reason for that can be because of a lack of technique allowing to prove their interactions with
 522 the metals. AFM is an ideal tool for that, but as far as we know, it has not been used in such studies
 523 except in one, where the role of EPS in the adsorption of Ag nanoparticles was demonstrated by
 524 imaging at high-resolution the EPS produced by cells after exposure to the nanoparticles (Pletikapić
 525 et al., 2012). Here, by functionalizing Zn particles at the edge of AFM cantilevers, it is possible to
 526 access their interactions with cells, and this way understand the molecular basis of the



527 bioremediation mechanism.

528 **Fig. 5. Probing the interactions between Zn particles and *P. kessleri* cells.** A) scheme showing the different
 529 parameters that can be extracted from the force curves obtained. B) Force curves obtained in the case of cells
 530 grown with nitrate and C) in the case of cells grown with ammonium. D) Boxplot showing the distribution of the
 531 work of adhesion values obtained for cells in both nutritive conditions, E) of the adhesion force values, and F) of
 532 the rupture distances values.

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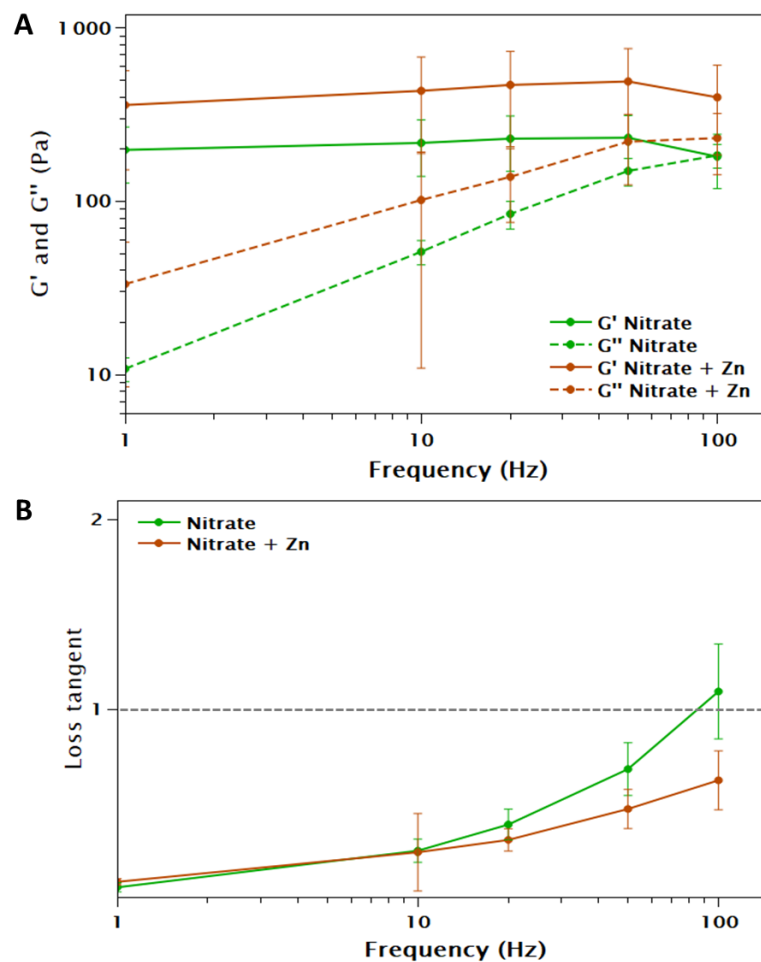
To further prove this point and confirm that the EPS produced by *P. kessleri* grown in the

534 presence of nitrate indeed absorb the zinc present in the medium, we next conducted dynamic

535 mechanical analysis (DMA) experiments using AFM. In this type of experiment, the idea is to

536 superpose low-amplitude sinusoidal oscillations to an initial indentation by a colloidal probe (Abidine

537 et al., 2013). Classic nanoindentation experiments, such as those previously performed (Fig. 2F and
 538 3F), assume the material is purely elastic. However, since the EPS forms a soft gel-like structure
 539 around the cells, it is reasonable to assume that the viscous component is not negligible. Performing
 540 DMA experiments allows obtaining this information on the viscosity, by making it possible to
 541 decompose the elastic and the viscous parameters. In a first step, the shear storage (G') and the
 542 shear loss (G'') are determined, corresponding respectively to the elastic (solid) and to the viscous
 543 (liquid) behavior. These results presented in Fig. 6A show that in both conditions (cells grown in
 544 nitrate with or without Zn(II)), G' is higher than G'' at frequencies lower than 100 Hz, meaning that
 545 the EPS show a solid-like behavior in this regime. While for cells treated with Zn, this remains the
 546 case, for control cells (no Zn(II) exposure), G' and G'' values intersect at 100 Hz. This indicates that at
 547 these high frequencies, EPS are more viscous when cells were not exposed to Zn(II) than when they
 548 are. This is confirmed by looking at the loss tangent (ratio of G'' over G' , Fig. 6B) that becomes
 549 superior to 1 at 100 Hz for control cells, while it is inferior to 1 for cells that have been exposed to
 550 Zn(II). Thus, these experiments show that the EPS do not behave the same way regarding their
 551 mechanical properties when cells have been exposed to Zn. In control conditions, the increase in the
 552 oscillating frequency causes the EPS to become viscous, while this is not the case for cells exposed to
 553 Zn. Most likely, this difference is caused by the presence of Zn particles within the EPS layer, thus



554 further proving our hypothesis that the Zn present in the medium is adsorbed by the EPS. Further
 555 experiments could be conducted to analyze the composition of extracted EPS before and after Zn(II)
 556 exposure, in order to understand if and how the heavy metal affects the EPS layer's composition.

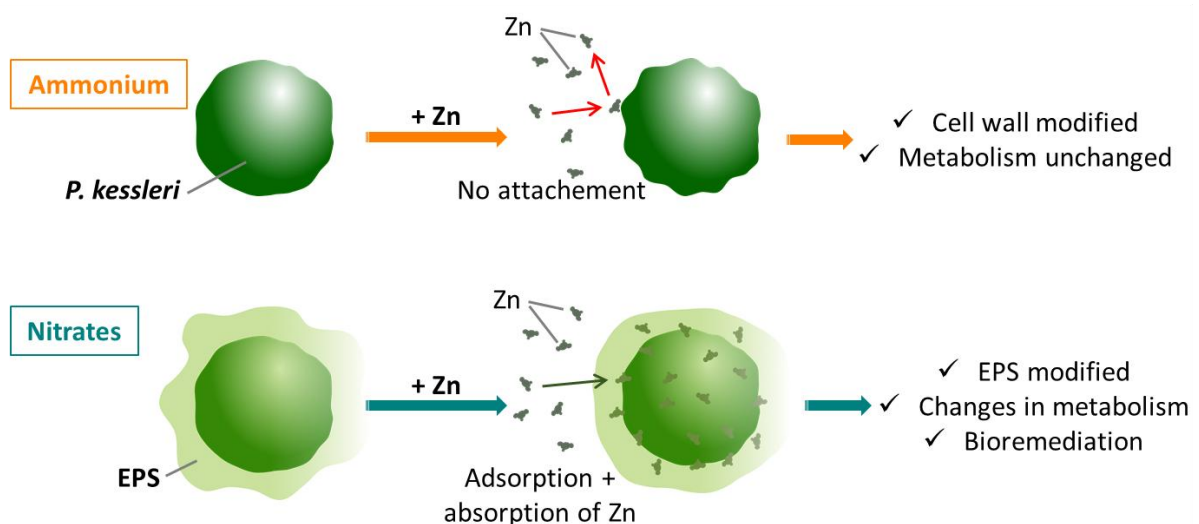
557 **Fig. 6. AFM dynamic mechanical analysis (DMA) of the EPS of *P. kessleri* cells grown with nitrate.** A)
 558 Viscoelastic modulus G' and G'' as a function of frequency for cells grown in the presence of nitrate, exposed
 559 (red lines) or not to Zn(II) (green lines). B) Loss tangent parameter (G''/G') for cells grown in the presence of
 560 nitrate, exposed (red line) or not to Zn(II) (green line).

561 4. Conclusions

562 Microalgae are valuable for remediating pollutants, including heavy metals, from aquatic
 563 environments. To harness this potential for biotechnological applications, it is crucial to understand
 564 the conditions and mechanisms of their remediation abilities. This study used *P. kessleri* cells from
 565 the polluted Reconquista River, adapted to extremely polluted environments. We employed Raman
 566 spectroscopy and atomic force microscopy (AFM) to image these cells with different nitrogen
 567 sources, analyze their biophysical properties, and study their interactions with zinc particles. This
 568 original approach allowed to obtain the key following results:

- 569 • Cells grown with nitrate produce EPS, while those grown with ammonium do not
- 570 • AFM analysis showed that exposure to Zn(II) altered the surface roughness and nanomechanical
 571 properties of the cell wall and EPS.
- 572 • Raman spectroscopy indicated significant metabolic differences based on the nitrogen source,
 573 with Zn impacting cells grown with nitrate more than those grown with ammonium.
- 574 • AFM force spectroscopy results further demonstrated that Zn binds strongly to polymers in the
 575 EPS of nitrate-cultured cells, whereas interactions with ammonium-grown cells were weaker and
 576 less frequent.
- 577 • DMA experiments showed changes in the mechanical behavior of the EPS when cells have been
 578 exposed to Zn(II).

579 These results are summarized in Fig. 7. Altogether, they allow understanding the mechanism
 580 by which *P. kessleri* cells are able to remediate Zn, through its absorption in the EPS that cells
 581 produce when grown with nitrate as a nitrogen source. This study constitutes a unique contribution
 582 to the microalgae bioremediation of heavy metals by underscoring the pivotal role of ~~extracellular~~
 583 ~~polymeric substances (EPS)~~ in the adsorption of Zn from the culture medium by the cells. It has also
 584 shed light on the significance of regulating the nitrogen source to induce the production of EPS in the
 585 cells. Consequently, this research offers crucial fundamental insights that could be applied in



586 subsequent biotechnological endeavors aimed at decontaminating aquatic environments.

587 ***Fig. 7. Schematic representation of the Zn remediation mechanism by P. kessleri cells.***

588

589

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597

598 **Statements & Declarations**

599 The authors declare no competing financial interests

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605 **Data availability**

606 The data will be available upon request.

607 **Ethical approval**

608 Not applicable

609 **Consent to participate**

610 All authors of this manuscript have provided their informed consent to participate in the study. They
611 have agreed to contribute their expertise, data, and analysis to the research project. Final version
612 was evaluated by all authors, who have read and approved the final manuscript.

613 **Consent to publish**

614 All authors of this manuscript have granted their consent for its publication. They acknowledge and
615 agree to be held responsible for the content presented in the manuscript and affirm that it does not
616 violate any copyright laws. Furthermore, they have reviewed and approved the final version of the
617 manuscript before submission.

618 **Competing interests**

619 The authors have no relevant financial or non-financial interests to disclose.

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