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The role of microplastics in microalgae cells aggregation: a study at the molecular scale using atomic force microscopy

Irem Demir-Yilmaz, Nadiia Yakovenko, Clément Roux, Pascal Guiraud,
Fabrice Collin, Christophe Coudret, Alexandra ter Halle, Cécile
Formosa-Dague

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1 **The role of microplastics in microalgae cells aggregation: a study at the**
2 **molecular scale using atomic force microscopy**

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5 Irem Demir-Yilmaz,^{a,b} Nadiia Yakovenko,^c Clément Roux,^c Pascal Guiraud,^{a,d} Fabrice Collin,^c
6 Christophe Coudret,^{c,d} Alexandra ter Halle,^c and Cécile Formosa-Dague,^{a,d*}

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9
10 ^a TBI, Université de Toulouse, INSA, INRAE, CNRS, Toulouse, France.

11 ^b LAAS, Université de Toulouse, CNRS, Toulouse, France.

12 ^c UMR 5623 IMRCP, CNRS, Toulouse, France.

13 ^d Fédération de Recherche Fermat, CNRS, Toulouse, France.

14
15
16
17
18 *corresponding author: Cécile Formosa-Dague, formosa@insa-toulouse.fr

19 INSA de Toulouse, Toulouse Biotechnology Institute

20 135 avenue de Rangueil,

21 31400 Toulouse, France

22

23

24 **Abstract**

25 Plastic pollution has become a significant concern in aquatic ecosystems, where photosynthetic
26 microorganisms such as microalgae represent a major point of entry in the food chain. For this
27 reason an important challenge is to better understand the consequences of plastic pollution on
28 microalgae and the mechanisms underlying the interaction between plastic particles and cell's
29 interfaces. In this study, to answer such questions, we developed an interdisciplinary approach to
30 investigate the role of plastic microparticles in the aggregation of a freshwater microalgae species,
31 *Chlorella vulgaris*. First, the biophysical characterization, using atomic force microscopy, of the
32 synthetic plastic microparticles used showed that they have in fact similar properties than the ones
33 found in the environment, with a rough, irregular and hydrophobic surface, thereby making them a
34 relevant model. Then a combination of optical imaging and separation experiments showed that the
35 presence of plastic particles in microalgae cultures induced the production of exopolysaccharides
36 (EPS) by the cells, responsible for their aggregation. However, cells that were not cultured with
37 plastic particles could also form aggregates when exposed to the particles after culture. To
38 understand this, advanced single-cell force spectroscopy experiments were performed to probe the
39 interactions between cells and plastic microparticles; the results showed that cells could directly
40 interact with plastic particles through hydrophobic interactions. In conclusion, our experimental
41 approach allowed highlighting the two mechanisms by which plastic microparticles trigger cell
42 aggregation; by direct contact or by inducing the production of EPS by the cells. Because these
43 microalgae aggregates containing plastic are then consumed by bigger animals, these results are
44 important to understand the consequences of plastic pollution on a large scale.

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48 **Keywords:** microplastic, microalgae, aggregation, interaction, atomic force microscopy

49 **1. Introduction**

50 Plastic is a revolutionary discovery of the early twentieth century that changed our way of life
51 forever. It has become an integral part of all consumer goods such as packaging, clothing, electronic
52 devices, medicine, etc., (Andrady and Neal, 2009). However, high demand, massive production,
53 extensive use, and poor plastic waste management contributes to plastic release and accumulation in
54 the environment, which has become one of the most pressing environmental problems of our time
55 (Geyer et al., 2017). Plastic waste accounts for 60 to 80% of all solid waste present in the aquatic
56 environment (Gregory and Ryan, 1997), most of them being microplastics particles (MPs) (EPA US,
57 2016). MPs are plastic particles ranging in size from 1 μm to 5 mm (Horton et al., 2017), which are
58 characterized by a variety of physical, chemical, and morphological properties such as different types
59 of polymers and composition, size, shape, density, colour, etc. In the environment, MPs represent a
60 group of persistent synthetic pollutants consisting of primary particles, manufactured at the
61 millimetric or sub-millimetric scale under the form of pellets or microbeads, and secondary particles,
62 resulting from the fragmentation of larger plastic debris through thermal, photo-oxidative,
63 mechanical, and biological degradation processes (Cole et al., 2011). Because of their small size and
64 ubiquitous distribution in all environmental compartments (Horton et al., 2017; Peng et al., 2020;
65 Zhang et al., 2020), MPs are of great concern with respect to their bioavailability, toxicity and
66 potential adverse effect on living organisms and ecosystem as a whole. Ingestion of MPs by aquatic
67 living organisms from zooplankton (Cole et al., 2013) to mammals (Zantis et al., 2021), and the wide
68 range of possible negative effects of plastic particles uptake are well documented. (EPA US, 2016;
69 GESAMP, 2016; Peng et al., 2020).

70 However, there is a gap of knowledge on the interaction and the effects of MPs on the basic
71 organisms of the trophic chain, such as microalgae, which are a major source of food for aquatic
72 animals. Microalgae are photosynthetic microorganisms that are the most numerous primary
73 producers in the entire aquatic ecosystem (Barbosa, 2009; Beardall and Raven, 2004). They are key
74 organisms in a wide range of ecosystem functions, where they have an impact on ocean's carbon

75 sequestration (Singh and Ahluwalia, 2013), oxygen production, nutrient cycling, etc., (Hopes and
76 Mock, 2015). Being ubiquitous, sensitive to environmental disturbances, and easy to cultivate in
77 laboratory, microalgae are an ideal model to study the effects of different pollutants in the
78 environment including MPs (Cid et al., 2012). The interaction between MPs and microalgae is a
79 complex process that can lead to a multitude of effects acting on the further fate and behaviour of
80 both MPs and microalgae, and thus potentially affecting the entire ecosystem (Nava and Leoni,
81 2021). For instance, in the environment, microalgae tend to colonize and form biofilms on abiotic
82 surfaces (Irving and Allen, 2011), among them plastic surfaces, using them as an abiotic substrate to
83 grow in a biofouling process (Bravo M et al., 2011; Carson et al., 2013; Jorissen, 2014; Reisser et al.,
84 2014). While colonising plastic surfaces or other types of surfaces, microalgae cells secrete
85 extracellular polymeric substances (EPS), which play an important role in biofilm formation. EPS
86 consist of polysaccharides, lipids, nucleic acids, proteins, and other polymeric compounds
87 (Wingender et al., 1999a; Xiao and Zheng, 2016), which favours cells cohesion and future adhesion to
88 the substrate' surface (Wingender et al., 1999b). In addition, biofouling changes the density of plastic
89 particles, affecting their buoyancy (Nava and Leoni, 2021; Oberbeckmann et al., 2015; Rummel et al.,
90 2017) and thus leading to the dissemination of plastic particles through the water column by sinking
91 to the bottom or moving to the surface. This widespread abundance of MPs particles consequently
92 increases their bioavailability for various living organisms. Another effect of biofouling is a decrease
93 in the hydrophobicity of the particle surface (Lobelle and Cunliffe, 2011). As a result, adsorption of
94 toxic pollutants from the aquatic environment to the surface of the plastic particles can be enhanced
95 (Bhagwat et al., 2021; Dong et al., 2017), which can amplify the toxicity of MPs. Moreover,
96 extracellular polymeric substances (EPS) produced by microalgae promote the heteroaggregation of
97 MPs and microalgae. The resulting aggregates become easy food for the aquatic organisms and are
98 also more prone to sediment, thus here also affecting their dissemination through the water column
99 as mentioned above (Lagarde et al., 2016; Long et al., 2015; Rummel et al., 2017). Then, MPs were
100 also found to have a number of adverse effects on microalgae, including inhibition of growth

101 (Sjollema et al., 2016; Zhang et al., 2017; Liu et al., 2020; Song et al., 2020), decrease in chlorophyll
102 content (Tunali et al., 2020; Wu et al., 2019) and photosynthetic activity (Zhang et al., 2017), physical
103 and morphological damages (Mao et al., 2018), oxydative stress (Xiao et al., 2020). Finally, due to
104 constant movement in the aquatic environment, plastic is a potential vector of geographic transport
105 for the migration of microalgae (Rowenczyk et al., 2021). This phenomenon creates a risk of
106 introducing pathogenic species (e.g. harmful algal blooms) into a new environment where native
107 species are not adapted to defend themselves (Masó et al., 2003; Glibert *et al.*, 2014; Oberbeckmann
108 et al., 2015).

109 For all these reasons, scientists are making increasing efforts to study the mechanism of MPs
110 and microalgae interaction, to better understand its negative impact on a global scale. For instance, a
111 recent review published by Nava *et al.* reports on the different effects that microplastics can have on
112 microalgae cells. Their study shows that the effects on cell growth, photosynthesis and cell
113 morphology are the most commonly reported effects, although this is highly dependent on both the
114 type of plastic and the microalgae species considered. On the contrary, microalgae, by colonizing
115 microplastics, also alter the plastic polymer, notably their density and sinking behaviour (Nava and
116 Leoni, 2021). However, most of the ecotoxicological studies under laboratory conditions are using
117 commercially manufactured models of MPs, in a vast majority of the studies the model plastic are
118 polystyrene micro- or nano- spheres, which are not representative of plastic particles found in the
119 environment (Gigault et al., 2021; Kokalj et al., 2021; Phuong et al., 2016). Thus, there is a need for
120 research based on the use of an environmentally relevant model of plastic particles. For this purpose,
121 we used a top-down method based on mechanical degradation to prepare more environmentally
122 relevant model of MPs (Yakovenko et al., 2022). Particles were prepared from polyethylene as it is
123 the most produced (Plastics Europe, 2020) and frequently found plastic type in the environment
124 (Peng et al., 2020). The model particles were characterized by polydispersity, irregular shapes, and
125 negative surface charge thereby representing several characteristics in common with the MPs
126 formed in the environment. However, being small in size, these particles are hard to observe in the

127 biotic and abiotic matrices without destructive methods such as density separation or digestion.
128 Thus, to overcome this limitation and make these particles easy to track and image in microalgae, we
129 doped them by Lanthanide-based upconverting nanoparticles (UCNPs) as a luminescent tag. These
130 recently developed phosphors are inorganic materials, very stable and able to convert low energy
131 Near-Infrared photons into visible light (Gu and Zhang, 2018). This allows their detection even in
132 thick samples such as in a tissue or a small animal. The studied model of MPs (Model-MPs) is
133 represented by two types of particles: i) microparticles of PE itself (μ -PE); and ii) labelled
134 microparticles of PE with UCNPs luminescence tag (μ -Upcon-PE).

135 In this study, we investigated the interaction between these Model-MPs and freshwater
136 microalgae cells and their further role in the aggregation of cells using nano- and molecular scale
137 experiments performed with an atomic force microscope (AFM) equipped with fluidic force
138 microscopy (FluidFM). AFM, first developed in 1986 (Binnig et al., 1986), has proven over the years to
139 be a powerful tool for surface characterization at the nanoscale (Pillet et al., 2014; Xiao and Dufrêne,
140 2016). In addition to high-resolution imaging capacities, down to the nanometer scale, AFM is also a
141 sensitive force machine able to record piconewton level forces, thus making it possible to access the
142 nanomechanical and adhesive properties of samples, as well as their interactions with their
143 environment (Formosa-Dague et al., 2018). In the particular context of microalgae, AFM has been
144 used to understand the morphology, nanostructure, nanomechanics and adhesive behavior of cells
145 (Demir-Yilmaz et al., 2021), but most importantly their interactions with particles or molecules
146 present in their environment (Besson et al., 2019; Demir et al., 2020; Demir-Yilmaz et al., 2021). In
147 FluidFM, a micro-sized channel is integrated into an AFM cantilever and connected to a pressure
148 controller, thus creating a continuous and closed fluidic conduit that can be filled with a solution or
149 with air, while the tool can be immersed in a liquid environment (Meister et al., 2009). An aperture at
150 the end of the cantilever allows air or the liquids inside the cantilever to be dispensed locally. In the
151 first part of this study, Model-MPs were first characterized using AFM to visualize the particles and
152 obtain information on their roughness. Then Fluidic force microscopy (FluidFM) was used to probe

153 their hydrophobic properties and probe their interaction with *Chlorella vulgaris* cells, a model green
154 microalgae species. Finally, we describe these interactions by evaluating the possible contribution of
155 EPS in the aggregation of cells.

156

157 **2. Materials and Methods**

158 **2.1. Microalgae strain and culture.**

159 The green freshwater microalgae *Chlorella vulgaris* strain CCAP 211/11B (Culture Collection of Algae
160 and Protozoa) was cultivated in sterile conditions in Wright's cryptophyte (WC) medium prepared
161 with deionized water (Guillard and Lorenzen, 1972). Cells were cultivated at 20°C, under 120 rpm
162 agitation, in an incubator equipped with white neon light tubes providing illumination of
163 approximately 40 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, with a photoperiod of 18h light: 6h dark. All experiments
164 were carried out with 7 days exponential phase batch cultures. Cells were first harvested by
165 centrifugation (3000 rpm, 3 min at 21 °C), washed two times in phosphate buffered saline (PBS) at pH
166 7.4 and directly used for the AFM experiments and for some flocculation/flotation experiments
167 (condition 3).

168 **2.2. Microparticles model.**

169 The synthesis and full characterization of the high-density polyethylene (HDPE) microparticle model,
170 are described elsewhere (Yakovenko et al., 2022). Briefly, two bulk polymeric materials were
171 prepared, including UCNPs-labelled HDPE (Upcon-PE) and UCNPs-free HDPE (Blank-PE). The oleate-
172 capped NaREF4 (RE= rare earth, 2% Er; 30% Yb; 68% Y) with a diameter of 20 nm were used to
173 provide a green luminescent plastic that can be directly observed by eye under 976 nm irradiation.
174 UCNPs were incorporated into the HDPE (CAS 9002-88-4, Sigma Aldrich, Saint Louis, MO, USA) matrix
175 by dissolving the polymer in boiling o-xylene ($\geq 99.0\%$ (GC grade); Sigma Aldrich, Saint Louis, MO,
176 USA), containing UCNPs in a 10 wt.% HDPE:UCNPs ratio. The composite was separated from the
177 reaction mixture by precipitation in the ice bath. The Blank-PE batch of polymer containing only

178 HDPE was prepared following the same protocol. Microparticles model was obtained by exposing
179 each bulk material to a cryogenic grinder (SPEX™ SamplePrep 6775 Freezer/Mill™, Delta Labo,
180 Avignon, France). The resulting polymer particles were dispersed in ethanol and fractionated by
181 subsequent cascade filtration to micro- and nanosized particles. The collected microparticles were
182 named as μ -PE (HDPE microparticles), and μ -Upcon-PE (HDPE labelled with the inclusion of UCNPs as
183 a luminescent tag). The full characterization of the Model-MPs in terms of particle size, shape,
184 crystallinity, chemical composition, surface charge, and luminescence properties, are described
185 elsewhere (Yakovenko et al., 2022).

186 **2.3. Zeta potential measurements.**

187 Zeta-potential measurements for Model-MPs were carried out at 25°C on a Zetasizer Nano-ZS
188 (Malvern Instruments, Ltd, UK) equipped with a He-Ne laser ($\lambda = 633 \text{ nm}$) at an angle of 173°.
189 Samples were prepared by dispersion of particles in 10 mM NaCl solution, to provide minimum level
190 of conductivity in the samples, following ISO and ASTM standard guides (ASTM E2865-12, 2018).
191 Before analysis, pH of every sample was measured. Zeta-potential and standard deviation (SD) were
192 obtained from 5 measurements of 11 runs of 10 seconds using the Smoluchowski model (Yakovenko
193 et al., 2022).

194 **2.4. Model-MPs size measurements.**

195 Size measurements for Model-MPs were carried out by Granulometry analysis using a Mastersizer
196 MS3000 (Malvern Panalytical, UK) as described elsewhere (Yakovenko et al., 2022). Briefly, samples
197 were prepared by dispersing the polymer powder in ethanol using HYDRO MV device with stirring at
198 2500 rpm. The refractive index used for ethanol and MPs were 1.36 and 1.52 respectively with an
199 absorption index for particles of 0.1. Results are expressed as a percentage number. From these
200 results, 90% of particles have a size below $\pm 15 \mu\text{m}$, 50% of particles have size below $\pm 6 \mu\text{m}$, and 10
201 % of particles have a size below $\pm 4 \mu\text{m}$. These are approximate average value obtained from the
202 duplicates. The results are summarized in Table 1.

203

204

205

206 **Table 1:** Summary of Model-MP characterization.

Sample	Zeta potential, (mV)	Size distribution Dn (10)	Size distribution Dn (50)	Size distribution Dn (90)
μ -PE	-71 ± 7	4.3 ± 0.0	5.6 ± 0.1	11.9 ± 1.1
μ -Upcon-PE	-73 ± 8	4.12 ± 0.3	5.7 ± 0.5	13.25 ± 0.9

207 ** 90%, 50% and 10% of the particles were observed to have sizes below the value given in the table.

208 2.5. Flocculation/flotation experiments.

209 To quantify the effects of Model-MPs on cell aggregation, flocculation/flotation separation of *C.*
210 *vulgaris* was performed in dissolved air flotation (DAF) experiments in a homebuilt flotation device,
211 shown in detail in Supplementary Figure S1. The depressurization at atmospheric pressure of water
212 saturated by air at 6 bar induced the formation of bubbles. Water free of algae was pressurized for
213 30 min before injection into the jars. The injection was controlled by a solenoid valve and 20 mL of
214 pressurized water was added to each beaker sample. Prior to bubble injection, cells were left to
215 flocculate during 15 min. Flocculation/flotation tests were conducted in three different conditions, in
216 each case in triplicate, with cells coming from 2 independent culture.

217 • **Condition 1:** *C. vulgaris* cells were cultured 7 days together with different concentrations of μ -PE
218 and μ -Upcon-PE (final concentrations of 0, 5,10 and 40 mg/L for μ -PE and 40 mg/L for μ -Upcon-
219 PE) until they reached mid-exponential phase. Then 100 mL of cell suspension was directly
220 poured into the test-jars with an initial OD₇₅₀ nm of 1.

221 • **Condition 2:** *C. vulgaris* cells were grown for 7 days until they reached mid-exponential phase.
222 After that, 100 mL of cell suspension was directly poured into the test-jars with an initial OD₇₅₀
223 nm of 1. Then μ -PE and μ -Upcon-PE were separately added (final concentration of 40 mg/L) to
224 the suspension, which was stirred at 100 rpm for 15 min to homogenize it before introduction of
225 the bubbles.

226 • **Condition 3:** *C. vulgaris* cells were grown for 7 days until they reached mid-exponential phase.
227 After that, cells were harvested by centrifugation at 3000 rpm for 3 min, then washed twice with
228 PBS buffer at pH 7.4. After that, 100 mL of cell suspension was directly poured into the test-jars
229 with an initial OD₇₅₀ nm of 1. Particles of μ-PE and μ-Upcon-PE were directly added (final
230 concentration of 40 mg/L) to the suspension, which was stirred at 100 rpm for 15 min to
231 homogenize it before introducing bubbles.

232 For all condition, after bubbles were introduced, the algal suspension was retrieved from the bottom
233 of the test-jars: the first 5 mL of treated phase were discarded, the next 20 mL were used for
234 quantifying flocculation/flotation efficiency. For that, the optical density of the withdrawn
235 microalgae suspension was measured and compared to the optical density of the microalgae
236 suspension measured before the experiments. The flotation efficiency (*E*) was calculated according to
237 the following equation 1.

$$E = \frac{OD_i \cdot V_i - OD_f \cdot V_f}{OD_i \cdot V_i} \quad (1)$$

238 **2.6. Optical imaging experiments.**

239 Flocculation was directly observed after resuspension of the cells in Phosphate Buffer Saline (PBS) at
240 a pH 7.4 containing μ-PE or μ-Upcon-PE at a concentration of 40 mg/L. Flocculation levels were
241 observed using an Axio Observer Z1 microscope (Zeiss, Germany) at high magnification (×50). To
242 color EPS, an Alcian Blue staining was used as described previously (Vergnes et al., 2019). To this end,
243 a solution containing Alcian blue at a final concentration of 0.018% and 0.036% of acetic acid solution
244 was deposited on the glass surfaces where cells were immobilized and allowed to stand for 15 min.
245 Glass surfaces were then immersed in distilled water for 5 min in order to remove the nonfixed
246 staining. Finally, images were recorded at high magnification (×50) using an Axio Observer Z1
247 microscope (Zeiss, Germany).

248 **2.7. AFM imaging.**

249 For μ -PE and μ -Upcon-PE imaging, particles were immobilized on PDMS substrates. In each case at
250 least 4 different particles were imaged in PBS at pH 7.4, using the Quantitative Imaging mode
251 available on the Nanowizard III AFM (Bruker, USA), with MSCT cantilevers (Bruker, nominal spring
252 constant of 0.01 N/m). Images were recorded with a resolution of 256 pixels \times 256 pixels, at an
253 applied force of <1.0 nN and a constant approach/retract speed of 90 μ m/s (z-range of 3 μ m). In all
254 cases the cantilevers spring constants were determined by the thermal noise method prior to
255 imaging (Hutter and Bechhoefer, 1993).

256 **2.8. Roughness analyses.**

257 Roughness analyses were performed on 9 different μ -PE and μ -Upcon-PE particles immobilized on
258 PDMS and on 10 different *C. vulgaris* cells coming from at least 2 independent cultures after
259 incubation with and without Model-MPs for 7 days, immobilized on positively charged glass slides
260 (SuperfrostTM Plus adhesion, Eprelia, USA). Individual μ -PE and μ -Upcon-PE images were recorded in
261 PBS whereas for cells, after incubation with Model-MPs, samples were directly imaged in culture
262 medium using contact mode with a Nanowizard III AFM (Bruker, USA), using MSCT cantilevers
263 (Bruker, nominal spring constant of 0.01 N/m). Images were recorded in contact mode using an
264 applied force of < 1 nN for micro-HDPE-(UCNPs) and of < 0.5 for cells and the cantilever spring
265 constants were determined by the thermal noise method prior to imaging (Hutter and Bechhoefer,
266 1993).

267 **2.9. Hydrophobicity measurements.**

268 To measure the hydrophobic properties of materials, a recently developed method was used, which
269 consists in measuring using Fluidic force microscopy (FluidFM), the interactions between a bubble
270 (hydrophobic surface) and materials (Demir et al., 2021). For that, FluidFM probes with an aperture
271 of 8 μ m of diameter (Cytosurge AG, Switzerland) were hydrophobized by coating with self-assembled
272 monolayers (SAMs) of silanes via SAMs vapor deposition technique. FluidFM cantilevers were
273 functionalized with 1H,1H,2H,2H-Perfluorodecyltrichlorosilane (FDTs) using an Orbis-1000

274 equipment (Memsstar, Livingston, UK) to make their external surface and inside microchannel
275 hydrophobic. The deposition was realized under vacuum at 40 torrs and -40°C, for 5 min. Then the
276 microchannel of these silanized cantilevers was filled with air and the probe was immersed in PBS 1X.
277 To eliminate any particle or dust contamination or to prevent clogging of the FluidFM cantilever, a
278 slight over pressure of 20 mbar was applied. Then to produce a bubble at the aperture of the
279 cantilever, a positive pressure of 200 mbar was applied inside the microfluidic cantilever in buffer.
280 The silanized probes were calibrated using the thermal noise method before each measurement
281 (Hutter and Bechhoefer, 1993). The interactions between the bubbles produced and MPs were then
282 recorded in force spectroscopy mode using a maximum applied force of 2 nN, a constant approach
283 retraction speed of 3 µm/s, and a delay time of 1.0 s. For each conditions, areas of 0.5 x 0.5 µm on 5
284 different MPs were probed. Adhesion forces were obtained by calculating the maximum adhesion
285 force on the retract force curves obtained; data are presented in Supplementary Table 1-3.

286 **2.10. Force spectroscopy experiments using FluidFM technology.**

287 Force spectroscopy experiments were conducted using a NanoWizard III AFM (Bruker, USA),
288 equipped with FluidFM technology (Cytosurge AG, Switzerland). In each case, experiments were
289 performed in PBS, using micropipette probes with an aperture of 2 µm (spring constant of 0.3, and 4
290 N/m, Cytosurge AG, Switzerland). First, PBS at a pH of 7.4 was used to fill the probe reservoir (5 µL);
291 by applying an overpressure (100 mBar) the PBS then filled the entire cantilever microchannel. The
292 probe was then immersed in PBS and calibrated using the thermal noise method prior to
293 measurement (Hutter and Bechhoefer, 1993). A single *C. vulgaris* cell was then aspirated from the
294 surface of the Petri dish by approaching the FluidFM probe and applying a negative pressure (-200
295 mBar). The presence of the cell on the probe was verified by optical microscopy. The cell probe was
296 then used to measure the interactions with Model-MPs. For that, µ-PE and µ-Upcon-PE solutions at a
297 concentration of 40 mg/L were deposited on polydimethylsiloxane (PDMS) surfaces and left for 30
298 min. After that, the PDMS surfaces were rinsed using PBS and directly used. Interactions between
299 single *C. vulgaris* cells aspirated at the aperture of FluidFM cantilevers and Model-MPs were

300 recorded at a constant applied force of 2 nN, force curves were recorded with a z-range of up to 2
301 μm and a constant retraction speed of 2.0 $\mu\text{m/s}$ to 20 $\mu\text{m/s}$. In each case, at least 6 cells coming from
302 2 independent culture were probed. Data were analyzed using the Data Processing software from
303 Bruker; they are presented in Supplementary Tables 4-6. Adhesion forces were obtained by
304 calculating the maximum adhesion force for each retract curves. Experiments were repeated three
305 times with ten different cells coming from at least three different cultures.

306 **2.11. Two-photon microscopy.**

307 Samples were imaged on a Zeiss LSM-710 two-photon microscope, equipped with a Ti: Sapphire
308 Chameleon Vision II laser (Coherent, Santa Clara, California), operating at 980 nm (2-3% of max
309 power for UCNPs and 50% for *C. vulgaris* and μ -Upcon-PE samples, 140 fs pulses, 80 MHz repetition
310 rate), and a Zeiss Plan-Apochromat 63x/1.40 oil immersion lens. Upconverted light was collected
311 between 490 and 600 nm for the green band, and 630 and 710 nm for the red band. Emission
312 spectrum of analyzed samples were collected between 418 and 729nm with a 10 nm step. Images
313 were processed using ImageJ (Fiji) software (Yakovenko et al., 2022).

314 **2.11 Statistical analysis.**

315 Experimental results represent the mean \pm standard deviation (SD) of at least three replicates. For
316 each experiments, the number of replicates is indicated both in the Material and Methods section in
317 the corresponding paragraphs, and in the Results and Discussion section. For large samples ($n > 20$
318 values) unpaired student t-test was used to evaluate if the differences between the conditions are
319 significant. For small samples ($n < 20$ values) non-parametric Mann and Whitney test was used to
320 assess the differences. The differences were considered significant at $p < 0.05$.

321

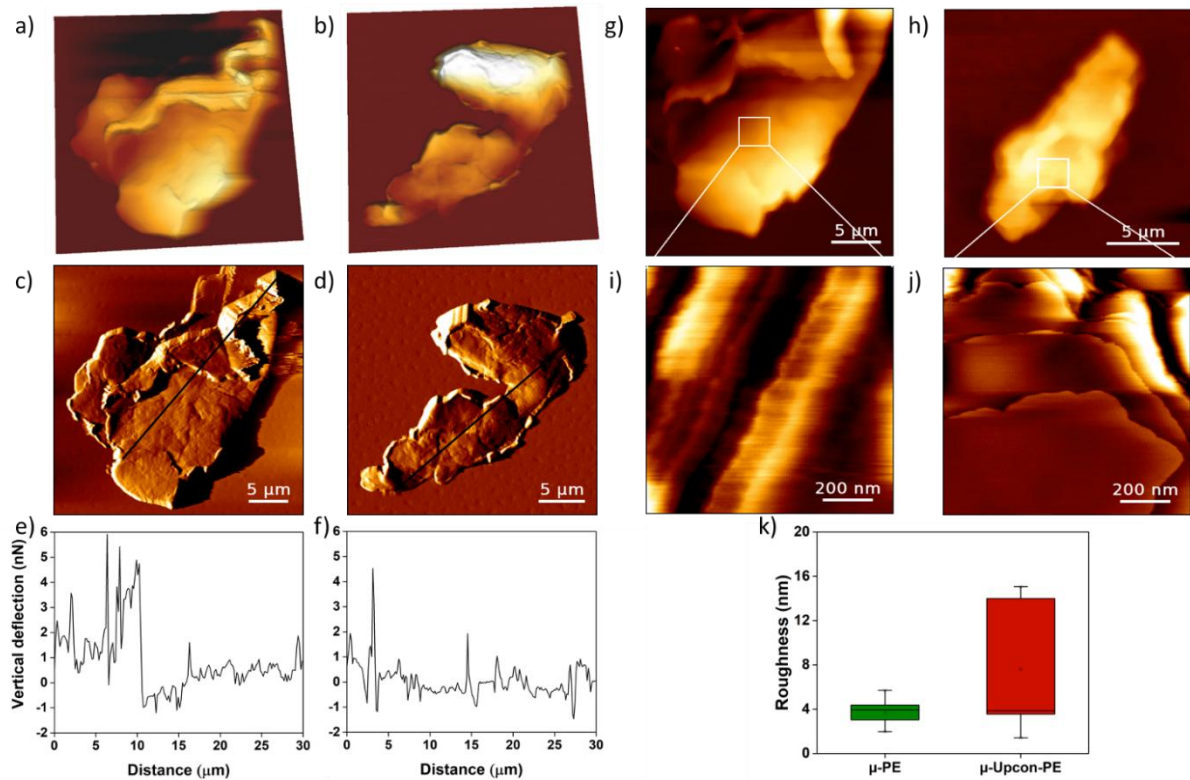
322 **3. Results and discussion**

323 **3.1. Model-MPs are irregular, have a rough surface and hydrophobic properties**

324 The Model-MPs were derived from previous work (Yakovenko et al., 2022), where they were
325 prepared from raw commercially available high-density polyethylene (PE). The top-down method
326 consisted in the cryogenic grinding of the polymer or the nanocomposite (PE loaded with 10% UCNPs
327 by high temperature swelling in xylene). This method is interesting because grinding is achieved at a
328 temperature below the vitreous transition of PE and elastic substances are generally then more
329 easily processed. The grinding at this temperature also prevents the chemical alteration of the PE
330 backbone (this was ensured by infrared measurements) or morphological modification (calorimetric
331 experiments) (Yakovenko et al., 2022). The size distribution of the particles obtained by
332 granulometric analysis showed that around 90% of the particles in number were smaller than 15 μm .
333 These Model-MPs (both $\mu\text{-PE}$ and $\mu\text{-Upcon-PE}$) were first characterized using AFM in contact mode.
334 The images obtained are presented in Figure 1. The 3D AFM height images (Figure 1a and b) and
335 vertical deflection (Figure 1c and d) images recorded show that both types of Model-MPs have an
336 irregular shape and a heterogeneous surface, like microplastics collected in the environment (ter
337 Halle et al., 2016). In addition, they are found mostly aggregated on top of each other, and not
338 present as single isolated particles. This is illustrated by the cross-section taken along the longer sides
339 of the particles in Figure 2c and d, which clearly show this irregularity, with height variations over 6
340 nm in Figure 1e and f. This tendency of Model-MPs to form aggregates could be explained by their
341 hydrophobicity. Such behaviour is not observed with model plastic micro- and nanospheres
342 commercially available, which are formulated with different surfactant additives allowing to prevent
343 this aggregation. However, these models are not reliable because their chemical properties and
344 further interactions are different from the plastic particles found in the environment (Phuong et al.,
345 2016).

346 We then acquired high resolution images on small areas ($5\mu\text{m} \times 5\mu\text{m}$) on top of the particles,
347 using advanced quantitative imaging (QI) mode; the resulting images are shown in Figure 1g-h. In this
348 case, QI mode was used instead of contact mode because of the complexity of Model-MPs surfaces.
349 QI being a force spectroscopy based imaging mode, there is no lateral forces exerted by the tip as

350 occurring in contact mode, which can damage the sample (Chopin et al., 2013). This way we could
351 obtain high-resolution images of the particles surface (Figure 1i and j), and quantify their roughness.
352 Roughness measurements were performed on 9 different particles for both type of Model-MPs (μ PE
353 and μ -Upcon-PE); the results of these analysis are presented in the boxplot Figure 1k. They show that
354 μ -PE have an average roughness of 3.7 ± 1.1 nm, which increases to 7.6 ± 5.4 nm when UCNPs are
355 incorporated in the particles (μ -Upcon-PE). Although there is an important heterogeneity in the
356 measurements in this last case, the difference with the μ -PE is not significantly different at 0.05 level
357 (non-parametric Mann and Whitney test). This result thus shows that the incorporation of UCNPs
358 may affect the structure of Model-MPs, by modifying their surface morphology; however, the
359 heterogeneity of the measurements performed on μ -Upcon-PE reflects the uncontrolled
360 incorporation of the UCNPs over the particles present in the sample (Yakovenko et al., 2022). The
361 surface roughness of particles is an important physical characteristic that plays a major role in how
362 they will behave in the environment and interact with microorganisms. Commercially available
363 models of micro- and nanospheres used for ecotoxicological studies are usually characterized by a
364 very smooth surface (Phuong et al., 2016; Rubin et al., 2021), whereas plastic particles found in the
365 environment are characterized by a rough surface (Roweczyk et al., 2020). Thus, our Model-MPs,
366 which have a high average surface roughness, can be considered as a more reliable model to study
367 the interactions between MPs and microorganisms such as *C. vulgaris*.



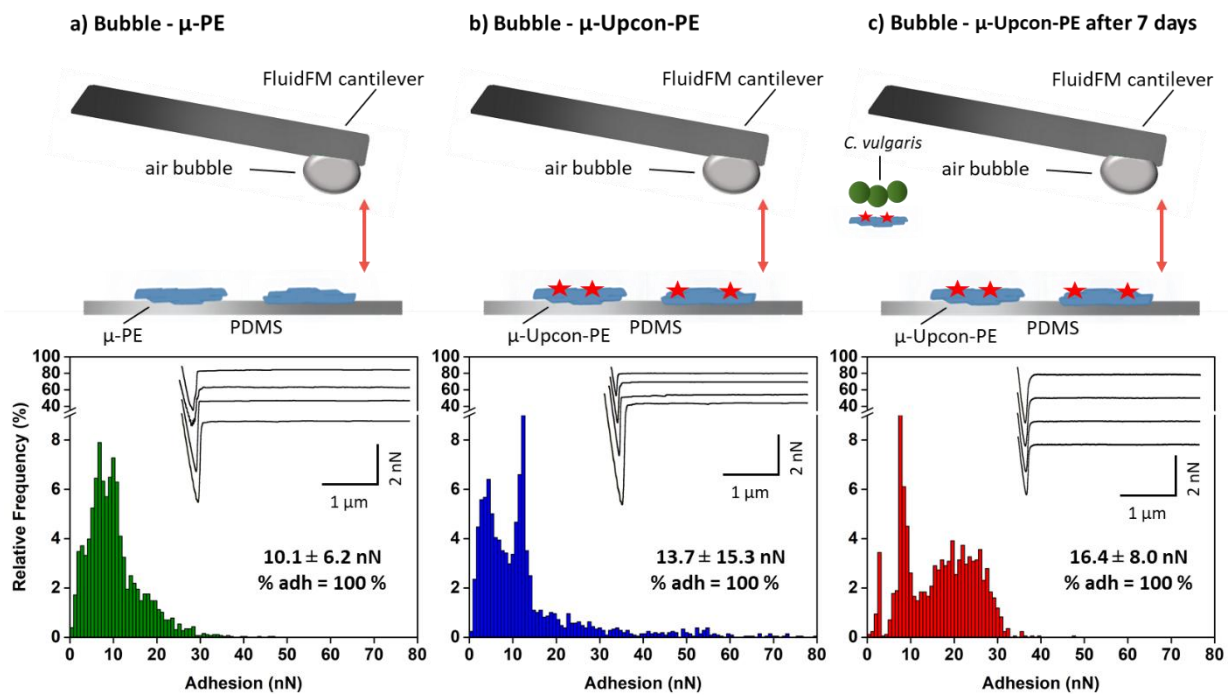
368 **Figure 1: Imaging and characterization of μ -PE surface before and after incorporation of UCNPs: a)**
 369 **3D AFM height image of μ -PE (color scale = 7 μm); b) 3D AFM height image of μ -Upcon-PE (color scale**
 370 **= 4 μm); c) Vertical deflection images of μ -PE; d) Vertical deflection images of μ -Upcon-PE; e) Cross**
 371 **section taken along the larger side in panel c and f) Cross section taken along the larger side in panel**
 372 **d; g) AFM height images of μ -PE (color scale = 7 μm) and h) AFM height images of μ -Upcon-PE (color**
 373 **scale = 5 μm); i) AFM height images of μ -PE surface (5 $\mu\text{m} \times 5\mu\text{m}$) (color scale = 23 nm) and j) AFM**
 374 **height images of μ -Upcon-PE surface (5 $\mu\text{m} \times 5\mu\text{m}$) (color scale = 19 nm); k) Quantification of μ -PE and**
 375 **μ -Upcon-PE surface roughness in a box plot.**

376 In the next step we then assessed the hydrophobic properties of the Model-MPs, which are
 377 an important physico-chemical factor that could greatly influence their interactions with microalgae.
 378 To this end, we used a recently developed method that consists in probing the interactions of
 379 samples with bubbles produced using FluidFM technology (Demir et al., 2021), which combines AFM
 380 with microfluidics (Meister et al., 2009). Air bubbles in water behave like hydrophobic surfaces. By
 381 producing them using FluidFM, it is then possible to probe their interactions with complex abiotic
 382 surfaces such as the MPs, and to measure their hydrophobic properties with accuracy. Such

383 measurements allow avoiding usual issues related to other tests like water contact angle
384 measurement (WCA). To perform these experiments, Model-MPs were immobilized on a PDMS
385 substrate and their interactions with bubbles were measured in PBS buffer at pH 7.4 (Figure 2a). For
386 both μ -PE and μ -Upcon-PE, 5 different particles were probed. In the case of μ -PE, the retract force
387 curves obtained (inset in Figure 2a) show a single peak occurring at the contact point, typical of a
388 hydrophobic interaction (Dague et al., 2007), with an average force of 10.1 ± 6.2 nN (Figure 2a, n=
389 2558 force curves obtained from 5 different particles, adhesion values can be found in
390 Supplementary Table 1). This force corresponds to the height of the adhesion peak, and thus to the
391 force needed to break the interaction between the bubble and the sample. As a hydrophobic
392 interface like bubbles interact with hydrophobic surfaces, then this force reflects the degree of
393 hydrophobicity of the sample, the stronger the adhesion, the higher the hydrophobicity. Similarly, in
394 the case of μ -Upcon-PE, a single peak occurring at the contact point is visible (inset in Figure 2b);
395 retract adhesion forces in this case were on average of 13.7 ± 15.3 nN (Figure 2b, n = 2107 force
396 curves obtained from 5 different particles, adhesion values can be found in Supplementary Table 2).
397 The large distribution of the adhesion values obtained in these experiments reflect the irregularities
398 of the Model-MPs used that were visible on the height AFM images in terms of nanostructure.
399 Indeed, in each case these irregularities change the contact area between the bubble and the
400 particle, which can have an impact on the adhesion force value recorded. Thus in conclusion the two
401 samples (μ -PE and μ -Upcon-PE) present hydrophobic properties as they are able to interact with
402 bubbles with a relatively important force (for comparison, the interaction between *C. vulgaris* cell
403 surface and bubbles give an average adhesion force of 4.2 nN, Demir et al., 2021). Plastic particles
404 found in the environment probably have more hydrophilic properties, caused by plastic aging and
405 oxidation (Liu et al., 2021). Thus the Model-MPs used in this study are a relevant model of plastic
406 that has just entered the environment, prior to the oxidation process.

407 The experiments were then repeated with Model-MPs that were incubated with cells during
408 their culture (7 days, Figure 2c). In this case also, hydrophobic interactions are recorded, with an

409 average adhesion force of 16.4 ± 8.0 nN ($n= 1685$ force curves obtained from 5 different particles,
410 adhesion values can be found in Supplementary Table 3), a value that is significantly different from
411 the two first conditions (p -value of 0.05, unpaired student test). Thus, the incubation of Model-MPs
412 with cells changes their hydrophobic properties. This is an important point because it means that our
413 Model-MPs, after seven days exposed to the cells, have their surface modified; a plausible hypothesis
414 could be that cells produce EPS in the culture medium, which then could coat the particles surface.
415 Finally, in order to confirm that the forces recorded are due only to the interactions between Model-
416 MPs and bubbles, we also probed the interactions between bubbles and the PDMS surfaces used to
417 immobilize the particles. The results presented in Supplementary Figure S2 show an average
418 adhesion force recorded of 1500 ± 100 nN ($n = 2500$ force curves). This adhesion force is much
419 higher than the ones obtained with Model-MPs (maximum around 50 nN), thus meaning that we
420 could precisely measure the interaction between bubbles and the particles without interfering with
421 the surface on which they are immobilized. In the natural environment, microplastics undergo
422 alterations, including among multiple factors, oxidation of the polymer that changes its polarity and
423 possibly its hydrophobicity (Andrady et al., 2011). FTIR analysis performed on the synthesized particle
424 did not show any oxidation bands (Yakovenko et al., 2022). However, Model-MPs have a negative
425 zeta potential and FTIR is most likely not sensitive enough to detect these functionalities if they are
426 present in small proportions. Small microplastics and nanoplastic are expected to be negatively
427 charged (Gigault et al., 2021). Because the particles synthesized here are also negatively charged,
428 they have supposedly a similar behaviour than weathered plastic particles. Thus, altogether the
429 biophysical characterization of the Model-MPs produced in this study show that they are aggregated,
430 have a rough surface and present hydrophobic properties. MPs found in the environment have
431 similar characteristics (Phuong et al., 2016), thus confirming the interest of our methodology to
432 obtain particles close to what can be found in the environment. Our Model-MPs are thus a reliable
433 model to understand the interactions that MPs can have with microalgae in the environment.



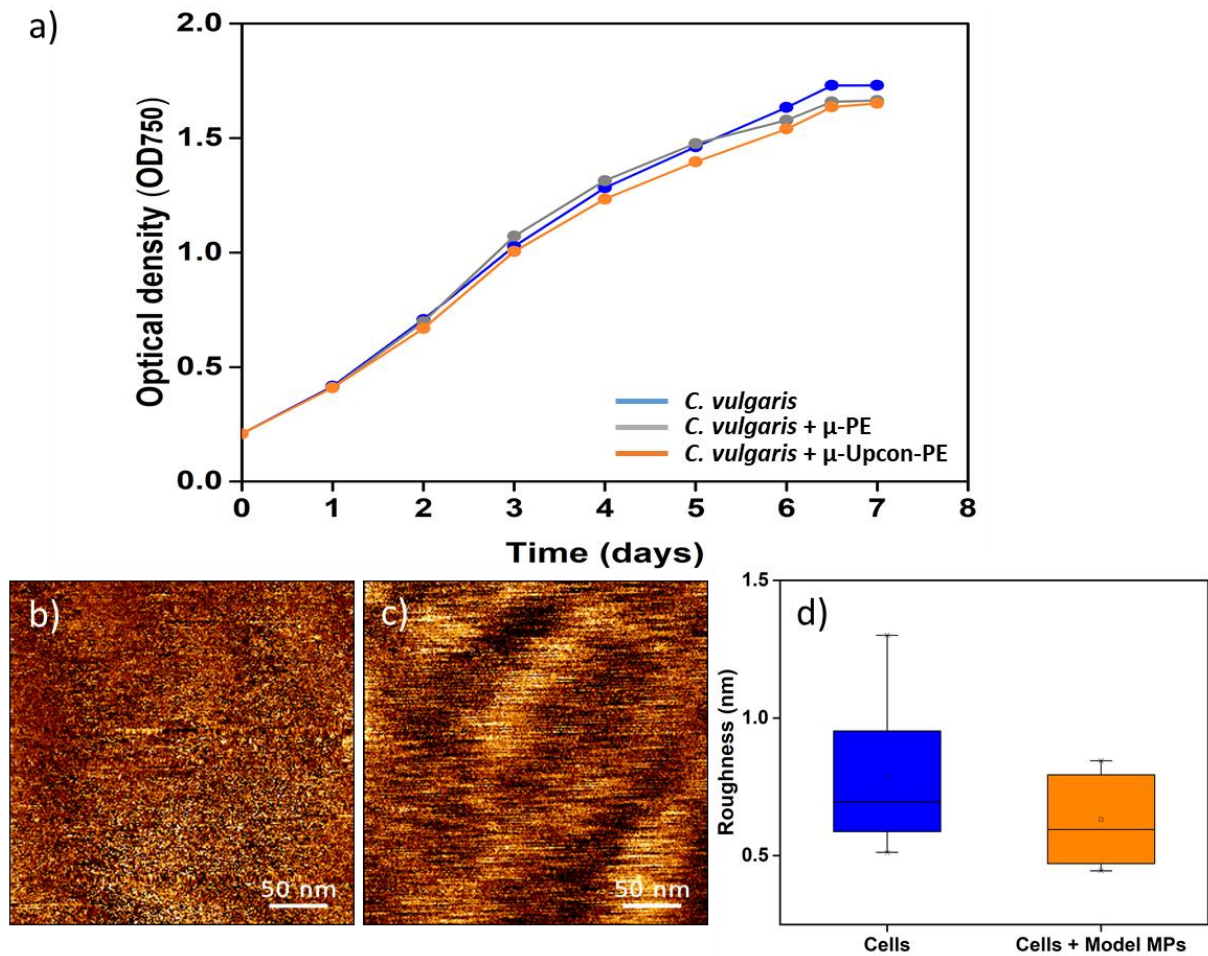
434 **Figure 2: Probing the interaction between bubble and Model-MPs: Adhesion force histogram**
 435 *obtained for the interaction between bubbles and a) μ -PE, b) μ -Upcon-PE, and c) μ -Upcon-PE after*
 436 *incubation for 7 days with *C. vulgaris* cells. Insets in a, b and c shows the representative force curves*
 437 *obtained during force spectroscopy experiments.*

438 **3.2. Model-MPs do not affect cell growth or morphology but have an effect on their aggregation.**

439 In a second part of the study, we evaluated the effects of Model-MPs on cell growth. In the
 440 literature, studies state that PS MPs of 100 μ m have no toxic effect up to 50 mg/L (Yan et al., 2021),
 441 the same was reported for MPs of 1-5 μ m, which had no effect on microalgae up to 41.5 mg/L (Prata
 442 et al., 2018). To verify whether it is the case for our Model-MPs and our microalgae strain, we
 443 monitored the cell growth of *C. vulgaris*, incubated or not with Model-MPs at a concentration of 40
 444 mg/L, which is the maximum concentration that we will use throughout this study. This
 445 concentration is most likely higher than the exposure concentrations encountered in the
 446 environment, however it allows us to observe and further characterize the effects on microalgae
 447 cells, which could not be detected otherwise. In addition, for small microplastics like the ones used in
 448 this study (5 μ m), no data on their environmental concentrations are provided in the literature, as
 449 most studies report data for particles between 300 μ m and 5 mm. It is thus difficult to predict the

450 concentrations of small particles in a natural sample. The growth curves obtained are presented in
451 Figure 3a; they show that in each case cell growth is similar, thereby showing that neither μ -PE nor μ -
452 Upcon-PE affect *C. vulgaris* cell growth. This is in good agreement with the data from the literature
453 although the type of plastic used was not the same. In addition, this also shows that UCNPs are not
454 toxic, as μ -Upcon-PE do not have an effect on cell growth. Moreover, incubation with Model-MPs do
455 not extend the exponential phase, meaning that *C. vulgaris* cells and Model-MPs do not have a
456 symbiotic relation either, as it was shown for other species. Indeed, Kang *et al.* observed that organic
457 intermediates resulting from MPs degradation can serve as a carbon source for algae (Kang et al.,
458 2019). Also in some cases, cell growth can be modified resulting from the EPS production (Casabianca
459 et al., 2020; Cunha et al., 2019; Long et al., 2017), but this is not the case here.

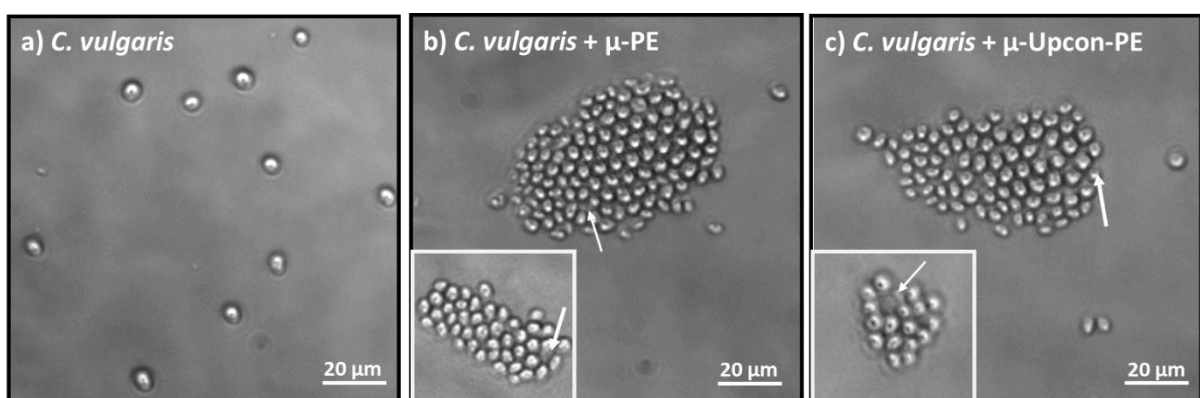
460 We then went down to the nanometer scale to evaluate if the presence of Model-MPs in the
461 culture medium had an effect on cell surface structure. For that, we incubated *C. vulgaris* during 7
462 days together with μ -PE, and took a close look at the cell surface and measured the roughness on
463 small areas ($0.3 \mu\text{m} \times 0.3 \mu\text{m}$) on top of the cells in contact mode, as shown in Figure 3b and c in
464 normal conditions or incubated with μ -PE for 7 days, respectively. These measurements were
465 repeated on 10 different *C. vulgaris* cells coming from at least 2 independent cultures in each case;
466 the results of these analysis are presented in the boxplot Figure 3d. They show that *C. vulgaris* cells
467 have an average roughness of $0.8 \pm 0.2 \text{ nm}$, which stays similar, of $0.6 \pm 0.2 \text{ nm}$, when cells are
468 incubated with μ -PE. Cell wall roughness of *C. vulgaris* was determined before at different pH values
469 (6 and 8) using AFM in a study by Demir *et al.* (Demir et al., 2020) and were in the same range.
470 Overall, these data show that the Model-MPs used at concentrations up to 40 mg/L have no effect on
471 either cell growth or cell nanostructure after 7 days of co-incubation.



472 **Figure 3: Characterization of *C. vulgaris* cells in interaction with Model-MPs.** a) Variations in optical
 473 density of *C. vulgaris* cells before and after incubated with Model-MPs (μ -PE and μ -Upcon-PE); b) AFM
 474 height images of *C. vulgaris* cell surface (0.3 μ m \times 0.3 μ m) in contact mode (color scale = 6 nm) and c)
 475 AFM height images of *C. vulgaris* cell surface (0.3 μ m \times 0.3 μ m) after incubated 7 days together with
 476 Model-MPs in contact mode (color scale = 8 nm); d) Quantification roughness values of *C. vulgaris* cell
 477 before and after incubation with Model-MPs for 7 days in a box plot.

478

479 Although our Model-MPs do not have an effect on *C. vulgaris* cell growth or cell



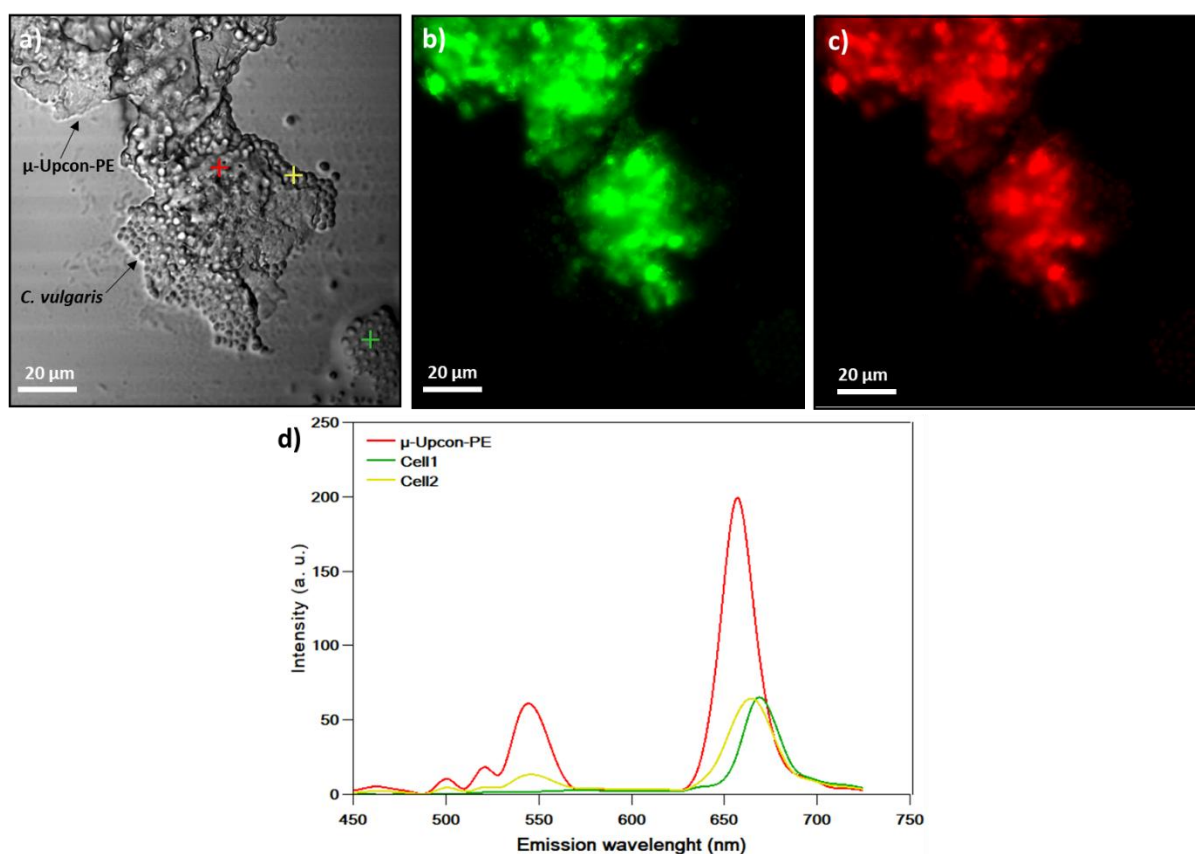
480 nanostructure, their addition to the culture medium could have an influence on the cell aggregation.
481 To evaluate this, we performed both optical microscopy imaging (Figure 4a-c) and two-photon
482 microscopy imaging (Figure 4d-f). In these experiments, cells were incubated 7 days with μ -PE and μ -
483 Upcon-PE at the concentration of 40 mg/L. In the control condition (Figure 4a, without Model-MPs),
484 we can see that cells are randomly distributed over the surface and no cell aggregation is observed.
485 In the cases cells were incubated with μ -PE and μ -Upcon-PE (Figure 4b and c), large aggregates of
486 cells are visible around what seems to be Model-MPs particles, indicated by the arrows on the
487 images.

488 **Figure 4: Images of *C. vulgaris* cells.** Bright field images of cells after incubated 7 days with: a)
489 nothing; b) μ -PE; c) μ -Upcon-PE. The arrow indicated the Model-MPs.

490 Thus, these first images suggest that Model-MPs cause the aggregation of cells. However,
491 because of the small size of Model-MPs, it is difficult to identify them with certainty and understand
492 their real implication in cell aggregation. In a next experiment, we thus took advantage of the
493 luminescence properties of μ -Upcon-PE, and made observations of cells incubated with μ -Upcon-PE
494 for 7 days (concentration 40mg/L) using a two-photon scanning microscope under an excitation at
495 980 nm (Yakovenko et al., 2022). On the bright field image (Figure 5a) big aggregate of cells can be
496 observed as well as what we expect to be μ -Upcon-PE particles. The composition of these aggregates
497 was confirmed by the green (Figure 5b) and red (Figure 5c) emissions under NIR irradiation. In the
498 case of μ -Upcon-PE, this emission corresponds to strong sharp green (515-575 nm) and red (630-680
499 nm) emission bands characteristic for Er-based UCNPs incorporated into μ -Upcon-PE, as visible on
500 the emission spectra in Figure 5d. *C. vulgaris* cells are characterized by a weak autofluorescence
501 (Takahashi, 2019; Tang and Dobbs, 2007), also visible on the spectra in Figure 5d. Upconversion and
502 autofluorescence spectra could be easily unmixed thanks to the limited spectra overlap especially in
503 the green, and the discrepancy in signal intensity (varying from different order of magnitude)
504 between upconversion and 2-photon emission (Figure 5d). Brightfield as well as bi-photon images of

505 UCNPs alone, μ -Upcon-PE alone and *C. vulgaris* cells alone can be found in Supplementary Figure S3.
506 These results allow us not only to accurately determine the presence and location of μ -Upcon-PE in
507 cell aggregates, but also to show two different things: i) the μ -Upcon-PE are in fact distributed over a
508 large part of the aggregate, which could not be visible on the standard optical microscopy images,
509 and ii) some cells are not directly bound to the particles (cells in the bottom-left part on the image
510 5d-f), which suggest that perhaps the aggregation in the presence of Model-MPs can occur through a
511 different mechanism than direct binding.

512 **Figure 5:** Two-photon microscopy imaging of μ -Upcon-PE and *C. vulgaris* aggregate after 7 days
513 inoculation together: a) Brightfield image; b) Green emission under NIR irradiation and c) red
514 emission under NIR irradiation observed for μ -Upcon-PE and *C. vulgaris* aggregate. Images show Z-

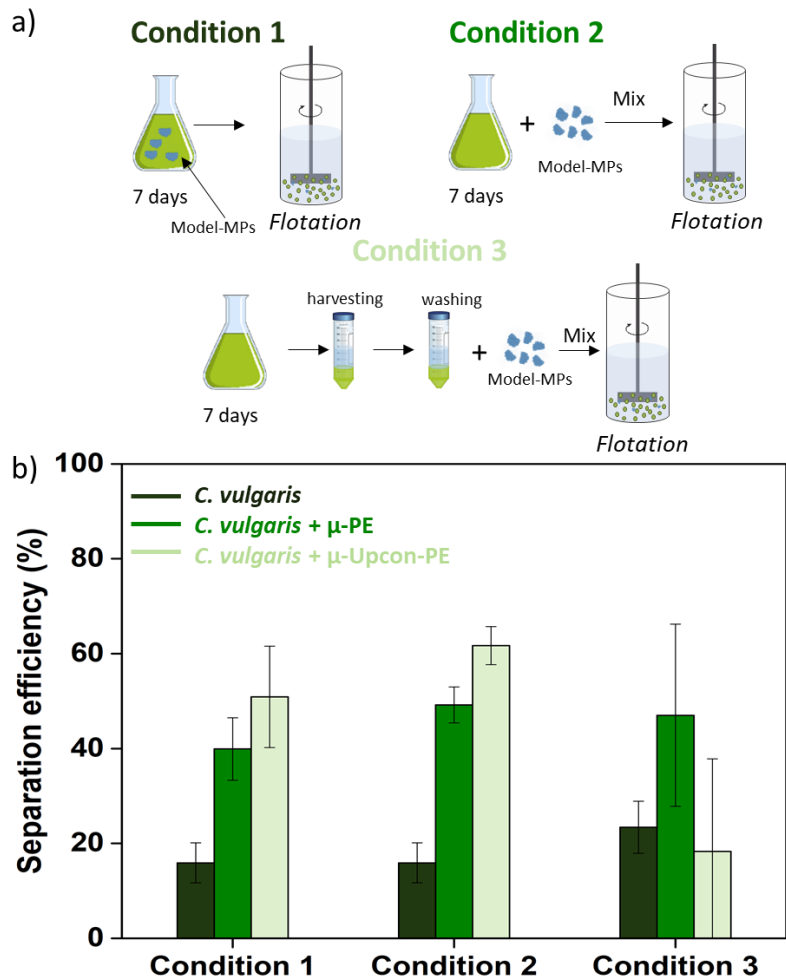


515 projection in a maximum intensity. d) Upconversion emission spectra of μ -Upcon-PE together with
516 two-photon emission spectra of *C. vulgaris* cells. The positions where the spectra were recorded on
517 the image is indicated by the coloured crosses on a).

518 Because these observations are qualitative, we then looked for a way to quantify the effect
519 of Model-MPs on cell aggregation, and performed flocculation/flotation experiments with different
520 μ -PE concentrations (final concentration of 0, 5, 10 and 40 mg/L) incubated 7 days together with *C.*
521 *vulgaris* cells. The results are presented in Supplementary Figure S4. In such experiments, cells can be
522 separated from the water by bubbles only if they are aggregated into flocs that are easily captured by
523 the rising bubbles and carried to the surface. The flotation step allows separating the aggregated
524 cells from the suspension, and thus to quantify the influence of Model-MPs on cell aggregation,
525 which is reflected by the separation efficiency percentage. In this case using bubbles was more
526 efficient than leaving the flocs to settle down because of their small size and low density. In the
527 absence of Model-MPs, the separation efficiency obtained is of 16 ± 5 %; this number reflects the
528 natural flocculation taking place in 7 days old-cultures, which is often the result of the natural
529 production of EPS by cells (Vergnes et al., 2019). When adding Model-MPs at a concentration of 5
530 mg/L and 10 mg/L into the culture medium for the 7 days of the culture, the separation efficiencies
531 are even lower, indicating that in these cases the addition of the MPs do not trigger any flocculation.
532 This is an interesting point because it means that to obtain aggregation, the concentration of MPs
533 must be important in the environment. Finally, as we expected from the optical microscopy
534 experiments, when cells are incubated with Model-MPs at a concentration of 40 mg/L, the
535 separation efficiency increases significantly, to around 50%, indicating that cell aggregation occurs,
536 but does not reach the entire cell suspension. Different hypotheses could explain the fact that at this
537 concentration only, cell aggregation occurs. The principal one could be that the presence of a certain
538 concentration of Model-MPs in the medium during culture triggers the production of EPS, which can
539 flocculate cells, as it has already been showed in the literature (Harrison et al., 2014; Lagarde et al.,
540 2016; Yan et al., 2021).

541 To test this hypothesis, flocculation/flotation experiments were repeated at 40 mg/L
542 concentration in different conditions (Figure 6). In the first condition, Model-MPs were incubated for
543 7 days together with *C. vulgaris* cells before conducting the experiments. In the second condition, the

544 cells in culture were not exposed to the Model-MPs, instead particles were added at the end of the
545 culture, for 15 minutes before flocculation/flotation experiments. The comparison of the results
546 obtained in these two conditions will help understanding if and how EPS interacts with Model-MPs,
547 or if cells produce more EPS when they are cultured in the presence of these particles. Finally in the
548 third condition, at the end of the cultures cells were washed in PBS to remove the EPS they may have
549 produced, and then only Model-MPs were added for 15 minutes before flocculation/flotation
550 experiments. The results obtained in each case are presented in Figure 6, they show that there is no
551 significant difference between condition 1 (flocculation efficiency of $51 \pm 11\%$ for μ -Upcon-PE) and
552 condition 2 (flocculation efficiency of $61 \pm 4\%$ for μ -Upcon-PE), meaning that even if cells are not
553 grown in the presence of Model-MPs, cell aggregation can still occur, and takes place rapidly as 15
554 minutes only are sufficient to obtain a separation efficiency similar to the one obtained in condition
555 1. An important point to note is that the modification of the Model-MPs used with UCNPs does not
556 have an effect on the flocculation/flotation efficiency, as similar efficiencies are observed with both
557 types of microparticles (μ -PE and μ -Upcon-PE). In condition 3, when EPS are removed from the cells
558 by centrifugation, the separation efficiency stays similar when μ -PE are used (flocculation efficiency
559 of $47 \pm 19\%$ for μ -PE), and decreases to $18 \pm 19\%$ when μ -Upcon-PE are used. Note that in this case,
560 the standard deviations obtained are large; non-parametric statistical test (Mann and Whitney test)
561 showed that the differences with condition 1 and condition 2 are in fact not significant when both
562 samples are used (μ -PE and μ -Upcon-PE). But still, these large standard deviations obtained in
563 conditions 3, even if the differences are not significant, tend to suggest that EPS could be involved in
564 the aggregation of cells, which may interact with MPs when they are added to the culture media.

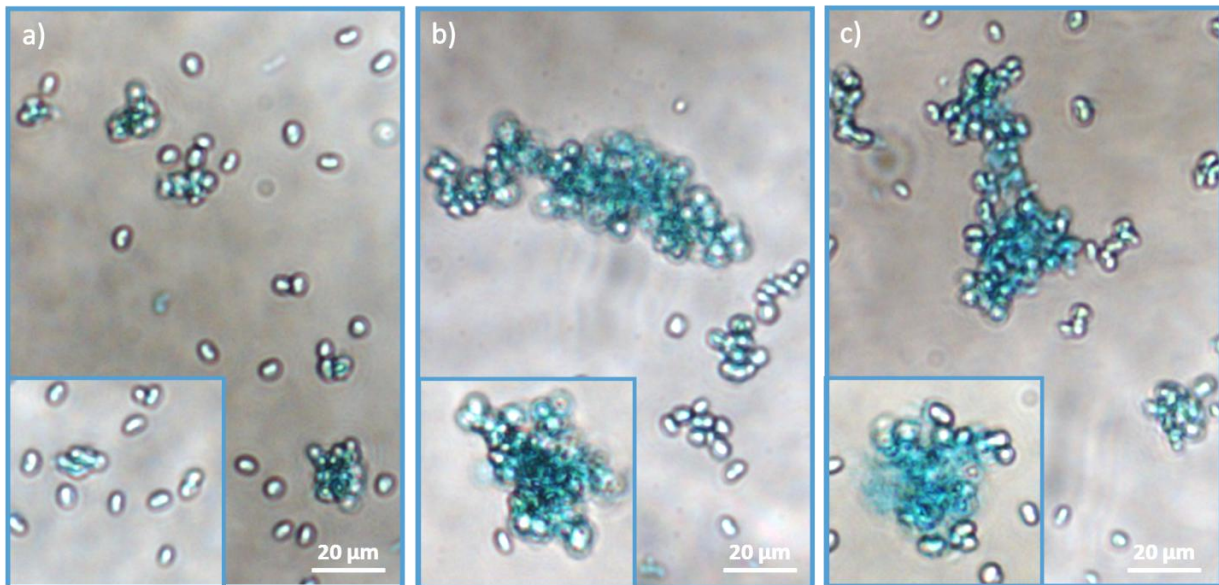


565 **Figure 6: Flocculation experiments of *C. vulgaris*.** a) Schematic representation of the conditions used
 566 for flotation/flocculation experiments. Condition 1: Model-MPs + cells after 7 days incubation
 567 together (no washing). Condition 2: Model-MPs are directly added to cells (no washing). Condition 3:
 568 Model-MPs are directly added to cells (washing PBS). b) *C. vulgaris* cell separation efficiency with
 569 Model-MPs (μ -PE and μ -Upcon-PE) at 40 mg/L concentration under the different conditions described
 570 in a).

571 3.3. Model-MPs induced aggregation can take place through different mechanisms

572 To understand if the presence of EPS is an important factor or not in the aggregation of cells
 573 in the presence of Model-MPs, we performed additional optical microscopy assays using Alcian blue
 574 staining. This dye is known to react specifically with acidic polysaccharides (Reddy et al., 1996;
 575 Shiraishi, 2015; Vergnes et al., 2019) present in the EPS excreted by microalgae cells, thus we
 576 selected this technique to qualitatively evaluate the presence of EPS excreted by cells grown in

577 presence of Model-MPs. The images obtained are presented in Figure 7. They show that when cells
578 are grown without Model-MPs (Figure 7a), cells produce EPS in a small amount. In particular, we can
579 see here the presence of small cell aggregates that are entrapped with the EPS visible on the image
580 (Figure 7a). This is in line with the flocculation/flotation results obtained that shows that cells
581 without MPs can still be separated with an efficiency of 16% (Supplementary Figure S3). When cells
582 have grown for 7 days in the presence of Model-MPs, large aggregates of cells are visible on the
583 images, on which large amounts of EPS can be observed (Figure 7b and c). These observations thus
584 suggest that the presence of Model-MPs in the culture medium triggers the production of EPS, as the
585 cells use them as a support to form biofilms around them (Yan et al., 2021), which is in line with the
586 previous literature on this subject (Harrison et al., 2014; Lagarde et al., 2016; Yan et al., 2021). When
587 we put these observations in perspective with the flocculation/flotation tests performed before, it
588 seems that the separation efficiency that we obtain when cells have been grown for 7 days with the
589 MPs is due to the increased production of EPS by the cells in a biofouling process. However, the
590 direct interaction of Model-MPs with cells seem also to induce aggregation (Figure 6 condition 2); as
591 it can be seen on these images (Figure 7b and c), cells do produce some EPS even if not grown with
592 MPs, and the decreasing trend of the separation efficiency obtained when cells are washed before
593 flocculation/flotation experiments (Figure 6, condition 3) would suggest that MPs can interact
594 directly with these EPS. The fact that the separations efficiencies when cells washed are not
595 significantly different could also suggest that this interaction could also be in part directly the cell
596 wall of cells. Another interesting point is that when cells have been grown with MPs, a certain
597 concentration is needed to induce cell aggregation: in this case this would mean that both the
598 production of EPS and the aggregation induced by contact between MPs and cells is concentration
599 dependent. When there are not enough MPs in the medium, the surface area of flocculant (MPs)
600 could be too small compared to the surface area of cells to aggregate them.

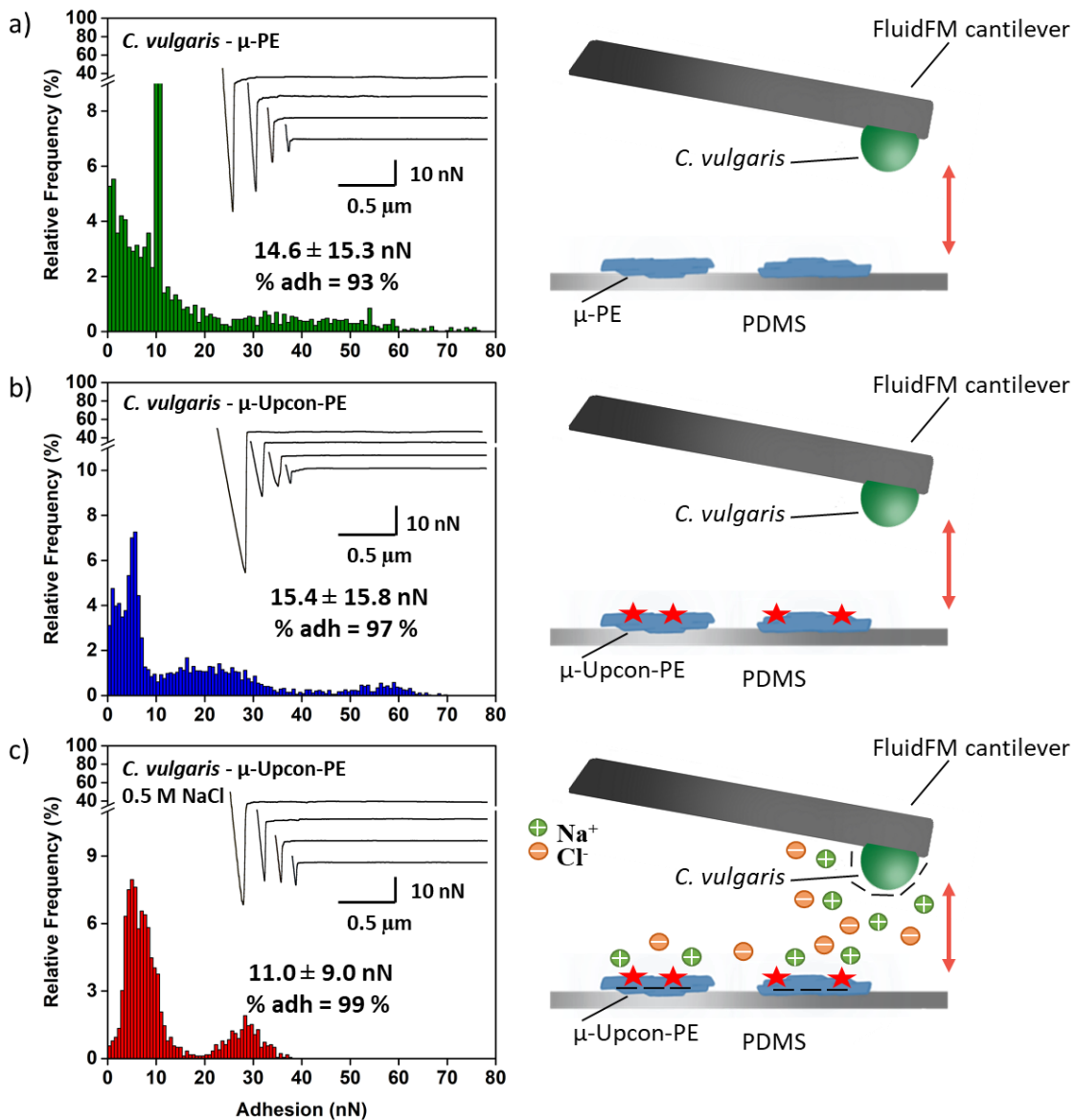


601 **Figure 7. Staining EPS produced by *C. vulgaris* with Alcian blue.** Optical images of cells dyed with
 602 Alcian blue, a) grown in normal conditions for 7 days, b) grown in the presence of 40 mg/L of μ -PE
 603 during 7 days, and c) grown in the presence of 40 mg/L of μ -Upcon-PE during 7 days.

604 While these results together bring explanations on the mechanism by which Model-MPs
 605 induce the aggregation of cells, a final point needs to be clarified; are the Model-MPs able to directly
 606 interact with cells? This would allow understanding why cells that have been in contact with MPs
 607 during 15 min only can be aggregated, and also why removing the EPS from cells does not decrease
 608 significantly the separation efficiency. To this end, we performed force-spectroscopy experiments to
 609 probe the interactions between single *C. vulgaris* cells and Model-MPs. In these experiments, cells
 610 have been washed to remove the EPS from the surface, this way it will be possible to directly probe
 611 the interactions between the cell's interface and the Model-MPs. For that, we used FluidFM
 612 technology, where single *C. vulgaris* cells are aspirated at the aperture of FluidFM probes by exerting
 613 a negative pressure inside the microfluidic cantilever. This negative pressure, compared to classic
 614 single-cell force spectroscopy methods using AFM, has the advantage of keeping the cells stable on
 615 the cantilever even when in contact with a strongly adhesive surface (Demir et al., 2020). The results
 616 of these experiments are presented in Figure 8. In the case of μ -PE (Figure 8a), the retract force
 617 curves obtained present a single retract peak happening close to the contact point, similar to what
 618 was observed with bubbles, with an average force of 14.6 ± 15.3 nN ($n = 2713$ force curves with 8

619 cells and particles coming from 2 different cultures, adhesion values can be found in Supplementary
620 Table 4). As for the interactions with bubbles, this force signature is typical of non-specific
621 interactions, and most likely reflect hydrophobic interactions, rather strong, between *C. vulgaris* cells
622 and μ -PE. Similar force curves were obtained for μ -Upcon-PE with a similar average adhesion force of
623 15.4 ± 15.8 nN ($n = 3470$ force curves with 10 cells and particles coming from 2 different cultures,
624 adhesion values can be found in Supplementary Table 5) shown in Figure 8b. The adhesion forces are
625 not significantly different at p -value of 0.05 (unpaired student test). This is in line with the previous
626 flocculation/flotation experiments, incorporating UCNPs to the μ -PE does not affect their interaction
627 with *C. vulgaris* cells. Thus, these results first show that there is indeed an interaction between cells
628 and Model-MPs, and that these interactions are nonspecific and hydrophobic. Recently, we
629 evaluated the hydrophobicity of *C. vulgaris* by measuring the interaction between air bubble and
630 single *C. vulgaris* cells (Demir et al., 2021), and found an average adhesion force of 4.2 nN (Demir et
631 al., 2021), showing that the surface of cells is not completely hydrophilic and has hydrophobic
632 properties. It thus means that Model-MPs can interact with cells directly through a hydrophobic
633 interaction. This is also in line with the bi-photon imaging experiment where microparticles directly
634 in contact with cells can be observed. To verify that no other type of non-specific interactions are
635 involved, like electrostatic interactions, additional force spectroscopy experiments were performed
636 between *C. vulgaris* and μ -Upcon-PE at higher salt concentrations (Figure 8c). When we increase the
637 salt concentration by adding 500 mM of NaCl in PBS buffer (0.137 M of NaCl) at pH 7.4, the charges
638 present on *C. vulgaris* cells and Model-MPs are shielded. Although the average adhesion force
639 recorded is 11.0 ± 9.0 nN ($n = 1785$ force curves with 6 cells and particles coming from 2 different
640 cultures, adhesion values can be found in Supplementary Table 6), given the wide distribution of the
641 values obtained, it is in the same range as for cells without salt addition. But still the difference is
642 significant (unpaired t-test, p -value of 0.05), meaning that electrostatic interactions are involved, but
643 they are not dominant compared to the hydrophobic interaction. An interesting point to note
644 concerns the wide distribution of the adhesion values obtained in each case. This heterogeneity can

645 be explained by the fact that in each case we aspirated a different cell. As we have no control over
646 the cell sizes depending on their age, the contact area in each case is different, resulting in different
647 adhesion values. Also, this heterogeneity in the results may be associated with the surface structure
648 of the Model-MPs which is irregular, perhaps modifying the contact area and the adhesion force
649 recorded. Indeed, when we look at the adhesion forces obtained throughout the surface of the
650 microparticles scanned, we can see that as the cantilever moves on the surface, the Model-MPs
651 adhesion force does not stay constant over consecutive measurements (decreases or increases,
652 Supplementary Figure S5). Finally, to confirm that the forces recorded are due to only interactions of
653 cells with microparticles, we probed the interactions between *C. vulgaris* cells and the surfaces of
654 Model-MPs are immobilized on, *i.e.*, PDMS. The results presented in Supplementary Figure S6a and
655 S6b show that neither *C. vulgaris* - PDMS nor FluidFM cantilever-Model-MPs interaction occurs,
656 confirming that the interactions described here indeed take place between cells and Model-MPs.



657 **Figure 8: Probing the interactions between *C. vulgaris* cells and Model-MPs.** Histogram showing the
 658 distribution of adhesion forces Adhesion force histogram obtained for the interaction between *C.*
 659 *vulgaris* cells and a) μ -PE, b) μ -Upcon-PE, and c) μ -Upcon-PE after 0.5 M NaCl addition. Insets in a, b
 660 and c shows the representative force curves obtained during force spectroscopy experiments.

661 Altogether, these results show that Model-MPs-induced aggregation can take place through
 662 different mechanisms. When cells have been grown in the presence of MPs, they use the MPs as a
 663 support for forming biofilms which triggers the production of EPS and the further aggregation of
 664 cells. However, MPs can also flocculate the cells by directly interacting with them, with their cell

665 surface directly and also with the small amount of EPS they produce at their surface in normal
666 conditions.

667 **4. Conclusions**

668 The ubiquitous presence of plastic in all environmental compartments raises great concern
669 about their potential negative impact on aquatic ecosystems in general. In recent decades, many
670 research efforts have focused on understanding the inclusion, transport, and effects of microplastics
671 on the aquatic trophic chain from zooplankton to mammals. However not much is known about the
672 interaction of MPs with primary producers such as microalgae, which are the base of trophic chain.
673 This study presents an original interdisciplinary work that allows for an understanding of the
674 interactions between environmentally relevant models of MPs and microalgae cells and the
675 consequences of such interaction. The biophysical characterization of the Model-MPs used in this
676 study showed that these particles are rough and irregular, similar to the ones found in the
677 environment, and also presents hydrophobic properties. Then, the combination of optical
678 microscopy imaging assays and population-scale flocculation/flotation experiments allowed us to
679 understand the role of MPs in the aggregation of cells. Our results showed that when cells are grown
680 in the presence of MPs, they produce more EPS responsible for cell aggregation. However, the
681 aggregation can also be induced by the direct contact between MPs and the cell surface or the EPS
682 they produce naturally in normal culture conditions. This was confirmed by single-cell force
683 spectroscopy experiments, which also led us to describe the physico-chemical nature of the
684 interactions between Model-MPs and cells. Altogether, the experimental approach developed in this
685 study has proven powerful to highlight the complexity of MPs-microalgae interactions and
686 understand the role of MPs in the formation of cell aggregates. This new information are important
687 to apprehend the impact of plastic pollution on aquatic ecosystems on a large scale.

688

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694

695 **Conflicts of interest**

696 The authors declare no conflicts of interest.

697

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