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Colonization of polystyrene microparticles by *Vibrio crassostreae*: light and electron microscopic investigation

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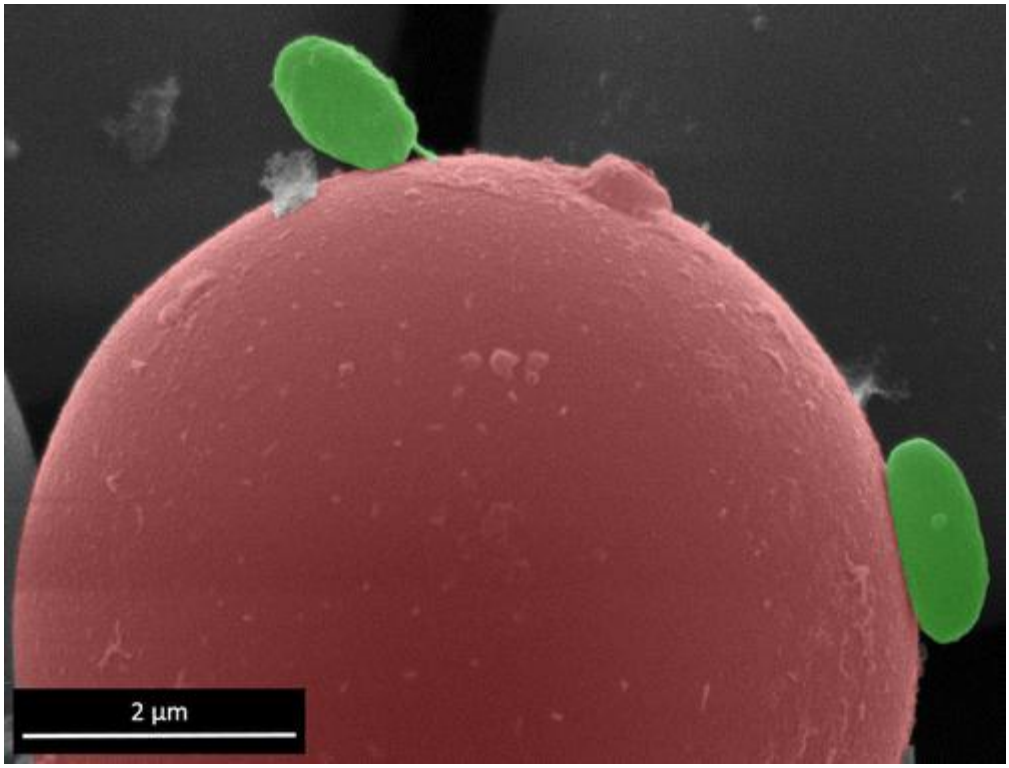
Abstract :

Microplastics collected at sea harbour a high diversity of microorganisms including some *Vibrio* genus members, raising questions about the role of microplastics as a novel ecological niche for potentially pathogenic microorganisms. In the present study we investigated the adhesion dynamics of *Vibrio crassostreae* on polystyrene microparticles (micro-PS) using electronic and fluorescence microscopy techniques. Micro-PS were incubated with bacteria in different media (Zobell culture medium and artificial seawater) with or without natural marine aggregates. The highest percentage of colonised particles (38-100%) was observed in Zobell culture medium, which may be related to nutrient availability for production of pili and exopolysaccharide adhesion structures. A longer bacterial attachment (6 days) was observed on irregular micro-PS compared to smooth particles (<10h) but complete decolonisation of all particles eventually occurred. The presence of natural marine agreggates around micro-PS led to substantial and perennial colonisation featuring monospecific biofilms at the surface of the aggregates. These exploratory results suggest that *V. crassostreae* may be a secondary coloniser of micro-PS, requiring a multi-species community to form a durable adhesion phenotype. Temporal assessment of microbial colonisation on microplastics at sea using imaging and omics approaches are further indicated to better understand the microplastics colonisation dynamics and species assemblages.

Graphical abstract



Keywords : microplastics, polystyrene, Vibrio, colonization

Introduction

While global plastic production was less than 2 million tons in the 1950s, worldwide production reached 311 million tons in 2014 [1]. Plastic is a persistent material that accumulates in land and water [2, 3] and the latest estimates are up to 51 trillion floating plastic particles on the ocean surface [4]. Plastics can be divided into three classes: macroplastics (> 20 cm), mesoplastics (0.5-20 cm) and microplastics (< 0.5 cm) [5]. Primary microplastics are synthesized as microbeads which are used in many cosmetics, synthetic fibers used in clothing manufacture, and industrial preproduction pellets [6]. Fragmentation of macro- and mesoplastic waste by physico-chemical and biological processes results in secondary microplastics [2, 7].

Microplastics can be transported over time over long distances by ocean currents and thus are subjected to intense biofouling by prokaryotic and eukaryotic organisms [8-13]. As a consequence, microplastics are suspected to disperse potentially invasive and harmful species [14], and may also represent a new ecological niche for microorganisms, also known as the "Plastisphere" [10]. Genomic studies on plastic samples collected in the Atlantic and Pacific oceans show a bacterial diversity that is different from the surrounding water [13, 15]. Zettler et al. (2013) reported that a significant proportion of the bacteria on a polypropylene microplastic were vibrios (24% of the total Operational Taxonomic Units of the plastic sample) [10]. The detection of *Vibrionaceae* members on marine microplastics was also demonstrated in other field studies conducted in the North Atlantic, the North Pacific Gyre, the Baltic Sea and the North Sea [16-18].

Vibrios are ubiquitous marine bacteria that are ecologically and metabolically diverse members of both planktonic- and animal-associated microbial communities [19, 20] and they represent one of the best studied models for the ecology and evolution of bacterial populations in the wild [21]. The study of the distribution of vibrios on fine phylogenetic and spatial scales

has demonstrated that vibrios coexisting in the water column can be divided into groups which pursue different lifestyles (free living, particle and animal-associated), defined as ecological populations [22]. Some populations are found in short lived blooms in the water column [21]. Rapid growth of vibrios has been correlated with a diatom bloom [23] or an association with algae [24]. This highlights, at microscale level, the importance of habitat occurrence and dynamic of vibrios population diversity in environment [25, 26]. Vibrios encompass the well-studied human pathogen, *V. cholerae*, as well as some very important, albeit less thoroughly characterized, animal pathogens [27]. For example, vibrios may participate actively in repeated mortality outbreaks in oyster beds (*Crassostrea gigas*) in France with losses of up to 80-100% of production [28, 29]. *Vibrio* species known for their pathogenic potential (*V. coralliilyticus, V. harveyi, V. splendidus, V. parahaemolyticus, V. alginolyticus and V. fluvialis)* were detected on microplastics [16, 18], suggesting that microplastics may constitute a niche for vibrios, influencing their population dynamics and ultimately pathogen emergence.

Previous studies demonstrating the interactions between MP and vibrio have been based on one shot 16S metabarcoding targeting hypervariable regions of the small subunit of the ribosomal RNA (rRNA) gene [10, 16]. However, this genomic approach at a single sampling time does not inform above the substrate specificity and the dynamics of the bacterial-plastic interaction. For instance, Datta et al. (2016) demonstrated that colonization of chitin microparticles result from a rapid succession of bacterial communities in which vibrios appear as secondary coloniser [30]. *Vibrionaceae* members are often stated to demonstrate habitat preference for plastics [10, 16, 17] whereas the colonization dynamics are still unclear. Whether vibrios are first colonisers exhibiting specific affinity for plastic polymer substrates or secondary opportunistic colonisers dependant of other bacteria that present on plastics remains unknown. As little is known about the distribution and dispersal mechanisms of most

pathogenic marine microbes, answering this question is of importance to understand the environmental conditions precluding *Vibrio* attachment on marine particles and the relative role of microplastics on their dispersal in regards with natural processes.

Vibrio crassostreae J2-9 has been associated to oyster disease (Pacific oyster *Crassostrea gigas* [28]) but the environmental reservoir of this pathogen remain to be elucidate [31]. The aim of this study was to investigate the ability of this *Vibrio* strain to colonise polystyrene microplastics in different conditions using fluorescent and scanning electron microscopy over 4 days. The results suggested that *Vibrio crassostreae* J2-9 was a secondary coloniser whose association with microplastics was favoured by prior formation of natural multi-species marine aggregates around microplastic particles.

Material and methods

1. Bacterial strain

Vibrio crassostreae, strain J2-9, was isolated during an mortality event in *C. gigas* in the Bay of Brest, France in 2011 [28]. A fluorescent-labelled bacterial strain constitutively expressing *gfp* (Green Fluorescent Protein) from a stable plasmid was established [32]. To reduce experimental variation among assays, the same bacterial culture was divided into aliquots in 20% glycerol for cryopreservation at -80°C. Eighteen hours before each experiment, a pre-culture of J2-9 GFP was prepared in Zobell liquid medium (4g peptone, 1g yeast extract, 0.1g Iron Phosphate and 30g sea salt added per litter of milliQ water) [33] supplemented with chloramphenicol 5 $\mu g.mL^{-1}$ at 18°C under mild stirring (130 rpm). The pre-cultures were then analysed with a flow cytometer (FACSVerse, Becton Dickinson, San Jose, CA, USA) to assess bacterial growth and concentration. Bacteria were detected on the green fluorescence channel (FITC channel, 527 / 32nm) after excitation by the blue laser at 488 nm, and the cell concentration was calculated based on the number of events (cells) recorded and the volume of samples analysed by the coupled Flow-sensor^{Tm} (Supporting Information - Figure S1).

118 2. Microplastics

119 Three types of polystyrene microparticles (micro-PS) were used as a colonization substrate:
120 (i) non-fluorescent smooth spherical microbeads (Phosphorex, Inc.) with a diameter of 6µm
121 (PS-s); (ii) fluorescent smooth spherical microbeads (Phosphorex, Inc.; excitation/emission
122 530/582nm) with a diameter of 5µm (PS-f); and (iii) non-fluorescent rough irregular particles
123 (Axalta) with a diameter of 45-60µm (PS-i).

124 3. Interactions between J2-9 GFP and PS microbeads

125 Bacteria and micro-PS were incubated in 15 mL Pyrex glass culture tubes in order to
126 minimise plastic particles sticking to the flask walls. Similarly, samples were systematically
127 collected using glass Pasteur pipettes to avoid a significant decrease in micro-PS
128 concentration as demonstrated in preliminary tests using plastic pipettes (up to 80% reduction;
129 data not shown). Two experiments were performed to test: (i) the effects of micro-PS shape
130 (spherical and smooth *vs.* rough and irregular) in two different media (artificial seawater – sea
131 salt (Sigma-Aldrich, Missouri, US), and Zobell culture medium) on J2-9 GFP colonization
132 processes in axenic conditions (Experiment 1); and (ii) the presence of natural microbial
133 communities on J2-9 GFP colonization processes (multi-species condition) (Experiment 2).

134 *3.1. Experiment 1 – Influence of micro-PS type in axenic conditions*

135 Bacteria and micro-PS (PS-s, PS-f and PS-i) were incubated in either artificial seawater at
136 $35 g.L^{-1}$ sea salt or in artificial seawater: Zobell media 50:50, all filtered at 0.22µm to remove
137 contaminating particles. Chloramphenicol was added to each medium at concentration of
138 $5 µg.L^{-1}$ to maintain the selection pressure and discourage contaminants (*e.g.* other bacteria) to
139 interfere with the experiment. The initial J2-9 GFP bacterial concentration was adjusted to
140 1.10^6 bacteria per mL in a final volume of 7mL of medium. The start of experiment
141 corresponds to the first contact between bacteria and microparticles, then all culture tubes
142 were immediately agitated at 300 rpm at 22°C. Experiment 1 was conducted in duplicate

tubes, and was replicated 3 times. The percentage of particles colonized by one or more J2-9 GFP bacteria was evaluated by epifluorescence confocal microscopy every 15-30 minutes during the first 10h of contact, at 24h post-incubation (PS-s and PS-f) and then every 24h for up to 4 days (PS-i only). For each sampling interval, 100 particles of PS-s and PS-f and 25 particles of PS-i were counted to estimate the percentage of colonized particles. Finally, detailed observations on the interaction between J2-9 GFP and PS microbeads were recorded using laser confocal microscopy and scanning electron microscopy (see details below § 4).

3.2. Experiment 2 - Colonization of micro-PS carrying a well-developed natural marine aggregate by V. crassostreae J2-9 GFP

To better simulate environmental conditions encountered *in situ*, PS-s, PS-f and PS-i (100 particles per mL) were incubated under gentle agitation for 7 days at 22°C in experimental tanks containing freshly collected natural seawater from the Bay of Brest. Well-developed aggregates were present around micro-PS within one week, as demonstrated in Wright et al. (2013)[34]. Then, 7mL of seawater containing micro-PS trapped in natural aggregates was incubated with J2-9 GFP bacteria (initial concentration: 1.10^6 bacteria per mL) in glass tubes. Tubes were placed at 22°C and gently stirred (300rpm) for up to 4 days. Qualitative observation using epifluorescence confocal microscopy was performed every 30 minutes for the first 10h and then every 24h until the end of the experiment (96h).

4. Microscopy

4.1. Confocal microscopy

Confocal microscopy allowed three dimensional visualization of micro-PS colonization by J2-9 GFP. This was monitored and measured using a Zeiss Axio Observer Z1 microscope (Carl Zeiss SAS, Jena, Germany) equipped with a mercury vapor lamp, coupled to the confocal module (spinning disc) VivaTome 3D. Observation of J2-9 GFP was performed by excitation through a blue filter 494/20 nm, and visualization of the green emission at 536/40 nm on the

GFP channel. Fluorescent micro-PS (PS-f) were shown through a red emission at 628/40 nm after excitation at 575/25 nm. All other microparticles were visible in white light. Images of particle colonization were obtained by a confocal laser scanning microscope (CLSM) Zeiss LSM 780 using 488nm and 561nm lasers. Colonized micro-PS were fixed with 6% glutaraldehyde for 24h at 4°C after 1h30 and 3h interaction. CLSM observations were only made in experiments performed in seawater, because there was green auto-fluorescence from Zobell diluted medium.

4.2. Scanning electron microscopy

Suspensions of colonized PS particles were fixed in 6% glutaraldehyde in 0.1M sodium cacodylate buffer (1.75% w/v of NaCl, pH 7.2). Suspensions were incubated for 10 minutes at 4°C before being filtered through polycarbonate filters with a 3µm pore size. Filters were rinsed with a solution of sodium cacodylate 0.1M (2% w/v of NaCl) in ultra-pure water MilliQ. After rinsing, samples were dehydrated by successive immersions in alcoholic hexamethyldisilazan (HMDS) (v:v): absolute ethanol:HMDS (3:1), absolute ethanol:HMDS (1:1), absolute ethanol:HMDS (1:3), and pure HMDS. Finally, samples were coated with gold palladium before being observed by scanning electron microscopy (SEM) (Hitachi S-3200N). Images of the particle colonization were obtained after fixation after 1h30 and 4h30 of interaction. Picture colorization was performed with the GNU Image Manipulation Program (GIMP 2).

5. Statistical analysis

Non-parametric (Wilcoxon-Mann Whitney) and parametric tests (2 way Student's t test with or without Welch correction) were carried out with R 3.2.3 data processing software (R Core Team, 2015). Mean comparisons were carried out on the maximum of colonization and on the

191 time needed to reach it between microparticles (PS-s, PS-f) and media (Zobell diluted media
192 and artificial seawater). A significant difference was observed for $p < 0.05$.

193 **Results and discussion**

194 **1. Fine scale micro-PS /*Vibrio crassostreae* interactions: pili formation and**
195 **exopolysaccharide production**

196 Rapid movements of J2-9 GFP from one particle to another prior to bacterial adhesion were
197 observed by confocal laser scanning microscopy (CLSM), suggesting active motility and
198 rapid dispersal ability, which are essential for the attachment of bacteria to microparticles [24,
199 30, 35]. After 1h30 of interaction, adherent J2-9 GFP cells were observed on 5µm fluorescent
200 smooth spherical polystyrene microbeads (PS-f) (figure 1A). Scanning electron microscopy
201 (SEM) observations confirmed the attachment of J2-9 bacteria to 6 µm smooth spherical
202 polystyrene microbeads (PS-s) *via* specific structures such as pili (arrow figure 1B). Pili are
203 tiny filaments (5-7 nm in diameter for 1-2 µm in length) linking bacteria to substrate by
204 specific proteins (pilin polymers and adhesins) [36-38]. It is well known that pathogenic *Vibrio*
205 species such as *V. cholerae*, *V. parahaemolyticus*, *V. fulnificus* and *V. mimicus* possess type
206 IV pili that are essential for adherence, colonization and pathogenicity [35, 39, 40]. In these
207 species, pili have predominantly a polar location, as it is observed here for *V. crassostreae*,
208 even though lateral pili can be also observed. When cells appeared attached sideways on
209 micro-PS, no specific structures were visible by SEM (figure 1C) suggesting that finer
210 structures were probably implicated in adhesion. After 3h of contact, CLSM observations
211 showed a higher proportion of bead aggregates, with J2-9 GFP bacteria being located around
212 PS-f particle aggregates (figure 1D). This was also confirmed by SEM observations made
213 after 4h30 of interaction, which demonstrated a high proportion of PS-s beads aggregated
214 alongside J2-9, and the presence of fibrous exopolysaccharide-like structures (EPS) (figure

1E). It was not possible to determine whether the presence of EPS was a cause or a consequence of micro-PS aggregation. EPS such as glycocalyx secreted around the bacterial wall are known to supplement reversible adhesion forces (electrostatic forces, Van der Waals, hydrophobic or ionic interaction) and may play a crucial role in the first stage of adhesion [41]. Datta et al. (2016) demonstrated the importance of particle attachment ability in the first hours of contact (attachment step) at the expense of bacterial growth on substrate [30]. Even though EPS may permit biofilm development leading to perennial settlement [19, 42, 43], the smooth micro-PS colonization observed in the present study was followed by a rather rapid decolonization as described below (§ 2).

2. *Vibrio crassostreae* exhibited rapid decolonization from smooth micro-PS

Colonization dynamics for PS-s and PS-f in each medium are shown in Figure 2. In all conditions (particle type and media) a dynamic in two phases was observed: first, the percentage of colonized smooth micro-PS increased to a maximum between 29 minutes and 7 hours of contact; then a decolonization phase occurred with a decrease in the number of colonized particles to zero after 24h of contact.

In seawater, the mean percentage of colonized particles was below 14% (except for one analytical PS-f replicate). The maximum percentages of colonized PS-s were 4.0 ± 1.4%, 14.5 ± 3.5% and 6.0 ± 1.4% (mean, n= 2, ± standard deviation, SD) for experimental replicates 1, 2 and 3, respectively (Figure 2A, Table S1). For PS-f, the maximum percentages of colonization were between 1.0 ± 1.4% and 1.5 ± 0.7% (mean ± SD), except for replicate 3 that exhibited a higher colonization of 58.0 ± 26.9% (Figure 2B, Table S1) despite identical experimental conditions (temperature, agitation, media, culture age and state). We cannot completely exclude experimental error to explain such high variability, but knowing that all cautions have been taken to avoid them, these results suggest instead that other parameters, such as fine level bacterial concentration dynamics, which were not controlled

and not addressed here, may influence the regulation of bacterial adhesion. The maximum percentage of colonized particles was significantly higher in Zobell culture medium than in seawater both for PS-s (p-value=0.0003) and PS-f (p-value=0.0049) (Figure 3A, Table S1). Colonization increased up to 38.0±2.8%, 72.0±11.3% and 95.5±3.5% for PS-s and 89.5±13.4%, 81±18.4 and 78.5±9.2% for PS-f in Zobell culture medium (Figure 2A-B, Table S1). No significant difference was observed between PS-s and PS-f for the maximum percentage of colonized particles and the time needed to reach the maximum percentage of colonization (Tm). Tm was between 4h20 and 6h09 in Zobell culture medium and this was significantly higher than the Tm measured in seawater (28min to 2h45) for both PS-s ($p = 0.0022$) and PS-f ($p = 0.0003$) (Figure 3B, Table S2). Higher nutrient availability is known to modulate the expression and maintenance of adhesion structures such as pili [44] and thus could enhance the final colonization success in Zobell culture medium with a higher percentage of colonized particles. Environmental reservoir of *Vibrio crassostreae* J2-9 remain unknown [31], as well as its carbon source preference in natural environment. An earlier colonization was observed in artificial seawater. The absence of nutrients may induce switch toward an active substrate/nutrient research phenotype, meaning increase dispersal ability, adhesion tentative or short adhesion on particulate matter present in the media (for instance microparticles). Indeed, adhesion was reported as a survival strategy for vibrios in nutrient-limited natural environment [45], and better biofilm formation was reported in nutrient limiting condition than in nutrient rich media [46]. Nutrient availability and/or quality might also explain the rapid decolonization observed for all smooth micro-PS. Decolonization and dispersion is well described in the literature for biofilms [44, 47] but is very poorly documented for the early stages of adhesion, notably for vibrios. In artificial seawater, the only source of organic matter is obtained from the bacterial inoculum by diluting the pre-culture, which was probably not sufficient for the deposition of a primary/conditioning film on the bead surface as observed by

CLSM and SEM for PS-s in Zobell culture medium (data not shown). The primary film changes surface properties (hydrophobicity, polarity, surface tension) and could provide sufficient nutrients at the substratum surface to be used by bacteria [36]. The absence of an artificial seawater primary film could explain the lower overall colonization. In Zobell culture medium, decolonization could be related to a limitation of some nutrients. Nutrient limitation is reflected by stationary phase emergence in bacterial growth. However, no relationship was demonstrated between the time of decolonization and the stationary phase of the culture. In order to further address this question, nutrient controlled experimental systems like fermenters equipped with a flow cell to measure bacterial concentration allowing maintenance of a stable bacterial population and nutrient supply could be used. In addition, the measurement of the numbers of bacteria per particle could not be quantitatively assessed in the present study by using microscopy techniques, and the development of cytometry methods is encouraged to assess this parameter as well as particle aggregation states, as demonstrated by Beloin et al. (2008) and Geng et al. (2014) [48, 49].

Additionally to other factors, adhesion structures are known to be regulated by multifactorial signals including quorum sensing [41, 43]. Quorum sensing involves inter- and intraspecific communication between bacterial cells by exchange of molecular signals involved in the expression of target genes [50, 51]. Therefore, an increase in cell density over time may regulate quorum sensing signals and lead to the production of adhesion modulator compounds like homoserine lactone causing cell detachment [19, 43, 47]. Physical parameters may also be involved in the decolonization dynamics. Indeed, recent studies in bacterial adhesion forces suggested that hydrodynamic movements associated with increased bacterial concentration might lead to detachment from substrates [52-54]. This is in agreement with real time CLSM observations that showed turbulences from rapid movement of bacteria. Collisions that occurred between them may have then generated offsets of adherent bacteria. This hypothesis

290 is also supported by the longer colonization observed for rough and irregular particles
291 (exposed in § 3) whose interstices may have provided a shelter supporting bacterial
292 settlement.

293 **3. Long term colonization was observed on irregular micro-PS (PS-i)**

294 PS-i colonization started within the first hours of contact, and the maximum percentage of
295 colonized particles was reached after 3h11 of contact in Zobell diluted medium (100% of
296 particles colonized) and 2h54 of contact in seawater (84%±17 of particles colonized) (Figure
297 2C) for one replicate. Particle colonization in the two other replicates was still increasing after
298 10h of contact in both media (Figure 2C). However, the percentage of colonized particles had
299 decreased by the next sampling observation (24h), therefore the time for which the maximum
300 percentage of colonized particles was reached was between 10h and 24h and cannot be
301 assessed more precisely. PS-i colonization remained higher than 30% after 24h of interaction
302 in seawater for all replicates while a complete decolonization was observed in Zobell culture
303 medium (data not shown). In seawater, even though a slight decrease of bacteria number per
304 particle, PS-i particles remained colonized for up to six days, after which complete
305 decolonization was observed. Substrate topography is a factor influencing bacterial adhesion
306 [36, 55]. The presence of interstices on the irregular PS microparticles may have provided shelter
307 for bacteria, as suggested by qualitative microscopical observations showing that bacteria
308 were located in deep cavities on the PS-i. Bacteria were thus less exposed to hydrodynamic
309 shearing forces [55]. The difference in size between PS-s (6µm) and PS-i (45-60µm) particles
310 may also explain the difference in colonization intensity and duration. Even though the
311 commercial polystyrene particles used in the present study are supposed to be free of
312 additives or any other chemicals, their full composition is unknown and other chemicals could
313 also influence the patterns of colonization reported in this study.

Overall, an efficient and lasting colonization with biofilm formation on micro-PS by *V. crassostreae* J2-9 GFP was not observed, regardless of particle type and media. This may suggest that this strain does not demonstrate any specific affinity for PS particles and is not able to use particle resources, which is crucial for primary colonisers [30]. Consequently J2-9 may be more of a secondary coloniser, requiring other microbial communities (first colonisers) that provide alternative carbon sources to durably colonise the polystyrene surface. As the substrate specificity of *Vibrio* on synthetic polymers is still not investigated [18], further experiments using other vibrio strains and other plastic polymers (e.g. polyethylene and polypropylene) should be conducted.

4. Natural aggregates enhanced perennial colonization of *Vibrio crassostreae* J2-9

PS-s, PS-f and PS-i incubated in natural seawater were rapidly subject to biofouling in the first 24h (figure 4A). After 7 days of incubation, no free micro-PS were detected in the natural seawater. All micro-PS were entrapped in aggregates of significant size (± 200µm) formed by debris and various microorganisms (figure 4B). Similar aggregates were also present with identical characteristics (size, shape, appearance) in the control tank containing seawater only. The rapid and efficient incorporation of the micro-PS within natural - probably multi-species - aggregates observed here after 7 days of incubation in natural seawater is in accordance with the rapid incorporation of micro-PS in monospecies marine phytoplankton aggregates (also called marine snow) previously demonstrated under controlled laboratory conditions [34, 56, 57]. Similarly, in the marine environment, plastic microparticles host a complex association of prokaryotic and eukaryotic microbial communities [8-10, 12, 13, 16]. Organism collaboration and competition result in aggregate formation and trapping of plastic microparticles [34, 56, 58]. When aggregates containing micro-PS were incubated with J2-9 GFP, a rapid and strong colonization of the surfaces of the aggregates by J2-9 GFP was observed with some patches of intense colonization (figure 4C). The vibrio J2-9 was located vertically in groups of 3 to 5

339 cells forming corolla structures (figure 4D - head arrow), or was organized into monospecific
340 biofilms at the surface of the aggregates (figure 4C - head arrow). This possibly resulted from
341 (i) communication between J2-9 GFP and organisms in the aggregate and (ii) alternative
342 source of nutrients produced as by-products by the organisms in the aggregate. This
343 colonization on aggregates was durable and still observed after 96 hours of contact at higher
344 intensity compared to that observed in all particle types used in experiment 1. However,
345 beyond time of contact (96h), it could not be established if J2-9 GFP disappearance was due
346 to loss of bacterial fluorescence or another factor. Predation of J2-9 GFP by ciliates feeding
347 on biofilms at the particle surface was observed as suggested by the appearance of
348 fluorescence in ciliates within a few hours (figure 4E). As J2-9 GFP was not visible
349 swimming in media, there was indirect evidence of lack of decolonization, contrary to all
350 other conditions (experiment 1 – particle colonization without pre-incubation in natural
351 seawater). Resulting of agitation, shear stress could also lead to an "erosion" of the aggregate
352 surface and the loss of J2-9 GFP bacteria. In natural environment, rafting communities on
353 plastic particles are exposed to currents and wave action, potentially resulting in their
354 dispersion. But shear stress was also reported as a factor which could increase cells' adhesion
355 capacity [59], suggesting complex processes acting in colonization and decolonization.

356 The presence of natural aggregates around the particles seemed to have favoured the perennial
357 colonization of J2-9 GFP on micro-PS suggesting that *V. crassostreae* J2-9 might be
358 classified as a second coloniser of micro-PS, as it was demonstrated for *Vibrionacea*e on
359 chitin microparticles [30]. These authors suggested that most particle attached bacteria may be
360 secondary consumers that recycle waste products from primary consumers.

361 These exploratory results obtained under controlled laboratory conditions open new fields for
362 research addressing the dynamics of microplastic colonization by populations of vibrios.

Indeed, our study concerning one strain of *Vibrio* and one polymer invite to investigate other strains with other type of plastic. The presence of vibrios detected *via* genomic approaches on microplastics collected at sea at one time point [10, 13, 16, 18] may result from secondary colonization enabled by primary bacterial colonisers or more specific chemotactic attraction to plastic polymer compounds. The identification of direct interaction as primary coloniser using different synthetic polymers should be carefully assessed for both pathogenic, non-pathogenic *Vibrio* species [18]. To test the hypothesis of secondary coloniser suggested by the present *in vitro* study, further studies mimicking more closely the natural environment and including omic approaches together with microscope observations, as it was done by Datta et al. (2016) [30], are clearly required. Sequential sampling over time on different plastic polymers and natural particles is necessary to characterize the temporal dynamics of first microbial – eukaryote – and *Vibrio* – colonization in natural marine environments. If *Vibrio* are truly second coloniser, the populations of microorganisms present on plastic and allowing for *Vibrio* colonization must be characterized for various plastic polymers in different environmental compartment and condition [13-15]. In addition, laboratory experiments using diverse ecological populations of vibrios would be useful to investigate species relationships (cooperation, competition, mutualism) involved in plastic colonization and their potential effects upon ingestion by marine organisms [22]. Given the relatively low proportion of microplastics in regards with other particulate matter (organic and inorganic) in marine ecosystems [60], the apparent complexity in the habitat preference of *Vibrio* species for plastic, and the relative lack of knowledge regarding their natural dispersal mechanisms, its makes difficult to postulate on the clear role of microplastics as vector for pathogenic vibrios in marine environment [13]. Studies aiming to better understand the colonization dynamics of microplastics by marine bacteria are required to investigate the role of microplastics as vectors of harmful bacteria for marine organisms.

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Supporting Information Available

Figure S1 shows cytogram of fluorescent bacteria counting by flow cytometry. Tables S1 and S2 provide detailed statistical test results. This information is available free of charge via the Internet at http://pubs.acs.org.

Figure captions

Figure 1. Electronic and photonic microscopical observations of Vibrio crassostreae J2-9 GFP adhesion in PS microparticles. A, D: confocal laser scanning microscopy (CLSM) of the colonization of Vibrio crassostreae J2-9 GFP (green) on fluorescent polystyrene microparticles (PS-f) (red) in artificial seawater. B, C, E: scanning electron microscopy (SEM) observations of colonization of V. crassostreae J2-9 GFP (green) on polystyrene microparticles (PS-s) (red) in Zobell diluted media. SEM pictures were artificially colorized using the GIMP software. Sample fixation times were 1h30 (A, B), 3h (D) and 4h30 (C, E). Scale bars: 2µm (A, D, E) ; 1µm(B) ; 0.5 µm (C).

Figure 2. Percentage of colonized polystyrene microparticles by Vibrio crassostreae J2-9 GFP over time in seawater (● - blue) and Zobell diluted media (○ - orange). These data were based on duplicate measurements from three independent experiments. For each sampling interval, 100 particles of PS-s and PS-f and 25 particles of PS-i were counted to estimate the

410 percentage of colonized particles. A: smooth non fluorescent polystyrene microparticles (PS-
411 s); B: fluorescent polystyrene microparticles (PS-f); and C: irregular polystyrene
412 microparticles (PS-i).

413 Figure 3. A: maximum percentage of colonization (mean ± SD; n = 6) for smooth polystyrene
414 microparticles (PS-s) and fluorescent polystyrene microparticles (PS-f) in both media
415 (artificial seawater and Zobell diluted media). B: time after which the maximum of
416 colonization was reached (mean ± SD; n = 6) for PS-s and PS-f in both media. Letters a, b
417 indicate significant difference between groups, $p < 0.05$.

418 Figure 4. Smooth non fluorescent polystyrene microparticles (PS-s) colonization in natural
419 seawater. A: PS-s were rapidly subject to biofouling in the first 24h observation – DAPI
420 staining. B: PS-s particles (shown by black arrows) entrapped in aggregates formed by debris
421 and microorganisms after 7 days of incubation. C: colonization of the aggregates containing
422 PS-s by Vibrio crassostreae J2-9 GFP after 8 hours of contact; PS-s are shown by black
423 arrows, J2-9 GFP biofilm is shown by arrowhead. D: corolla positioning of Vibrio
424 crassostreae J2-9 GFP on aggregates; PS-s are shown by black arrows, J2-9 GFP corolla
425 structure is shown by arrowhead. E: GFP fluorescent ciliate observed 2 hours after Vibrio
426 crassostreae J2-9 GFP addition. Scale bars: 10µm (A, E); 20µm (D); 50µm (B, C).

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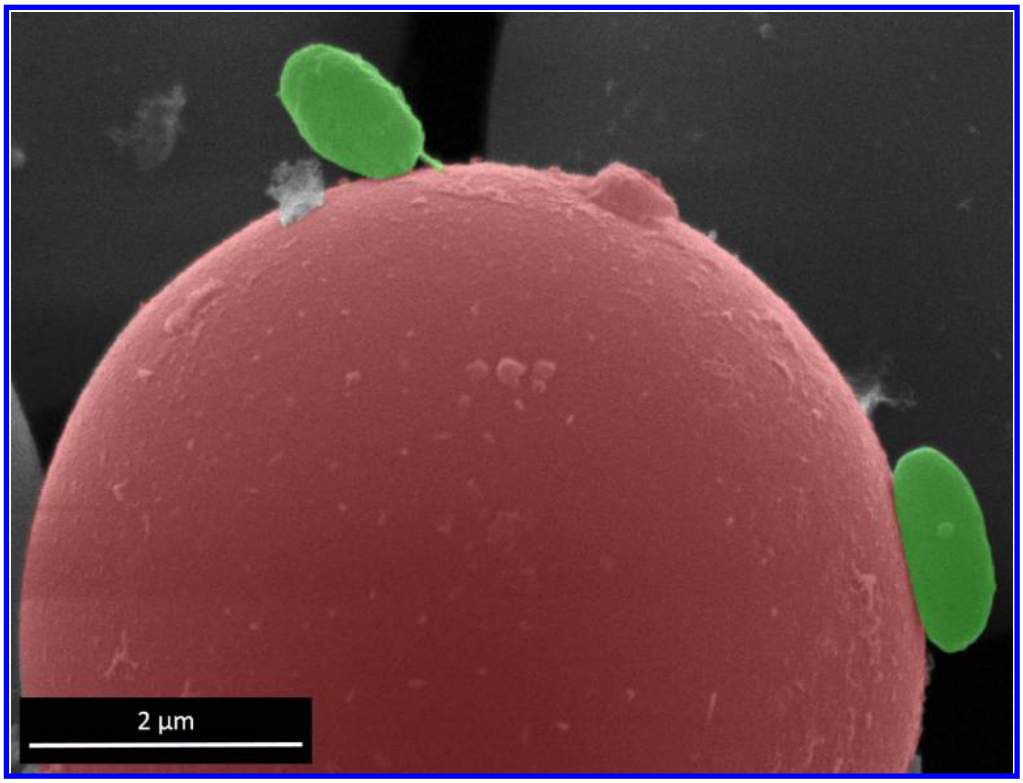


TOC abstract graphic

229x174mm (150 x 150 DPI)

Figure captions

Figure 1. Electronic and photonic microscopical observations of Vibrio crassostreae J2-9 GFP adhesion in PS microparticles. A, D: confocal laser scanning microscopy (CLSM) of the colonization of Vibrio crassostreae J2-9 GFP (green) on fluorescent polystyrene microparticles (PS-f) (red) in artificial seawater. B, C, E: scanning electron microscopy (SEM) observations of colonization of V. crassostreae J2-9 GFP (green) on polystyrene microparticles (PS-s) (red) in Zobell diluted media. SEM pictures were artificially colorized using the GIMP software. Sample fixation times were 1h30 (A, B), 3h (D) and 4h30 (C, E). Scale bars: 2µm (A, D, E) ; 1µm(B) ; 0.5 µm (C).

Figure 2. Percentage of colonized polystyrene microparticles by Vibrio crassostreae J2-9 GFP over time in seawater (● - blue) and Zobell diluted media (○ - orange). These data were based on duplicate measurements from three independent experiments. For each sampling interval, 100 particles of PS-s and PS-f and 25 particles of PS-i were counted to estimate the percentage of colonized particles. A: smooth non-fluorescent polystyrene microparticles (PS-s); B: fluorescent polystyrene microparticles (PS-f); and C: irregular polystyrene microparticles (PS-i).

Figure 3. A: maximum percentage of colonization (mean ± SD; n = 6) for smooth polystyrene microparticles (PS-s) and fluorescent polystyrene microparticles (PS-f) in both media (artificial seawater and Zobell diluted media). B: time after which the maximum of colonization was reached (mean ± SD; n = 6) for PS-s and PS-f in both media. Letters a, b indicate significant difference between groups, $p < 0.05$.

Figure 4. Smooth non-fluorescent polystyrene microparticles (PS-s) colonization in natural seawater. A: PS-s were rapidly subject to biofouling in the first 24h observation – DAPI staining. B: PS-s particles (shown by black arrows) entrapped in aggregates formed by debris and microorganisms after 7 days of incubation. C: colonization of the aggregates containing PS-s by Vibrio crassostreae J2-9 GFP after 8 hours of contact; PS-s are shown by black arrows, J2-9 GFP biofilm is shown by arrowhead. D: corolla positioning of Vibrio crassostreae J2-9 GFP on aggregates; PS-s are shown by black arrows, J2-9 GFP corolla structure is shown by arrowhead. E: GFP fluorescent ciliate observed 2 hours after Vibrio crassostreae J2-9 GFP addition. Scale bars: 10µm (A, E); 20µm (D); 50µm (B, C).

Figure 1

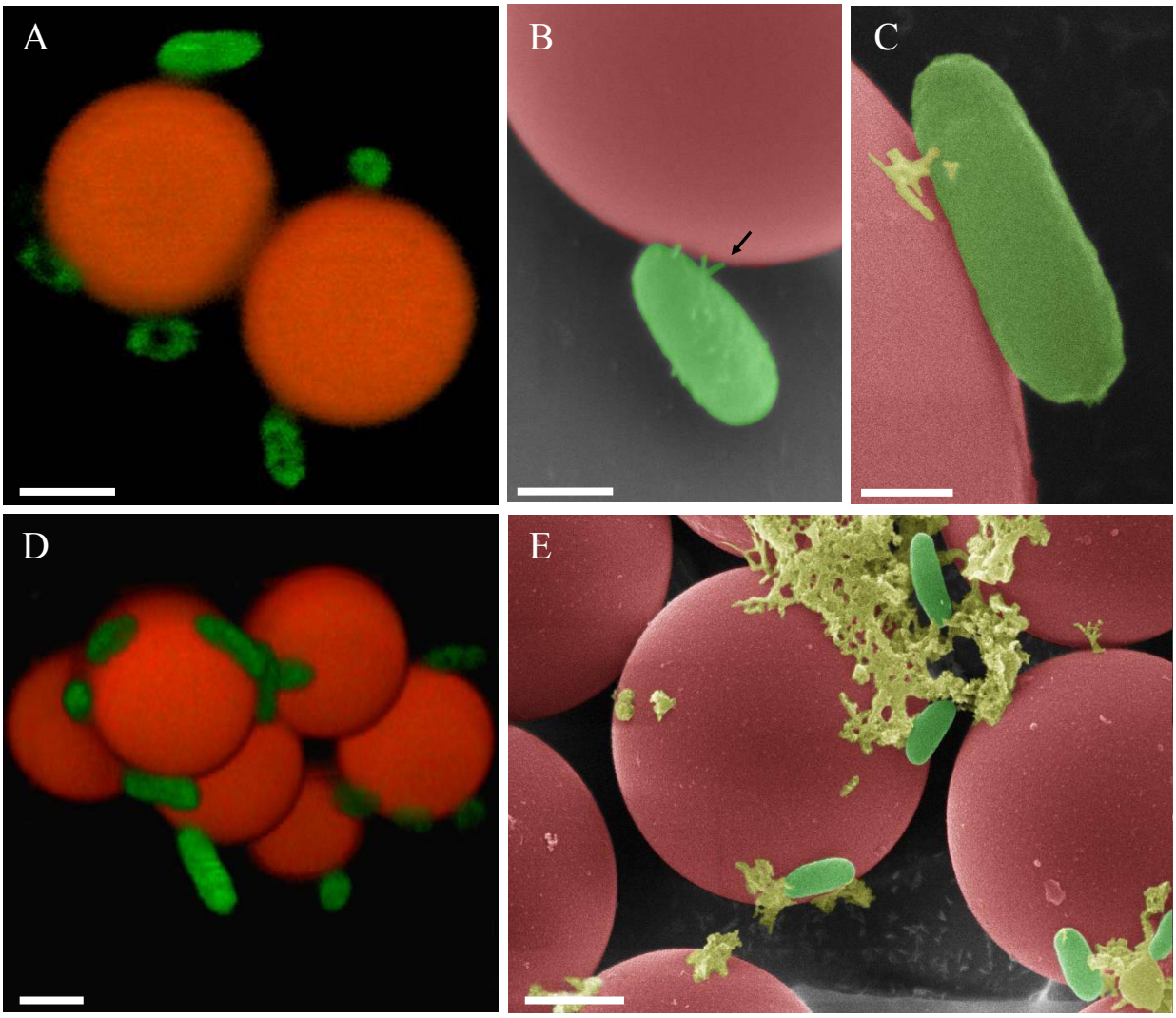
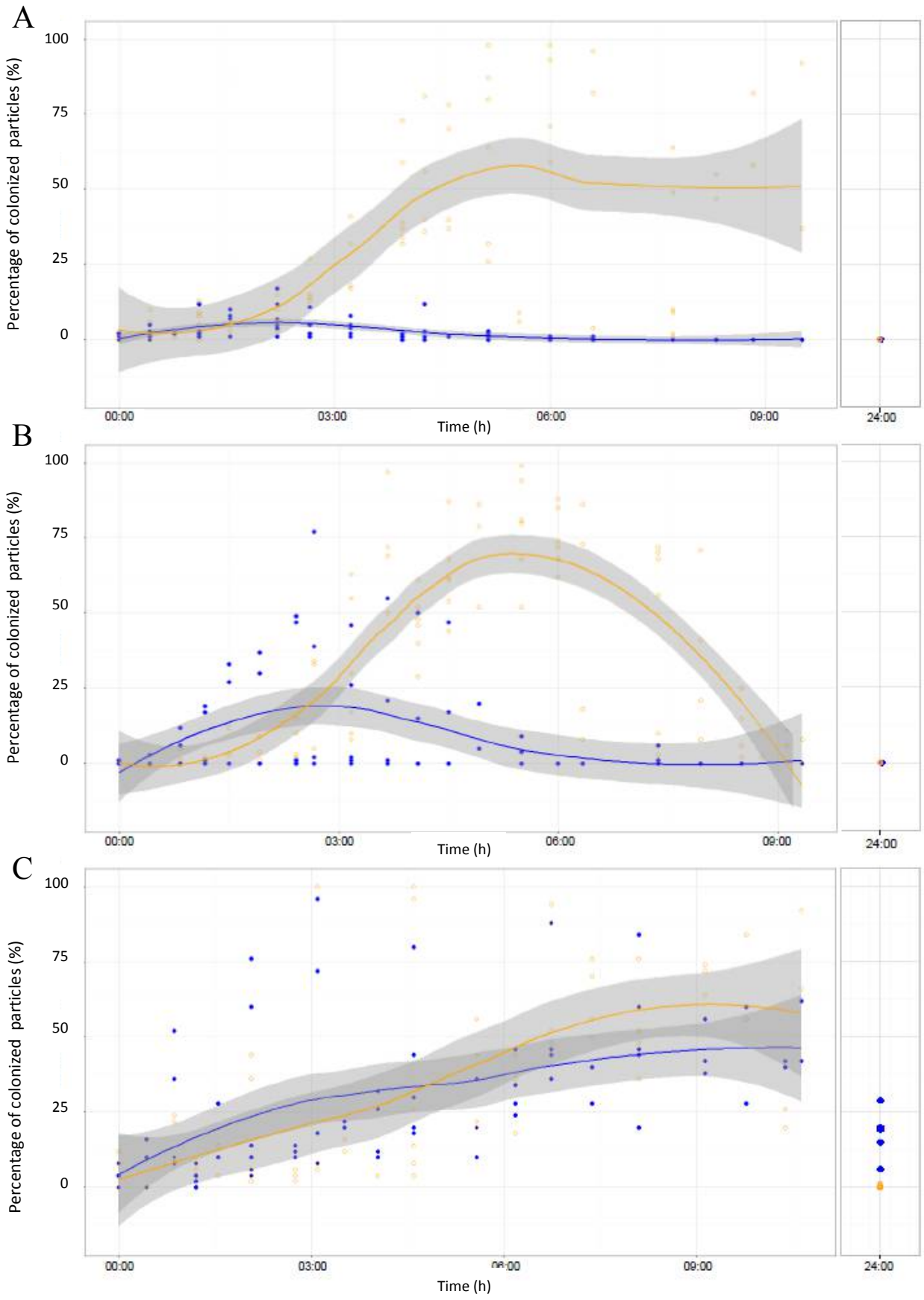


Figure 2


A
B
C
Percentage of colonized particles (%)
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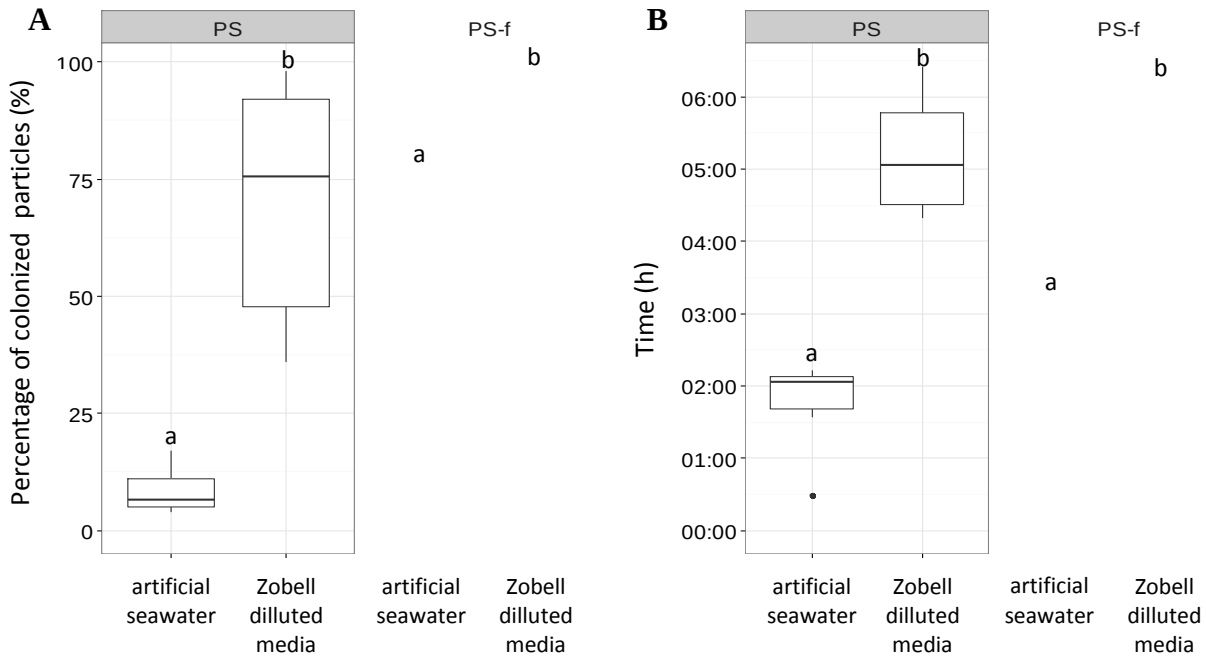
Time (h)

Figure 3


A
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Percentage of colonized particles (%)
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artificial seawater
Zobell dilluted media
artificial seawater
Zobell dilluted media

Figure 4