Biofilm formation by marine bacteria is impacted by concentration and surface functionalization of polystyrene nanoparticles in a species-specific manner

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Originality-Significance Statement

This work provides the first experimental evidence of how biofilm formation by marine bacteria is impacted by polystyrene nanoplastics and demonstrates that surface charge and concentration play important roles. The results of this study are significant to the scientific community, as they demonstrate the concentrations at which nanoplastics impact biofilm formation of marine bacteria.

Summary

The world's oceans are becoming increasingly polluted by plastic waste. In the marine environment, larger plastic pieces degrade into nanoscale (<100 nm in at least one dimension) plastic particles due to natural weathering effects. We observe that the presence of 20 nm plastic nanoparticles at concentrations below 200 ppm had no impact on planktonic growth of a panel of heterotrophic marine bacteria. However, the presence of plastic nanoparticles significantly impacted the formation of biofilms in a species-specific manner. While carboxylated nanoparticles increased the amount of biofilm formed by several species, amidine-functionalized nanoparticles decreased the amount of biofilm of many but not all bacteria. Further experiments suggested that the aggregation dynamics of bacteria and nanoparticles were strongly impacted by the surface properties of the nanoparticles. The community structure of an artificially constructed community of marine bacteria was significantly altered by exposure to plastic nanoparticles, with differently functionalized nanoparticles selecting for unique and reproducible community abundance patterns. These results suggest that surface properties and concentration of plastic nanoparticles, as well as species interactions, are important factors determining how plastic nanoparticles impact biofilm formation by marine bacteria.

Introduction

Marine environments are becoming increasingly polluted by plastics. By the year 2025, it is estimated that the oceans will contain more than 25 Mt of plastic litter (Jambeck et al., 2015). Research into the environmental impacts of plastic litter has predominately focused on plastics at the macro (>5 mm) and micro (<5 mm) scales. Concentrations of these plastics in the marine environment vary widely (Auta, Emenike, & Fauziah, 2017), with one study calculating that over a period of three days, more than two billion macro and microplastics entered coastal waters from two southern Californian rivers alone (Moore, Lattin, & Zellers, 2011). The fragmentation of microplastics into nanoplastics (<100 nm in at least one dimension) is anticipated in marine environments (Andrady, 2011), with studies confirming the presence of submicron plastic particles in ocean water beginning to emerge together with advances in detection technology (Ter Halle et al., 2017). In the marine environment, natural weathering effects caused by sand abrasion, waves and UV radiation (Gigault, Pedrono, Maxit, & Ter Halle, 2016), as well as digestive fragmentation (Dawson et al., 2018) degrade plastic waste into plastic nanoparticles. The process of larger plastic pieces degrading into the nanoscale has been observed in simulated weathering conditions (Lambert & Wagner, 2016; Shim et al., 2014). Placed in a weathering chamber, 1 cm^2 coupons from the polystyrene lids of disposable coffee cups released billions of submicron particles to the surrounding liquid within two months (Lambert & Wagner, 2016). Similarly, a recent report looking at nylon and polyethylene terephthalate teabags steeped in 95 °C water for 5 min observed the formation of billions of micro and nanoplastics (Hernandez et al., 2019). Degraded microplastics are associated with surface defects such as microcrack formation and bubbling or pitting, while the generated nanoplastics are frequently close to spherical in shape (Cooper & Corcoran, 2010; Corcoran, Biesinger, & Grifi, 2009; Hernandez et

al., 2019; Hüffer, Weniger, & Hofmann, 2018; Lambert & Wagner, 2016; Yousif & Haddad, 2013). The formation of nanoplastics in simulated marine environments has been shown to lead to the formation of fractal aggregates, and critical coagulation constants are often observed in the order of 10^{-3} – 10^{-2} M and 10^{-2} – 10^{-1} for multivalent and monovalent salts, respectively (Alimi, Farner Budarz, Hernandez, & Tufenkji, 2018; Gigault et al., 2016; Koelmans, Besseling, & Shim, 2015). This suggests that nanoplastics will readily aggregate in marine environments, with this aggregation expected to depend on surface charge and the presence of coatings, such as exopolymeric substances produced by bacteria (Alimi et al., 2018; Summers, Henry, & Gutierrez, 2018).

Despite evidence that the oceans are becoming contaminated with small plastic particles, very few studies have examined the ecological impact of nanoscale plastic waste on marine organisms. The majority of studies that do exist, focus on macroorganisms such as mussels (*Mytilus edulis*) and oysters (*Crassostrea virginica*), which have been shown to take up 100 nm polystyrene beads (Ward & Kach, 2009). Recently, the commercially important mollusc *Pecten maximus* was shown to uptake polystyrene nanoparticles at environmental concentrations of less than 0.015 ppm (Al-Sid-Cheikh et al., 2018).

Studies conducted to date on the impact of plastic nanoparticles on marine microorganisms have largely focused on photosynthetic microorganisms. For example, it has been shown that polystyrene nanoparticles modified with carboxyl groups (carboxyl nanoparticles; CNP) attach onto the surface of microalgae, while polystyrene nanoparticles functionalized with amino groups (amidine nanoparticles; ANP) can inhibit the growth of microalgae at sufficiently high

concentrations (Bergami et al., 2017). Another study showed that algal photosynthesis was hindered by the adsorption of 1.8–6.5 ppm of 20 nm polystyrene nanoparticles, mostly present as aggregates (Bhattacharya, Lin, Turner, & Ke, 2010).

Heterotrophic bacteria recycle waste and contaminants in marine environments. The water column is full of bacteria in planktonic form, and marine bacteria form biofilms on all available marine surfaces, including ship hulls and the surfaces of marine animals and plants (Dang & Lovell, 2016). Marine bacteria interact with eukaryotic microorganisms such as diatoms to form aggregates called marine snow (Gärdes, Iversen, Grossart, Passow, & Ullrich, 2011), which bring organic carbon and nutrients to the sea floor. Despite the ecological importance of heterotrophic marine bacteria, the impact of plastic nanoparticles on their ecology and physiology is completely unknown.

Bacteria have been shown to readily attach to and form biofilms on waste plastic in marine environments (Cooksey & Wigglesworth-Cooksey, 1995; Lobelle & Cunliffe, 2011), with different types of plastic selecting for distinct bacterial phyla (Roager & Sonnenschein, 2019). A recent study modeling the effects of biofouling on marine plastics predicted that biofilm formation decreases the onset time of particle settling, as attached organisms weigh down the particles (Kooi, Nes, Scheffer, & Koelmans, 2017). Biofouling of plastic also decreases its hydrophobicity, which can facilitate its passage below the air-water interface of the ocean surface (Lobelle & Cunliffe, 2011). Biofilm formation by marine bacteria on plastic in the oceans could therefore increase the speed with which this plastic makes its way to the sea floor. The effect of plastic nanoparticles on the dynamics of this biofilm formation, however, has not

yet been investigated. One study focused on polysaccharides produced by biofouling phytoplankton observed that polystyrene nanoparticles caused an increase in aggregation of polysaccharides (Chen et al., 2011), which suggests that the presence of plastic nanoparticles might stimulate biofilm formation by marine bacteria.

To test this hypothesis, we investigate the effect of varying concentrations of polystyrene nanoparticles on planktonic growth and biofilm formation of seven species of heterotrophic marine bacteria, both individually and as a community. Marine bacteria were selected to represent a range of biofilm forming abilities. Nanoparticles with two different surface characteristics were selected in order to investigate the potential importance of surface charge and functionality.

Results and Discussion

Characterisation of nanoparticle and bacteria physical properties

Nanoparticle suspensions of ANPs (Invitrogen™ amidine latex beads, 4% w/v, 0.02 μm; Thermo Fisher catalogue number A37309) and CNPs (Invitrogen ™ carboxyl latex beads, 4% w/v, 0.02 μm; Thermo Fisher catalogue number C37261) were prepared in both artificial seawater (Millipore Sigma Sea Salts catalogue number S9883, 40 g/L) and marine broth (Bacto Marine Broth, DIFCO 2216). While the individual particles were small (20 nm), ANPs and CNPs exhibited significant aggregation, with dynamic light scattering (DLS) (Zetasizer Nano ZS, Malvern, Massachusetts, USA) indicating that populations of nanoparticles greater than 1 μm existed under all conditions (Figure 1). Additionally, polydispersity indexes (PdIs) were frequently high, suggesting that several populations of aggregate sizes exist within a given suspension. Table 1 lists average size and PdI, together with size distributions. Nanoparticles

were characterized in both marine broth, in which experiments were conducted, and artificial seawater, to determine if the characteristics of nanoparticles suspended in nutrient media differed from what could be expected in the open ocean.

Broadly, nanoparticles in seawater were aggregated to a larger extent than in broth. This was most pronounced for CNP, with a Z-average diameter of 6,704 ± 1,746 nm in seawater, but only 202 ± 142 nm in broth (Table 1). Looking at the intensity weighted histograms in Figure 1, this latter value appears to be the average of two distributions, one at 44 nm and the second at approximately 1 μm. Given that the primary particles are 20 nm, the presence of this smallest peak likely suggests there are a significant fraction of CNP monomers, dimers, and trimers that have been stabilized by organic components of the marine broth. In seawater, this stabilization was not observed, and the smallest detected population occurred at approximately 460 nm. For ANPs, the disparity in Z-average sizes between marine broth and seawater was not as large, and the intensity weighted distributions remain similar.

Measurements of zeta potential (an estimate of surface charge) largely followed expected trends in seawater. ANPs were near neutral though slightly positive (2.1 ± 5.4 mV), while the CNPs were more negatively charged (-21.7 ± 5.8 mV). In broth, no significant difference between the zeta potentials of ANPs and CNPs was observed, with all nanoparticles falling between -4.9 and -9.9 mV. This supports the possibility of constituents of the broth adsorbing to the nanoparticle surface, forming a corona, and thereby influencing the surface charge (Fatisson, Quevedo, Wilkinson, & Tufenkji, 2012; Pulido-Reyes, Leganes, Fernández-Piñas, & Rosal, 2017).

For tested marine bacteria (Table 2), few differences were observed, with zeta potential falling between -1.7 and -11.1 mV in seawater and marine broth. Under all conditions, the bacteria had a

147 slightly negative charge. The magnitudes of the calculated zeta potential values were all less than
148 ± 30 mV, which is not sufficiently charged to provide a strong barrier to aggregation. As a result,
149 nanoparticle interactions with bacteria would be expected under all conditions.

150 **Impact of nanoplastic on planktonic growth and aggregation of marine bacteria**

151 Neither growth rate nor OD_{600} at stationary phase of tested marine bacteria (Table 2) were
152 impacted by the presence of most tested concentrations of nanoparticles, with the exception of
153 the highest concentration of 200 ppm (Figure 2). At 200 ppm CNP (equivalent to approximately
154 4.5 $x10^{13}$ 20 nm particles per mL), *C. marina* exhibited an increased growth rate and OD_{600} at
155 stationary phase, while the maximum optical density attained by *O. kriegii* at stationary phase
156 with this treatment was decreased. The impact of ANPs at the same concentration was more
157 consistent across bacteria, with most species demonstrating a decrease in OD_{600} at stationary
158 phase. Such a decrease can be interpreted either as an increase in aggregation of cells, or as a
159 decrease in total cell numbers due to impaired cell division or cell death.

160 To determine whether the presence of nanoparticles was causing an increase in cell-cell
161 aggregation, we examined the nanoparticle-treated cultures under the microscope after 48 h, and
162 quantified the percentage of total aggregation (Figure 3). Treatment with CNP had little impact
163 on aggregation, with only 200 ppm CNP treatment significantly increasing aggregation of *P.*
164 *carrageenovora* and *C. marina*. In contrast to CNP, treatment with 200 ppm ANP increased
165 aggregation of all bacterial species. Aggregation between negatively charged bacteria might be
166 facilitated by electrostatic interactions with the positively charged amine groups on ANP
167 particles.

To determine if treatment with nanoparticles was killing bacterial cells, a dead/live staining assay (BacLight® dead/live stain, ThermoFisher) was conducted on nanoparticle-treated samples after 48 h. No impact on the ratio of dead to living cells could be observed up to a nanoparticle concentration of 20 ppm (data not shown). However, fluorescent imaging of planktonic cells was not possible at 200 ppm due to the high concentration of nanoparticles in the suspension. Other studies have observed decreased growth rates of microalgae exposed to polystyrene nanoparticles, although this observation in a photosynthetic organism is most likely due to decreased light penetration caused by high concentrations of nanoparticles in suspension or attached to the cell surface (Baudrimont et al., 2019; Bergami et al., 2017). A study on the marine bacterium *Halomonas alkaliphile* observed that 80 ppm of polystyrene nanoparticles inhibited its growth, which the authors attributed to oxidative stress caused by the generation of intracellular reactive oxygen species (Sun et al., 2018). In the Sun et al. study, it was further observed that positively charged nanoparticles induced higher intracellular levels of reactive oxygen species and thereby oxidative stress than negatively charged ones. This observation offers a possible explanation for why we observed decreased growth in the presence of the neutral/positively charged ANPs but not the more negatively charged CNPs. Most likely, a combination of increased aggregation and decreased growth is responsible for the observed impact of nanoparticles on OD_{600}, with the relative contribution of each factor dependent on the bacterial species**.**

Impact of CNP and ANP on biofilm formation of marine bacteria

Lower concentrations of CNP had no impact on the amount of biofilm formed (Figure 4a). At 200 ppm, however, significantly more biofilm was formed by *M. adhearens*, *M. algicola*, *C. marina* and *O. kriegii*. This same concentration of CNP resulted in a small but significant

decrease in the amount of biofilm formed by *P. inhibins*. Confocal laser scanning microscopy of biofilms revealed that nanoparticles were integrated into the biofilm itself (Figure 4b), potentially accounting for the increase in total biomass revealed by crystal violet staining. However, *P. inhibins*, which showed a decrease in total biomass with CNP treatment, also integrated CNP into the biofilm. Treatment with 200 ppm CNP had no impact on the ratio of living (green) to dead (red) cells present in any of the biofilms, suggesting the presence of CNP does not kill the cells. Thus, even though the fluorescently-labeled CNP used in the biofilm experiments were not washed to remove any additives present in the stock suspension (Pikuda, Xu, Berk, & Tufenkji, 2018), those additives remaining did not impact cell viability.

Lower concentrations of ANP had no impact on the amount of biofilm formed (Figure 5). At 200 ppm, however, significantly less biofilm was formed by *M. hydrocarbonoclasticus*, *P. inhibins*, *P. carrageenova*, *M. algicola*, and *C. marina.* This is in contrast to the impact of CNP, which tended to increase total biofilm amount. Treatment with ANP did increase the amount of biofilm formed by *O. kriegii*.

Fluorescently labeled ANP are not commercially available, so the presence of ANP in the biofilm could not be assessed using fluorescence microscopy. The ratio of living (green) to dead (red) cells in the ANP treated vs untreated biofilms (Figure 5b) suggests that the presence of ANP did not kill the cells.

Concentrations less than 20 ppm of either type of polystyrene nanoparticle had no detectable impact on growth or biofilm formation of the seven tested marine bacteria. This raises the important question of environmentally relevant concentrations. Although it is possible to detect the presence of sub-micron plastic particles in environmental samples using pyrolysis GC-MS

(Ter Halle et al., 2017), the confounding influence of other environmental components currently hinders the direct quantification of plastic nanoparticles in environmental samples. In contrast to nanoplastics, microplastics (100 nm - 5 mm) have been well quantified in various marine environments, with the highest concentrations recorded in beach sediments (Besseling, Redondo-Hasselerharm, Foekema, & Koelmans, 2019). As this plastic degrades into nanoparticles, it can be assumed that the highest concentrations of nanoparticles will be achieved in areas rich in plastic debris with little mixing to the wider environment. For example, in sheltered microenvironments of the Great Pacific Garbage patch where plastic is exposed to the degrading effects of photooxidation and mechanical abrasion, and in beach sediments where plastic debris accumulates. To achieve a concentration of 200 ppm, 200 μg of plastic would need to fragment into approximately 4.5 $x10^{13}$ nanoparticles contained within 1 mL of seawater. While this does not sound unreasonable within localized microenvironments, the true concentrations of plastic nanoparticles in the environment will remain unknown until technology develops further. The fact that concentrations of less than 20 ppm had no impact on growth or biofilm formation of marine bacteria in our study suggests that plastic nanoparticles present in well mixed pelagic marine environments will most likely not have a significant impact on the formation of marine bacterial biofilms.

CNP and ANP had distinctly different impacts on the amount of biofilm formed by the seven tested marine bacteria, presumably due to the unique surface properties imparted by the amidine and carboxyl functionalization. Without control samples treated with non-polystyrene NPs, results of these experiments cannot be attributed to the fact that the NPs are made of polystyrene, but it is clear that surface functionalization of the NPs played an important role. Interestingly, in the presence of ANP, increased cell-cell aggregation was observed in planktonic cultures (Figure

3), yet the amount of surface-attached biofilm was decreased (Figure 5). This suggests that nanoparticles in the marine environment might impact pelagic microbial communities differently from sessile ones, although any extrapolations from the experiments performed here to the natural marine environment remain speculative at this point.

In the marine broth in which experiments were conducted, CNPs exhibited a large heterogeneity in particle aggregate sizes, with distinct populations clustering around 60 nm and 1100 nm, while ANP clustered exclusively around the 1100 nm range. The size of aggregates formed by the 20 nm nanoparticles might therefore be contributing to the observed differences in biofilm formation. When suspended in seawater, CNPs exhibited increased aggregation and lacked the smaller 60 nm population observed in marine broth. This highlights the importance of environmental factors on the behavior of plastic nanoparticles. Previous studies have shown that aggregation of nanoparticles is to be expected under the high-salt conditions of both natural/artificial seawater and marine broth (Alimi et al., 2018; Gigault et al., 2016; Koelmans et al., 2015; Summers et al., 2018). Our experiments were conducted in marine broth in which the surface charge of ANPs particles was decreased, possibly decreasing the impact of surface charge in our experiments compared to what might be expected in natural marine environments. This may at least partially explain why ANPs, which could be expected to facilitate electrostatic interactions between negatively charged bacterial cells and increase cell-cell interactions, actually decreased biofilm formation in most species, rather than increasing it.

Impact of nanoplastic on bacterial community structure

An artificial community was created by combining all seven marine bacteria used in this study (Table 2). The relative abundance of the seven species changed between the three treatments (Figure 6a), with the relative abundance of *M. hydrocarbonoclastic* increasing with nanoparticle

259 treatment, and the relative abundance of *P. inhibins* decreasing. nMDS analysis showed that the
260 species abundance patterns of replicate samples of untreated, CNP treated, and ANP treated
261 biofilms were similar to each other, but significantly dissimilar from other treatments, with no
262 overlap of 95% confidence ellipses (Figure 6b). Presence of either CNP or ANP therefore had a
263 significant impact on the community structure of biofilms, in a reproducible manner that was
264 unique to the nanoparticle type. Although starting communities were identical, the relative
265 abundance of the species comprising the resulting biofilm community that formed in the
266 presence of CNP was significantly different from the relative abundances in the presence of
267 ANP. These results suggest that not only the presence but also the type of nanoparticle can have
268 a significant impact on the biofilm community structure. Interestingly, the specific impacts on
269 community structure could not have been predicted from the results of monoculture experiments.
270 For example, in the presence of ANPs, *M. hydrocarbonoclasticus* dominated the resulting
271 biofilm community. Yet, when a monoculture of *M. hydrocarbonoclasticus* was exposed to
272 ANPs, the amount of biofilm decreased. This suggests that the impacts of plastic nanoparticles
273 on community structure depend not only on physical characteristics of the particles and the
274 bacteria, or on environmental parameters, but also on the interactions between species.
275 Considering our artificial community was composed of only seven species, interactions between
276 species in the natural marine environment can be expected to be even more complex.

277 An additional factor that was not investigated here is the substrate on which the biofilm forms.
278 How the presence of plastic nanoparticles impacts biofilm formation on a plastic substrate is not
279 necessarily indicative of how cell-cell aggregation in a particle of marine snow would be
280 impacted, or how a biofilm would form on the rough surface of a clam shell. The potentially
281 different impacts on free-floating aggregates such a marine snow and sessile marine biofilms is

well illustrated by our results showing that ANPs increased aggregation of planktonic bacterial cells (Figure 3), yet decreased surface-attached biofilm formation (Figure 5).

Conclusions

The fact that concentrations of less than 20 ppm had no impact on growth or biofilm formation of marine bacteria in our study suggests that plastic nanoparticles present in well mixed pelagic marine environments will most likely not have a significant impact on the formation of marine bacterial biofilms. Whether lower concentrations might nevertheless impact the community composition of biofilms remains to be determined. The results of our study demonstrate that nanoparticle surface characteristics and concentration both have an impact on biofilm formation by marine bacteria, albeit in a species-specific manner. The impact of plastic nanoparticles will differ between bacterial species, and become increasingly complex as the number of species increases. In order to understand how naturally occurring nanoparticles will impact marine microbial communities, it will be critical to understand the physical properties of naturally produced nanoparticles in the environment.

Acknowledgements

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Tables and Figures Captions

Table 1. Size and zeta potential of nanoparticles and bacteria used in this study, suspended in seawater or marine broth. CNPs and ANPs were obtained suspended in water by the manufacturer without any additives or preservatives. Dynamic light scattering (DLS) was performed to assess the aggregate size of nanoparticles suspended in artificial seawater or marine broth media at a particle concentration of 200 ppm. Size measurements are reported as both intensity-weighted and Z-average (cumulants fit) diameters. The heterogeneity in aggregate sizes within a suspension is indicated by the polydispersity index (PdI), which ranges from 0 to 1, with more polydisperse samples approaching unity. Electrophoretic mobility (EPM) measurements were collected via laser Doppler velocimetry and converted to zeta potential (ZP), providing an estimate of surface charge which gives insight into the stability of particles in suspension.

	Seawater					Marine Broth				
	Z- average Size (d.nm)			Zeta Potential (mV)		Z-average Size (d.nm)			Zeta Potential (mV)	
	avg	std	PdI	avg	std	avg	std	PdI	avg	std
CNP	6704	1746	0.36	-21.7	5.8	202	143	0.83	-9.9	4.6
ANP	2910	1731	0.83	2.1	5.4	1019	104	0.20	-6.6	5.1
M. adhaerens				-6.0	2.3				-8.9	1.4
O. kriegii				-6.7	4.8				-8.8	2.2
M. algicola				-6.5	1.2				-8.1	1.6
C. marina				-10.8	1.2				-9.7	1.1
M. hydrocarbonoclasticus				-6.3	2.6				-9.7	1.6
P. carrageenovora				-7.5	1.8				-11.1	3.2
P. inhibens				-1.7	3.8				-5.6	1.4

Table 2. **Marine biofilm-forming bacteria used in this study.** Strains are ordered by increasing biofilm-forming ability. All bacterial strains used in this study were obtained from the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ).

	bacterium	**Original isolation source**	**Biofilm formation in this study**
1	*Marinobacter adhaerens* DSM-23420	marine aggregates (0.1-1 mm in diameter) from surface waters, Wadden Sea (Germany)	poor
2	*Oceanobacter kriegii* DSM-6294 (ATCC 27133)	seawater (USA)	poor
3	*Marinobacter algicola* DSM-16394	lab culture of dinoflagellate *Gymnodinium catenatum* YC499B15 (Scotland)	good
4	*Cobetia marina* DSM-4741 (ATCC 25374)	seawater	good
5	*Marinobacter hydrocarbonoclasticus* DSM-11845 (ATCC 700491)	oil producing well (Vietnam)	good
6	*Pseudoalteromonas carrageenovora* DSM-6820 (ATCC 43555)	sample of seawater and marine algae (Halifax, Canada)	excellent
7	*Phaeobacter inhibens* DSM-17395	seawater from larval cultures of scallop, *Pecten maximus* (Spain)	excellent

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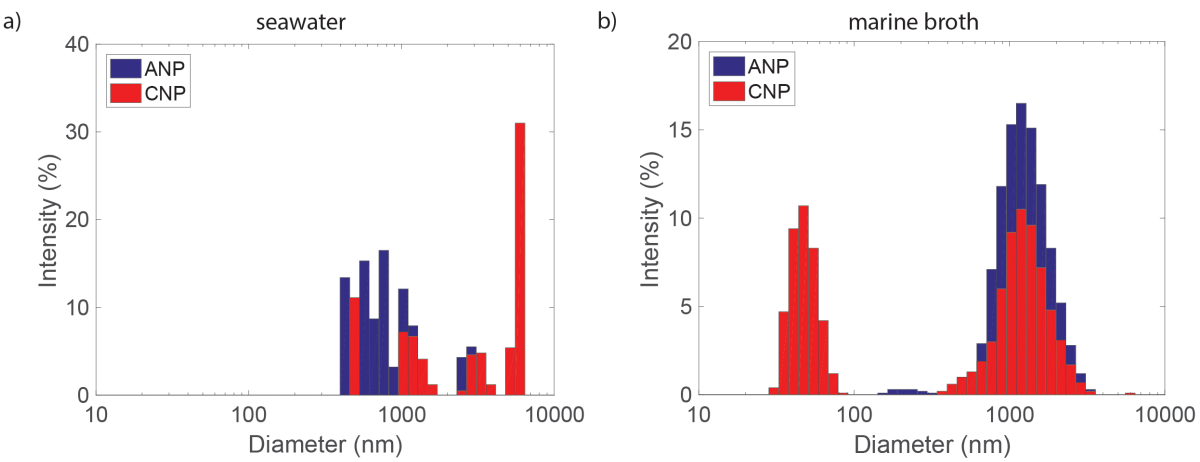


322

323 Figure 1. **Size distribution of 20 nm nanoparticle suspensions** in a) artificial seawater or b)
324 marine broth. Nanoparticles were suspended at a concentration of 200 ppm and corresponding
325 aggregate sizes were determined by DLS. The details of these and all other methods can be found
326 in supplementary data.

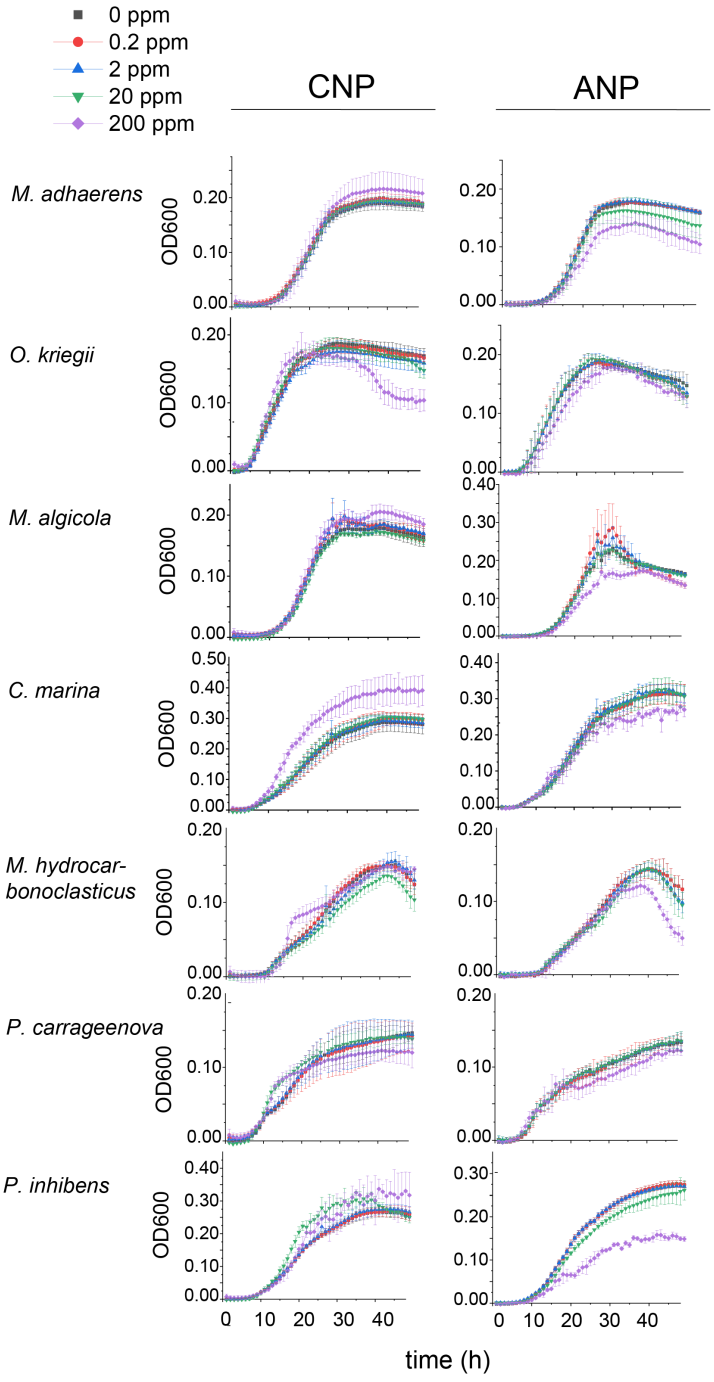
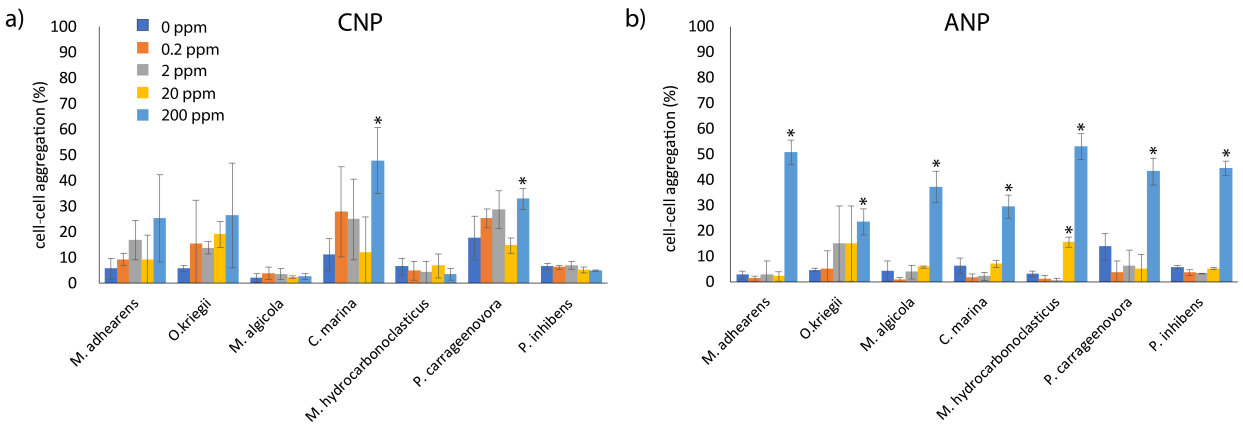


Figure 2. **Planktonic growth curves in the presence of CNP or ANP.** Bacterial strains were grown in a 96-well flat-bottom microtitre plate (Costar) over a period of 48 hours in marine broth containing concentrations of 0, 0.2, 2, 20 or 200 ppm of NPs. Growth curves were repeated three times and the average value at each time point is reported with error bars showing standard deviation. OD_{600} was recorded using a Tecan Infinite M200 Pro microplate reader (Tecan Group Ltd., Switzerland) with 30 s of shaking prior to OD measurement. Optical density of wells with nanoparticles but no bacteria were subtracted from all wells with bacteria.



336

337 Figure 3. **Percent aggregation of marine bacteria** in the presence of a) CNP or b) ANP after 48
338 h of incubation. Bacteria were grown in microfuge tubes on a shaking table at 180 rpm and at room
339 temperature. Samples were viewed under a compound light microscope at 600× total
340 magnification. Four independent randomly chosen fields of view were imaged and analyzed using
341 ImageJ. All groupings larger than 60 pixels ^{2} (representing approx. 4 bacterial cells) were
342 designated as aggregates. Area of aggregated cells was divided by total biomass area to determine
343 percentage of aggregated biomass. Reported values are the average of four measurements with
344 error bars showing standard deviation. Values significantly different ($p \leq 0.01$; Student's t-test)
345 from 0 ppm treatment are indicated by an asterisk.

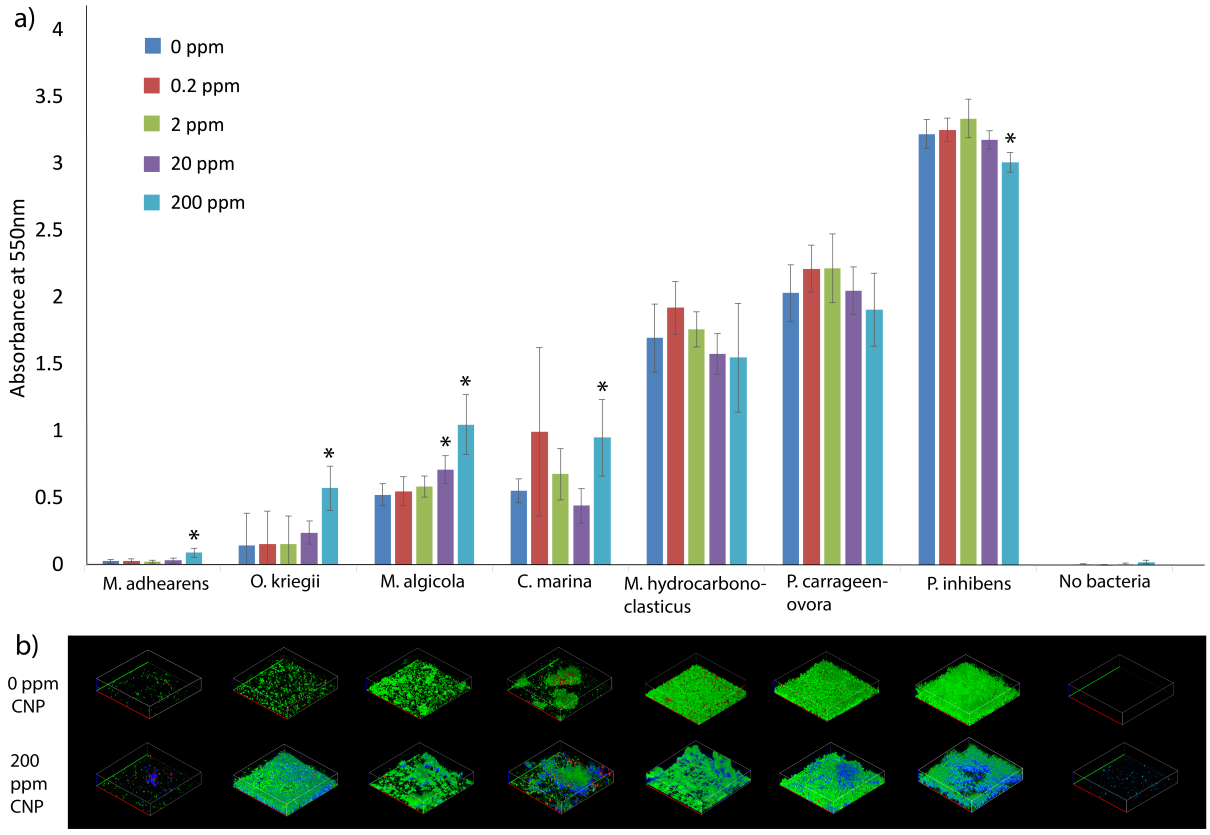


Figure 4. **Impact of CNP on bacterial biofilm formation**. a) Quantification of biofilm biomass after five days of incubation by crystal violet staining as per (O'Toole, 2011). Each bar shows the average of three replicates and error bars show standard deviation. Significant differences ($p \leq 0.05$; Student's t-test) in biomass compared to untreated (0 ppm) are indicated by an asterisk. b) Representative confocal laser scanning images of untreated biofilms and biofilms grown in the presence of 200 ppm CNP with blue fluorescence (FluoSpheres® carboxyl-modified microsphere, 0.2 µm, blue fluorescence, 2% solids; ThermoFisher catalogue number F8805). Living cells are shown in green (stained with SYTO60) and dead cells (defined as compromised membrane integrity; stained with TOTO-1) are shown in red. Nanoparticles are shown in blue. The length of red and green axes is 100 µm and the blue axis is 30 µm. Biofilms were imaged in uncoated 96-well microscopy plates (Ibidi) and using a Zeiss LSM800 confocal laser scanning microscope equipped with a 63× objective. Images were processed using Zen software version 2.3 (Zeiss), with 3D images rendered using a mixed surface projection.

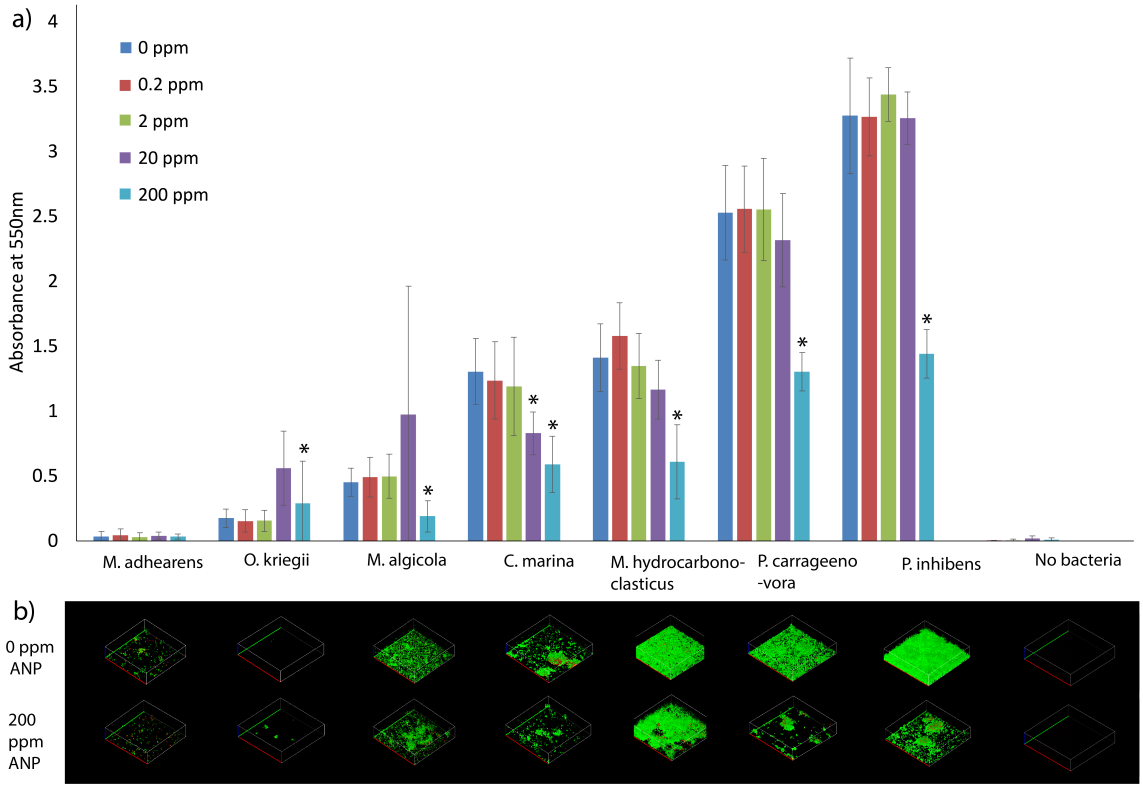
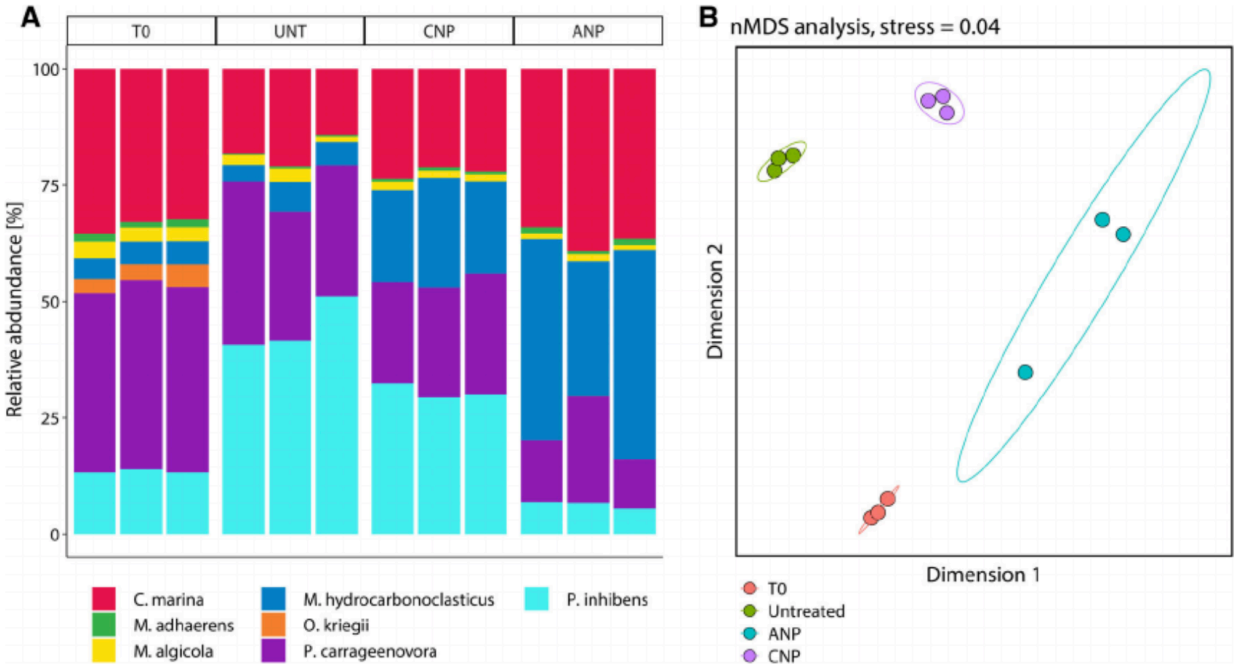


Figure 5. **Impact of ANP on bacterial biofilm formation**. a) Quantification of biofilm biomass after five days of incubation by crystal violet staining (O'Toole, 2011). Each bar shows the average of three replicates and error bars show standard deviation. Significant differences ($p \leq 0.05$; Student's t-test) in biomass compared to untreated (0 ppm) are indicated by an asterisk. b) Representative confocal laser scanning images of untreated biofilms and biofilms grown in the presence of 200 ppm ANPs. Fluorescent 20 nm ANPs were not available from any commercial manufacturer and could therefore not be used in this experiment. ANPs are thus present but not visible in the 200 ppm ANP images. Living cells are shown in green and dead cells (defined as compromised membrane integrity) are shown in red. The length of red and green axes is 100 µm and the blue axis is 30 µm. Fluorescent staining and imaging was performed as described for Figure 4B.



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373 Figure 6. **Impact of CNP and ANP on an artificial biofilm community of marine bacteria**. a)
374 Relative abundance of seven bacterial species prior to incubation (T0) and community structure of
375 the biofilm after 5 days incubation in the presence of no nanoparticles (untreated; UNT), CNP, or
376 ANP. Bacterial species were grown at room temperature on polypropylene squares (0.3 × 0.3 mm)
377 in marine broth in the presence of 200 ppm ANPs or CNPs. Relative abundances of community
378 members were determined by 16S rDNA amplicon sequencing with primer set 515f/806R
379 (Caporaso et al., 2011) and using an Illumina MiSeq. Obtained sequencing read counts were
380 corrected by the number of the rRNA operons of the respective community members. b) Non-
381 metric multidimensional scaling (nMDS) of the same communities based on the community
382 dissimilarity metric φ (Quinn, Richardson, Lovell, & Crowley, 2017). nMDS was carried out as
383 implemented in the R package vegan (Dixon, 2003). Confidence ellipses (95 % level) were
384 calculated using the R package ellipse (https://cran.r-project.org/web/packages/ellipse/) and
385 assuming a multivariate normal distribution. Relative abundances and the nMDS plot were
386 visualized using the R package ggplot2 (Wickham, 2016).

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