



Bacteria-mediated aggregation of the marine phytoplankton *Thalassiosira weissflogii* and *Nannochloropsis oceanica*

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Abstract

The ecological relationships between heterotrophic bacteria and marine phytoplankton are complex and multifaceted, and in some instances include the bacteria-mediated aggregation of phytoplankton cells. It is not known to what extent bacteria stimulate aggregation of marine phytoplankton, the variability in aggregation capacity across different bacterial taxa or the potential role of algogenic exopolymers in this process. Here we screened twenty bacterial isolates, spanning nine orders, for their capacity to stimulate aggregation of two marine phytoplankters, *Thalassiosira weissflogii* and *Nannochloropsis oceanica*. In addition to phytoplankton aggregation efficiency, the production of exopolymers was measured using Alcian Blue. Bacterial isolates from the *Rhodobacterales*, *Flavobacteriales* and *Sphingomonadales* orders stimulated the highest levels of cell aggregation in phytoplankton cultures. When co-cultured with bacteria, exopolymer concentration accounted for 34.1% of the aggregation observed in *T. weissflogii* and 27.7% of the aggregation observed in *N. oceanica*. Bacteria-mediated aggregation of phytoplankton has potentially important implications for mediating vertical carbon flux in the ocean and in extracting phytoplankton cells from suspension for biotechnological applications.

Keywords Environmental biotechnology · Microbial aggregation · *Thalassiosira* · *Nannochloropsis* · Microalgae-bacteria interactions

Introduction

The ecological interactions between marine phytoplankton and bacteria can vary from mutualistic to parasitic (Amin

et al. 2012) and can strongly influence the physiology, metabolic activity, abundance and growth of both partners (Lee et al. 2000; Grossart and Simon 2007; Buchan et al. 2014). These interactions have been shown to promote microalgal growth (Seyedsayamdost et al. 2011), protect phytoplankton from pathogens (Geng and Belas 2010) or alternatively to inhibit microalgal growth (Mayali and Azam 2004), while at the same time governing the productivity and biogeochemistry of aquatic ecosystems (Cole 1982; Landa et al. 2016).

One of the ways that bacteria can influence phytoplankton is by increasing cell stickiness and consequently causing aggregation (Decho 1990; Heissenberger and Herndl 1994; Grossart et al. 2006b). Physical attachment of bacteria to phytoplankton cells has been shown to influence aggregation in several species (Rodolfi et al. 2003; Grossart et al. 2006b; Gärdes et al. 2010), which has potential significance for biogeochemical cycling in the ocean, because the aggregation of phytoplankton and subsequent sinking of organic matter in the form of marine snow increases the flux of organic carbon from surface waters to the deep ocean (Alldredge and Gotschalk 1989; Grossart and Ploug 2001; Grossart et al. 2006b), meaning these microscale interactions can have ecosystem-level implications (Amin et al. 2012, 2015; Landa et al. 2016).

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Bacterial mediation of phytoplankton aggregation not only is important in natural ecosystems but also has the potential to lead to biotechnological advances. The widespread commercialisation of microalgal bioproducts is hindered by inefficiencies in biomass harvesting, which impacts the cost of production (Milledge and Heaven 2013; Vandamme et al. 2013). Therefore, the application of phytoplankton-bacteria aggregates has been proposed as an effective pre-concentration strategy, and it has been successfully applied in the water purification and wastewater treatment industries to extract biomass (de Godos et al. 2011). Given the emerging knowledge of phytoplankton-bacteria interactions within natural environments, a logical extension is to take advantage of these processes for biotechnological applications.

The impact of heterotrophic bacteria on phytoplankton aggregation rates can be highly variable. While bacteria may increase aggregation and the stability of aggregates (Heissenberger and Herndl 1994), attached bacteria may also reduce aggregation due to the hydrolysis of phytoplankton surface mucus (Grossart et al. 2006a, b). Moreover, the role of heterotrophic bacteria for phytoplankton aggregation can vary appreciably between phytoplankton species (Grossart et al. 2006b; Powell and Hill 2013) while the identity of the bacteria can also influence levels of aggregation (Kranck and Milligan 1988; Alldredge and Gotschalk 1989; Riebesell 1991; Alldredge et al. 1995; Gärdes et al. 2011; Nontembiso et al. 2011; Wang et al. 2012; Powell and Hill 2014).

Bacterial-induced phytoplankton aggregation is generally believed to be primarily governed by two key mechanisms: direct cell attachment and the secretion of aggregating polymers such as transparent exopolymer particles (TEP) (Grossart et al. 2006a). It is proposed that TEP constitute up to 40% of the POC pool in ocean surface waters, thus contributing to the marine carbon budget (Passow 2002a; Mari et al. 2017). Phytoplankton are significant producers of TEP, and diatoms have been shown to have particularly high rates of TEP production (Passow 2002a). Heterotrophic bacteria consume TEP (Grossart et al. 2006a), while on the other hand, their presence has also been shown to increase the production of TEP by phytoplankton (Decho 1990; Heissenberger and Herndl 1994; Grossart 1999; Gärdes et al. 2011), which may have important implications for the aggregation of, and enhanced sedimentation rates of senescent phytoplankton (Riebesell 1991).

The aim of the present study was to screen a range of bacteria from nine different taxonomic orders and determine their ability to aggregate two marine phytoplankton, *Thalassiosira weissflogii* and *Nannochloropsis oceanica*. Diatoms often dominate phytoplankton communities in coastal environments and the open ocean, supporting marine food webs through the organic carbon production; therefore, *T. weissflogii* was chosen for its ecological relevance (Armbrust 2009). *Nannochloropsis oceanica* was chosen for

its biotechnological importance owing to its high oil content and widespread use as a feedstock in aquaculture (Chiu et al. 2009). Our goal was to develop a framework to understand phytoplankton-bacteria interactions in the context of microbial aggregation and TEP production and identify bacterial species of interest for biotechnological exploitation, while improving our understanding of the potential contribution of TEP to the marine carbon cycle.

Materials and methods

Microorganisms and growth conditions

Two marine phytoplankton species, *Thalassiosira weissflogii* (Strain CS-871, synonym CCMP-1336, Australian National Algae Culture Collection) and *Nannochloropsis oceanica* (Strain CS-179; Australian National Algae Culture Collection) were grown in filtered artificial seawater (salinity 32‰) enriched with F medium as described by Guillard and Ryther (1962). Stock cultures of xenic *N. oceanica* and *T. weissflogii* were maintained at 20 °C under a 12 :12 h light:dark cycle with fluorescent illumination at a photon flux density (PFD) of 40 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$.

We selected 20 bacterial strains to screen for their capacity to aggregate *T. weissflogii* and *N. oceanica* (Table 1). These bacteria were chosen to represent a diverse range of taxonomic groups, and were isolated from a range of sources, including both natural environments and laboratory-maintained cultures. *Marinobacter adhaerens* HP 15 WT attaches to and aggregates *T. weissflogii* (Sonnenschein et al. 2012) and *Bacillus megaterium* has been shown to aggregate *N. oceanica* (Powell and Hill 2014); hence, both species were included as positive controls. All bacteria were grown in marine broth (BD Difco 279110, Becton Dickinson, USA) at 25 °C, agitated at 180 rpm and stored as glycerol stocks prior to experiments.

Co-culture experiments

Thalassiosira weissflogii and *N. oceanica* were grown for 6 days to mid-exponential phase, before being inoculated in 50 mL Tissue Culture Treated Flasks (Falcon, 25 cm² culture area) in F medium at an initial cell density of 6–8 $\times 10^5$ cells mL⁻¹ and 1–2 $\times 10^6$ cells mL⁻¹, respectively. Bacteria were grown from glycerol stocks in marine broth overnight (25 °C, 180 rpm). Bacterial cells were diluted with sterile F medium, and were added to the phytoplankton culture in a final concentration of 1.2–1.6 $\times 10^5$ cells mL⁻¹ for the *T. weissflogii* experiments and 2–4 $\times 10^5$ cells mL⁻¹ for the *N. oceanica* experiments in order to achieve an approximate final ratio of 1:5 bacteria:phytoplankton cells in 20 mL. Phytoplankton and bacterial cell numbers were determined using a haemocytometer

Table 1 Bacterial species used in this study, identified by class, order and genus, the site/source they were isolated from and accession number

Bacteria ID (confirmed 16S)	% ID	Class	Order	Genus	Site/source	Accession number
<i>Bacillus megaterium</i> QMB1551	100	Bacilli	Bacillales	Bacillus	American Type Culture Collection	CP1001983
<i>Roseovarius litoreus</i> GSW-M15	100	Alphaproteobacteria	Rhodobacterales	Roseovarius	Culture of <i>Alexandrium minutum</i> CS324	NR_109594
<i>Octadecabacter asciadiaceicola</i> RA1-3	99	Alphaproteobacteria	Rhodobacterales	Octadecabacter	Port Hacking, NSW Australia	NR_147751
<i>Sulfitobacter mediterraneus</i> CH-B427	98	Alphaproteobacteria	Rhodobacterales	Sulfitobacter	Culture of <i>Thalassiosira pseudonana</i>	NR_026472
<i>Phaeobacter porticola</i> P97	99	Alphaproteobacteria	Rhodobacterales	Phaeobacter	Culture of <i>Emiliana huxleyii</i>	NR_157650
<i>Shimia marina</i> CL-TA03	100	Alphaproteobacteria	Rhodobacterales	Shimia	Culture of <i>Synechococcus</i>	NR_043300
<i>Erythrobacter citreus</i> RE35F/1	100	Alphaproteobacteria	Sphingomonadales	Erythrobacter	Culture of <i>Emiliana huxleyii</i>	NR_028741
<i>Erythrobacter flavus</i> SW-46	100	Alphaproteobacteria	Sphingomonadales	Erythrobacter	Culture of <i>Thalassiosira pseudonana</i>	NR_025245
<i>Sphingorhabdus flavimaris</i> SW-151	99	Alphaproteobacteria	Sphingomonadales	Sphingorhabdus	Culture of <i>Synechococcus</i>	NR_025814
<i>Marinobacter adhaerens</i> HP15 WT	100	Gammaproteobacteria	Alteromonadales	Marinobacter	Kaepfel et al. (2012)	NR_074765
<i>Alteromonas macleodii</i> NBRC 102226	100	Gammaproteobacteria	Alteromonadales	Alteromonas	Port Hacking NSW Australia	NR_114053
<i>Shewanella baltica</i> 63	99	Gammaproteobacteria	Alteromonadales	Shewanella	Iron Cove, NSW Australia	NR_025267
<i>Acinetobacter bouvetii</i> EU40	99	Gammaproteobacteria	Pseudomonadales	Acinetobacter	Botany Foreshores Beach, NSW Australia	JF681285
<i>Vibrio gigantis</i> LGP 13	99	Gammaproteobacteria	Vibrionales	Vibrio	Culture of <i>Emiliana huxleyii</i>	NR_044079
<i>Maribacter lutimaris</i> KJ4	99	Flavobacteria	Flavobacteriales	Maribacter	Culture of <i>Alexandrium minutum</i> CS324	NR_148860
<i>Aquaticitalea lipolytica</i> Ar-125	99	Flavobacteria	Flavobacteriales	Aquaticitalea	Port Hacking, NSW Australia	NR_149769
<i>Maribacter dokdonensis</i> DSW-8	99	Flavobacteria	Flavobacteriales	Maribacter	Culture of <i>Thalassiosira pseudonana</i>	NR_043294
<i>Winogradskyella poriferorum</i> UST030701-295	99	Flavobacteria	Flavobacteriales	Winogradskyella	Culture of <i>Nannochloropsis oceanica</i>	NR_043230
<i>Roseivirga spongicola</i> UST030701-801	99	Cytophagia	Cytophagales	Roseivirga	Culture of <i>Nannochloropsis oceanica</i>	NR_043531
<i>Arcobacter venerupis</i> F67-11	97	Epsilonproteobacteria	Campylobacterales	Arcobacter	Botany Foreshores Beach, NSW Australia	NR_117569

and flow cytometry, respectively, as described in Tran et al. (2017). Phytoplankton cultures without bacterial addition were used as negative controls. Co-culture flasks were shaken for 24 h at 70 rpm, and optical density (OD) at 750 nm and pH were measured immediately after inoculation (t_0) and after 24 h (t_{24}). pH was measured to confirm that aggregation was due to the presence of bacteria as opposed to autoflocculation at pH > 9.5 (Sukenic and Shelef 1984).

Aggregation efficiency

The aggregation efficiency of bacterial isolates co-cultured with *T. weissflogii* and *N. oceanica* was determined by measuring changes in OD (Vandamme et al. 2012). Briefly, *T. weissflogii* and *N. oceanica* co-cultures containing bacterial isolates, and control cultures containing no bacteria, were left to settle for 30 min, before a 1-mL sample was taken from the

middle of each culture flask, and the OD was measured at 750 nm. The aggregation efficiency (η_a) was calculated according to the equation:

$$\eta_a = \frac{OD_{t_0} - OD_{t_{24}}}{OD_{t_0}} \times 100$$

where OD_{t_0} is the optical density of the culture immediately after inoculation with bacteria and $OD_{t_{24}}$ is the optical density of the same culture 24 h later. Three biological replicates were carried out for each phytoplankton-bacterium combination.

TEP determination

Transparent exopolymer particles (TEP) in the co-cultures were stained and quantified using the methodology of Passow and Alldredge (1995). Briefly, TEP were collected by filtering each sample through 0.2 μm pore-size 25 mm

polycarbonate Nucleopore filters (Whatman). The volume of sample necessary for each TEP analysis varied from 1 to 5 mL depending on the size of the aggregates and density of each culture, so as to avoid clogging the filter. After filtration, filters were stained for 3 s with 500 μL of a 0.02% working solution of Alcian Blue (pre-filtered through a 0.2- μm filter) in 0.06% acetic acid (8GX, Sigma). Alcian Blue binding was calibrated with Gum Xanthan (Sigma) according to the protocol described in Passow and Alldredge (1995). This colorimetric method measures all materials that adsorb Alcian Blue, which is directly related to the weight of a specific polysaccharide. The weight equivalent for the adsorption of Alcian Blue to TEP is given by using the reference exopolymer Gum Xanthan due to the heterogeneity of natural exopolymers (Engel and Passow 2001). Following staining, filters were rinsed twice with milliQ water to eliminate excess dye before storage at $-80\text{ }^{\circ}\text{C}$. The Alcian Blue stain was extracted by soaking the filters in 80% sulfuric acid for 2–3 h and analysed at 787 nm. Empty filters stained with Alcian Blue were used as blanks. The concentration of TEP ([TEP]) was determined in units of Gum Xanthan equivalents per volume of sample filter ($\mu\text{g XG L}^{-1}$).

Epifluorescence and TEP imaging

In order to visualise the proportion of bacteria attached to aggregated phytoplankton, and whether this coincided with a greater abundance of TEP, cultures were visualised by epifluorescence microscopy. Briefly, samples (1 mL) were fixed with glutaraldehyde (final conc. 1%), stained with SYBR-Green-I (final conc. 1:10,000) and incubated for 15 min in the dark. Stained samples were filtered onto 0.2 μm pore-size 25 mm polycarbonate Nucleopore filters (Whatman), stained with Alcian Blue as described above, and washed with 2 mL milliQ water to eliminate excess dye. Samples were imaged at $\times 40$ magnification for *T. weissflogii* and $\times 100$ magnification for *N. oceanica* using an epifluorescence microscope (Nikon Eclipse Ni-E equipped with a DS-Qi2 monochrome and DS-Fi2 colour camera), with cells visualised using 488 LP (excitation 460 \times 490 nm and emission > 500 nm) and mCherry (excitation 535 \times 555 nm and emission > 595 nm) filters for SYBR-Green-I stained bacteria and phytoplankton autofluorescence, respectively. Alcian Blue staining of TEP was visualised by brightfield microscopy. Image processing involved inverting the brightfield images in order to merge it with the fluorescence channels, and false colours were used to improve contrast.

Statistical analyses

For both phytoplankton, aggregation efficiency and TEP concentration, data were compared between the control and each bacterial species by permutational analysis of variance

(PERMANOVA) using the PRIMER v6 and PERMANOVA+ software and Euclidean distance. PERMANOVA Monte Carlo pairwise comparisons, corresponding to t-statistics, were performed where significant differences were observed. To determine the influence of TEP concentration on aggregation efficiency, a regression analysis was performed on all biological replicates for both phytoplankton.

Results

Aggregation efficiency and TEP production

Twenty different species of bacteria were screened for their ability to stimulate aggregation of the marine phytoplankton *T. weissflogii* and *N. oceanica*. Significant ($p < 0.05$) levels of aggregation of *T. weissflogii* were observed following the addition of 16 of these bacterial strains, while in *N. oceanica*, 17 bacterial strains caused aggregation. In the negative control cultures, where there was no addition of bacteria, negative aggregation efficiencies were observed: $-15.1 \pm 4.3\%$ for *T. weissflogii* cultures and $-4.1 \pm 2.0\%$ for *N. oceanica* cultures. Negative aggregation efficiency implies net phytoplankton culture growth, with the culture becoming more turbid during the experimental period. The degree of aggregation varied significantly ($p < 0.05$) across bacterial isolates, and the *Sphingomonadales* and *Erythrobacter citreus* caused the highest levels of aggregation of both *T. weissflogii* (Fig. 1a) and *N. oceanica* cells (Fig. 1b). When co-cultured with *E. citreus*, the aggregation efficiency within *T. weissflogii* cultures was $78.5 \pm 3.8\%$ ($p = 0.002$) and it was $77.7 \pm 1.8\%$ ($p = 0.001$) in *N. oceanica* cultures. A bacterium of the same genus, *Erythrobacter flavus*, was also responsible for stimulating high levels of aggregation of cells in co-culture with *T. weissflogii* ($60.4 \pm 1.7\%$; $p = 0.001$) and *N. oceanica* ($70.8 \pm 1.9\%$, $p = 0.001$). The *Flavobacteriales* were another taxonomic group showing high levels of aggregation of phytoplankton cells. In co-culture, *Maribacter lutimaris* aggregated $58.4 \pm 1.7\%$ ($p = 0.002$) of *T. weissflogii* cells and $72.8 \pm 2.5\%$ ($p = 0.002$) of *N. oceanica* cells. Two members of the *Rhodobacterales*, *Octadecabacter asciadiaceicola* and *Roseovarius litoreus*, were also capable of stimulating high levels of aggregation. In *T. weissflogii* cultures, *O. asciadiaceicola* aggregated $62.7 \pm 2.0\%$ ($p = 0.001$) of cells, while in *N. oceanica* cultures, *R. litoreus* aggregated $61.2 \pm 2.0\%$ ($p = 0.001$) of cells. The efficiency of bacteria-mediated aggregation was compared against high-pH aggregation induced by NaOH addition (Vandamme et al. 2012). Notably, the bacteria-mediated aggregation efficiency of *E. citreus* was only 15.4% lower than high-pH aggregation efficiency in *T. weissflogii* cultures ($93.9 \pm 0.2\%$), and 9.4% lower in *N. oceanica* cultures ($87.1 \pm 1.0\%$).

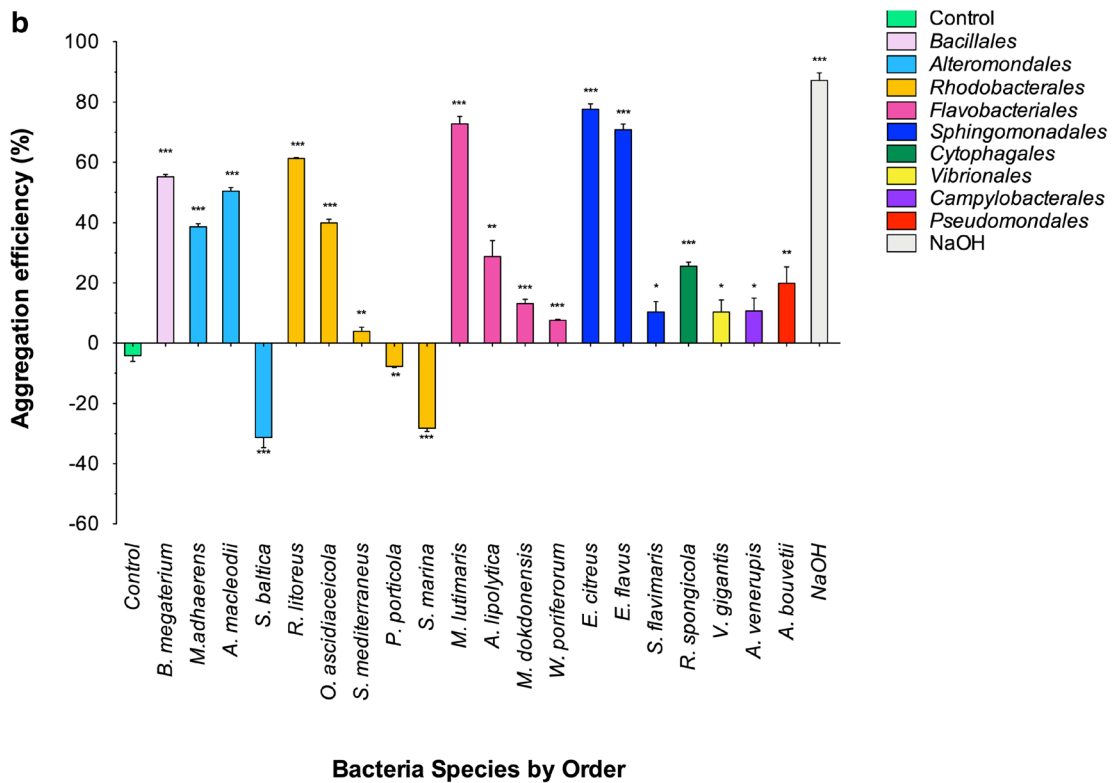
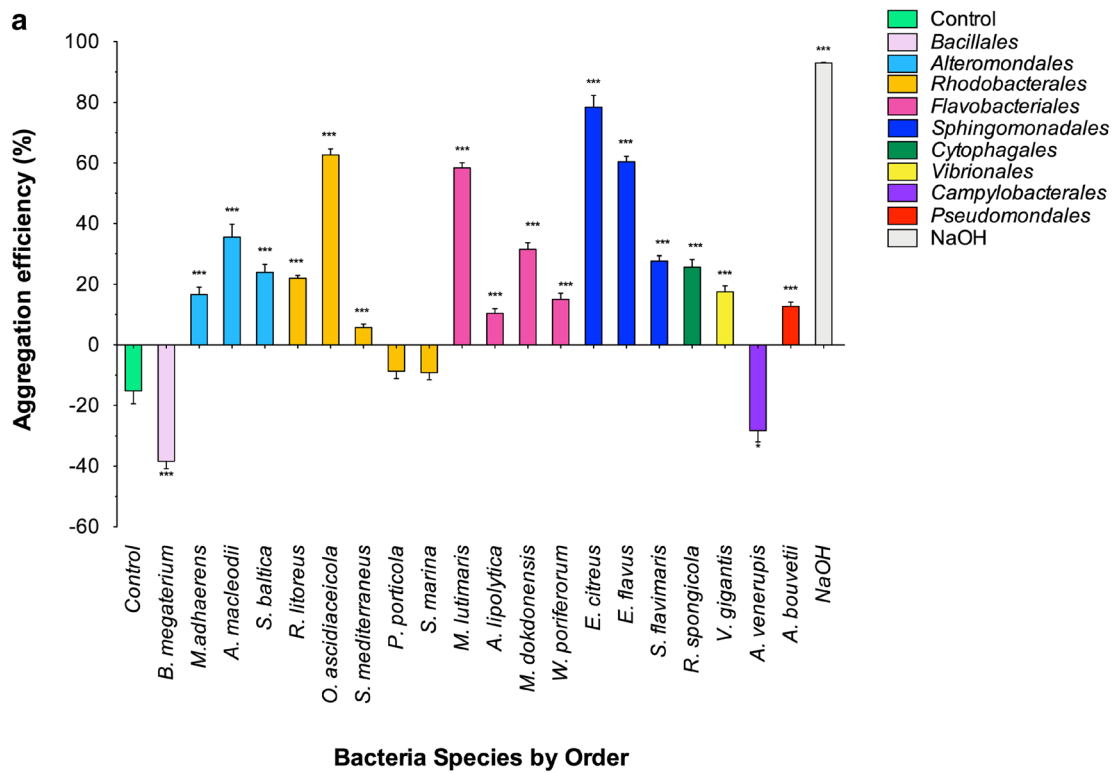


Fig. 1 Aggregation efficiency (%) within phytoplankton cultures after 24 h of co-incubation with different bacterial isolates **a** *T. weissflogii*. **b** *N. oceanica*. No bacteria were added to the negative control. High-pH aggregation was used as a positive control. Colours are representative of

different bacterial orders. Mean \pm SEM ($n = 3$). Significance level of $\alpha < 0.05$ was set, * < 0.05 , ** < 0.01 , *** < 0.001 . Negative aggregation efficiency implies net phytoplankton culture growth

Interestingly, the *Bacillales* bacterium *B. megaterium* stimulated significant levels of aggregation in *N. oceanica* cultures ($55.2 \pm 0.8\%$, $p = 0.001$), but did not cause aggregation of *T. weissflogii* ($-38.3 \pm 2.5\%$, $p = 0.003$). The same pattern was also observed for the *Campylobacteriales* bacterium *Arcobacter venerupis*, which stimulated aggregation in *N. oceanica* cultures ($10.7 \pm 4.3\%$, $p = 0.023$), but not in *T. weissflogii* cultures ($-28.2 \pm 3.7\%$, $p = 0.04$). In contrast, one of the *Alteromonadales* isolates, *Shewanella baltica*, caused aggregation of *T. weissflogii* ($24.0 \pm 2.6\%$, $p = 0.001$), but not of *N. oceanica* ($-31.2 \pm 3.4\%$, $p = 0.002$). Two of the *Rhodobacterales* isolates, namely *Phobacter porticola* and *Shimia marina*, led to negative aggregation efficiency in both *T. weissflogii* ($-8.7 \pm 2.4\%$, $p > 0.05$; $-9.1 \pm 2.4\%$, $p > 0.05$) and *N. oceanica* ($-7.7 \pm 0.4\%$, $p = 0.008$; $-28.2 \pm 1.1\%$, $p = 0.001$).

Overall, the three highest aggregating bacterial species of *T. weissflogii* or *N. oceanica* also generated some of the greatest concentrations of TEP in co-culture with these phytoplankton. During the co-culture experiments, concentrations of TEP varied significantly among bacterial species. In *T. weissflogii*, TEP concentrations ranged between 189 and 11,258 $\mu\text{g XG L}^{-1}$ (Fig. 2a), with the highest concentrations produced by co-cultures containing *Maribacter lutimaris* ($11,258 \pm 1122 \mu\text{g XG L}^{-1}$; $p = 0.001$) and *O. asciadiaceicola* ($10,063 \pm 440 \mu\text{g XG L}^{-1}$; $p = 0.001$). These values were significantly different to the TEP concentration of the control culture ($3339 \pm 379 \mu\text{g XG L}^{-1}$).

In *N. oceanica*, TEP concentrations ranged between -1551 and $8910 \mu\text{g XG L}^{-1}$ (Fig. 2b). When co-cultured with bacteria, the highest TEP concentrations were measured with *Acinetobacter bouvetii* ($8910 \pm 454 \mu\text{g XG L}^{-1}$; $p = 0.001$) and *Erythrobacter citreus* ($8503 \pm 986 \mu\text{g XG L}^{-1}$; $p = 0.001$), with significantly higher levels of TEP production than in the control ($2764 \pm 5 \mu\text{g XG L}^{-1}$) in both cases.

Bacteria of the order *Rhodobacterales*, *Sphingomonadales* and *Flavobacteriales* were some of the highest aggregating species in co-culture, and it is notable that co-culture with these isolates also corresponded with the greatest amount of TEP production. A regression analysis was performed to assess the impact of TEP concentration on aggregation efficiency. A significant positive relationship was observed between TEP concentration and aggregation efficiency for both *T. weissflogii* and *N. oceanica* when co-cultured with bacteria. TEP concentration accounted for 34.1% ($p < 0.0001$) of the aggregation observed in *T. weissflogii* (Fig. 3a) and 27.7% ($p < 0.0001$) of the aggregation observed in *N. oceanica* (Fig. 3b).

Microscopic characterisation of phytoplankton aggregates and TEP

Microscopic visualisation of co-cultures following the 24 h incubation period indicated that TEP was present in all

cultures. Similar amounts of TEP were present in the control cultures of *T. weissflogii* (Fig. 4a) and in non-aggregating co-cultures with *S. marina* (Fig. 4b). In contrast, when *T. weissflogii* was in co-culture with *E. citreus*, Alcian Blue-stainable material formed a significant component of aggregates (Fig. 4c). Similar results were observed in the same three conditions of *N. oceanica*, with minimal TEP in the control cultures (Fig. 4d) and in non-aggregating co-cultures of *N. oceanica* with *S. marina* (Fig. 4e) and greater amounts of Alcian Blue-staining material in aggregates if *N. oceanica* in co-culture with *E. citreus* (Fig. 4f). Within aggregates, there was a clear pattern of phytoplankton and bacterial cells surrounded by a matrix of TEP.

Discussion

There is evidence that microscale interactions between heterotrophic bacteria and phytoplankton in the ocean might play a role in facilitating aggregation of phytoplankton biomass (Gärdes et al. 2011; Sonnenschein et al. 2012). If widespread, this process will have a significant impact on ocean biogeochemistry by potentially contributing to enhanced sedimentation of particulate organic matter (Engel 2004). In addition, exploiting the relationship between bacteria and phytoplankton in co-cultures could lead to novel aggregation strategies that improve phytoplankton harvesting efficiency for biotechnological applications (Ummalyma et al. 2017). This study compared the aggregation efficiency of a diverse library of twenty marine bacterial isolates, derived from laboratory phytoplankton cultures and isolated from the natural environment, on two marine phytoplankton, *T. weissflogii* and *N. oceanica*, which were chosen due to their ecological and biotechnological relevance.

Key bacterial phylogenetic groups implicated in aggregation and TEP production

A range of bacteria, spanning nine different taxonomic orders, stimulated aggregation of *T. weissflogii* and *N. oceanica*. Of these, only three bacteria isolates had entirely different effects on the two different phytoplankton species. Specifically, *S. baltica* stimulated aggregation of *T. weissflogii* but not *N. oceanica*, while *B. megaterium* and *A. venerupis* caused aggregation of *N. oceanica* but not *T. weissflogii*. These bacteria comprise three different taxonomic orders, *Alteromonadales*, *Bacillales* and *Campylobacteriales*, respectively, which is indicative of species-specific effects on aggregation. Evidence for exopolymer production by *S. baltica* and *B. megaterium* may have contributed to the observed aggregation (Neal et al. 2007; Powell and Hill 2014); however, our understanding remains limited as little evidence exists for the ability of these bacteria to aggregate phytoplankton. However,

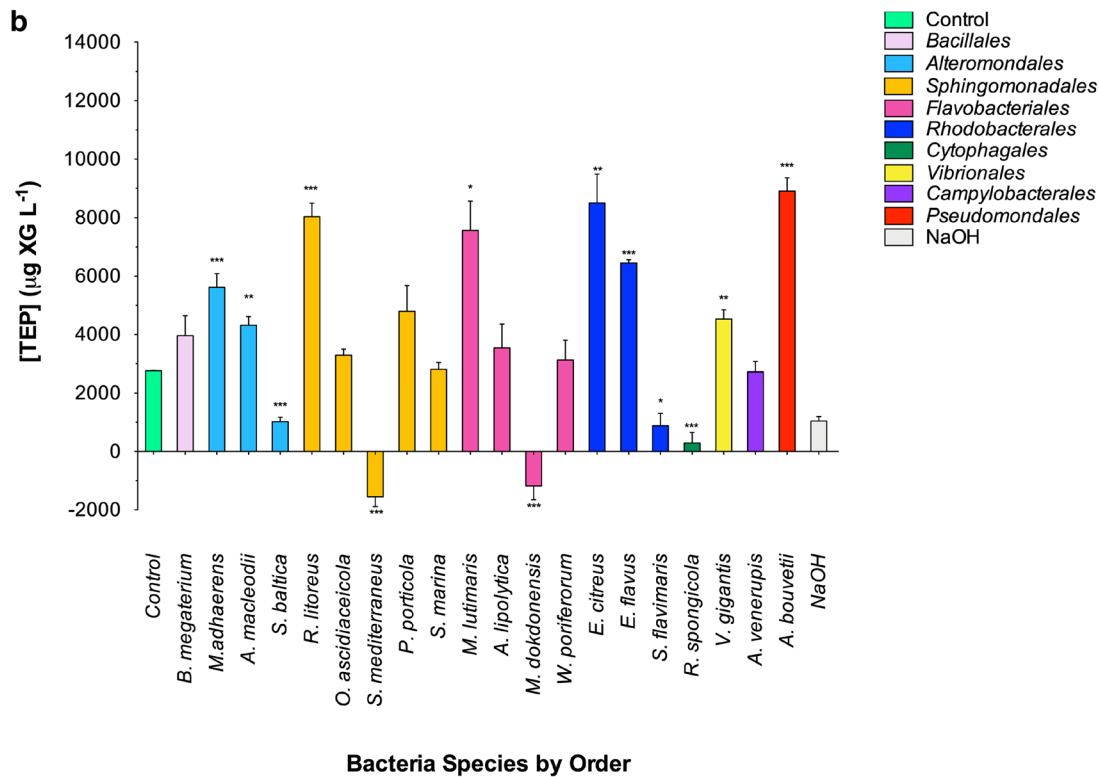
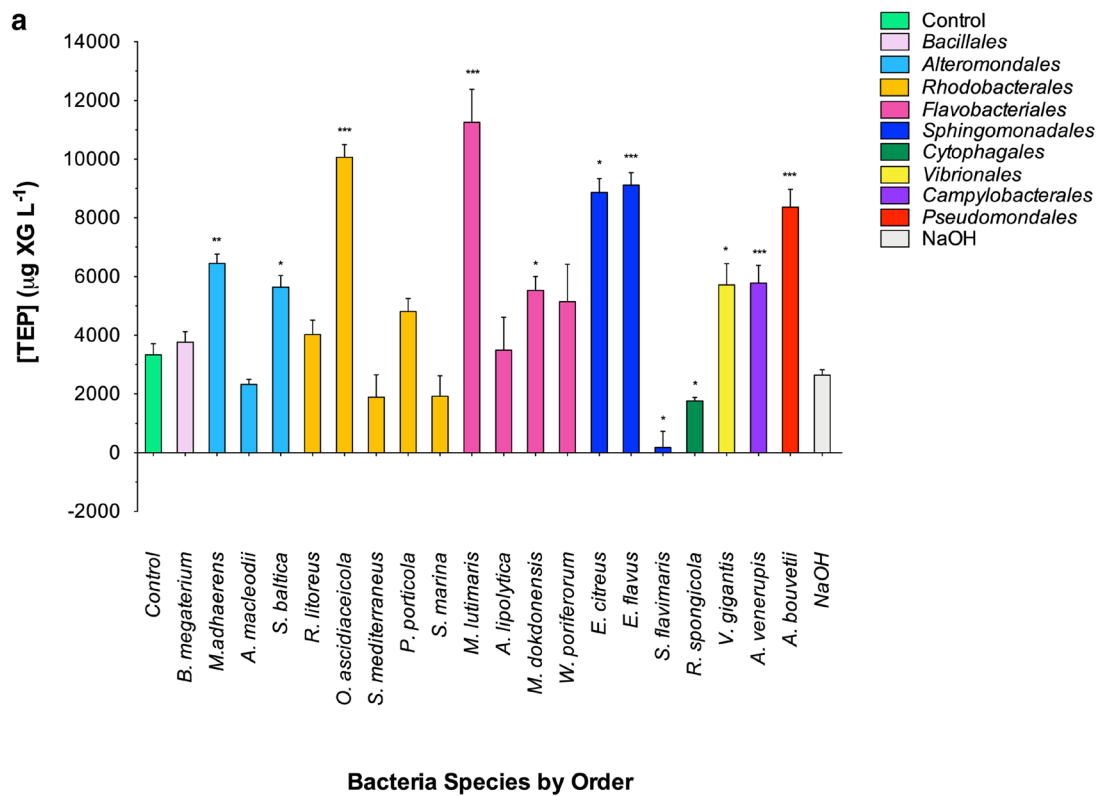


Fig. 2 TEP concentration ($\mu\text{g XG L}^{-1}$) within phytoplankton cultures after 24 h of co-incubation with bacterial isolates **a** *T. weissflogii*. **b** *N. oceanica*. No bacteria were added to the negative control. High-pH aggregation was used as a positive control. Colours are representative of

different bacterial orders. Mean \pm SEM ($n = 3$). Significance level of $\alpha < 0.05$ was set. * < 0.05 , ** < 0.01 , *** < 0.001 . Negative TEP concentration implies net TEP consumption by bacteria

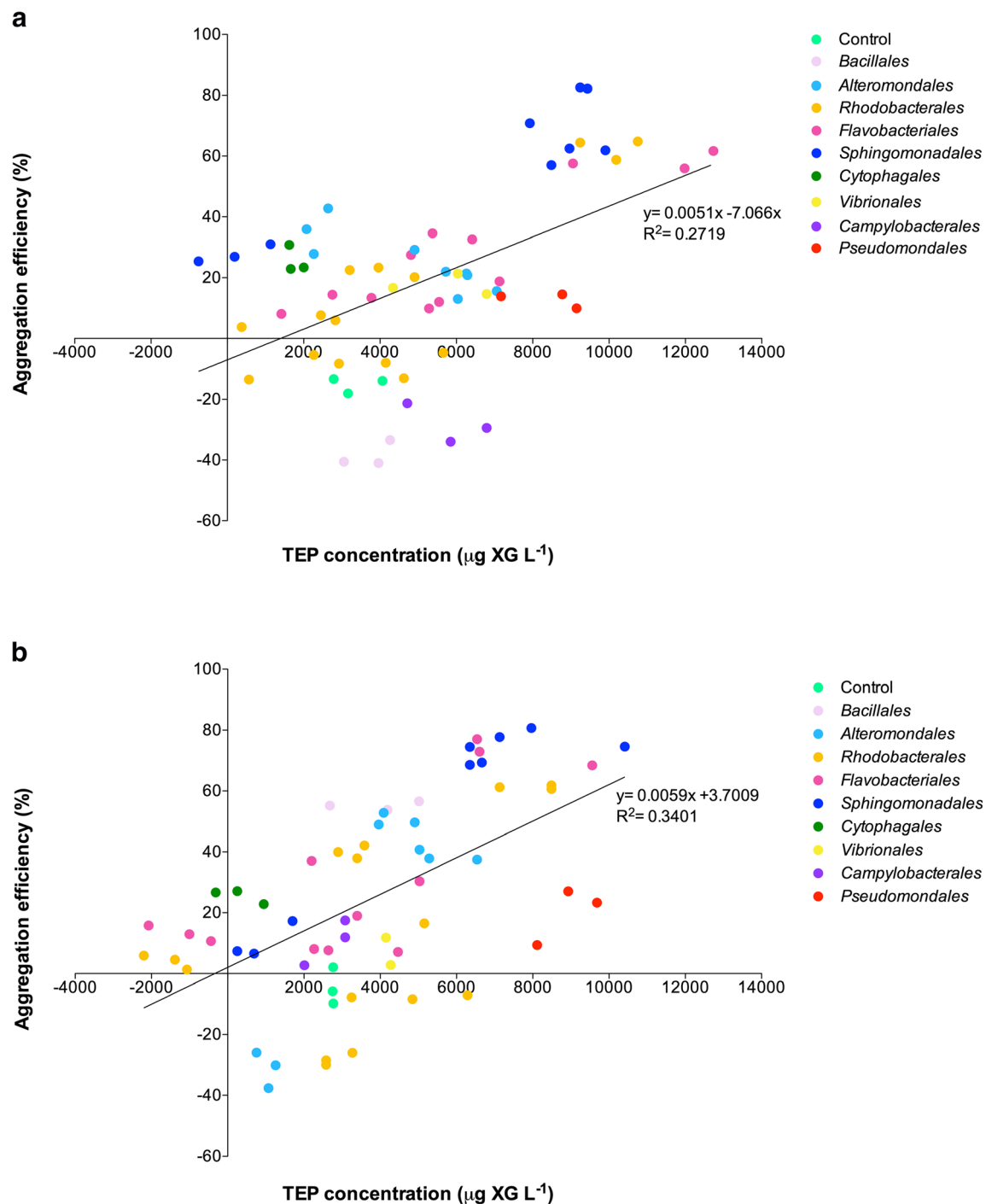


Fig. 3 The relationship between TEP concentration ($\mu\text{g XG L}^{-1}$) and aggregation efficiency (%) for **a** *T. weissflogii* and **b** *N. oceanica*. Each dot represents one biological replicate with three biological replicates for

each bacterial isolate, and colours are representative of different orders of bacteria

across the other 17 bacterial species, remarkably generalisable effects were observed across the two phytoplankton species.

When co-cultured with either *T. weissflogii* or *N. oceanica*, the bacteria that caused the highest levels of aggregation belonged to the *Sphingomonadales* and *Rhodobacterales* (class *Alphaproteobacteria*) and *Flavobacteriales* (phylum *Bacteroidetes*) orders. This result is consistent with previous

observations that bacteria of the *Alphaproteobacteria*, *Gammaproteobacteria* and *Bacteroidetes* groups are often associated with marine aggregates (Amin et al. 2012; Teeling et al. 2012; Milici et al. 2017) and they are regular members of phytoplankton microbiomes (Goecke et al. 2013; Krohn-Molt et al. 2013). Gärdes et al. (2011) demonstrated that certain bacteria in the *Gammaproteobacteria*, *Flavobacteria* and

Firmicutes groups were able to induce aggregation of *T. weissflogii*, with physical attachment of the bacterial cells to the diatom shown to be necessary. Similarly, other studies have identified members of the *Firmicutes*, including *Bacillus* sp. (Zheng et al. 2012; Powell and Hill 2013) and *Solibacillus silvestris* (Wan et al. 2013), and a member of the *Pseudomonadales* (Wang et al. 2012) as catalysts for aggregation in *N. oceanica*. Our experiments expand upon these previous studies by providing evidence of phytoplankton-aggregation ability among a broad diversity of bacterial species.

The highly variable levels of aggregation efficiency caused by bacteria within the same order were perhaps surprising. Among the *Rhodobacterales*, *R. litoreus* and *O. asciadiaceicola* caused high levels of aggregation of both *N. oceanica* and *T. weissflogii*, while *P. porticola* and *S. marina* did not stimulate any notable aggregation, suggesting that the aggregation phenotype is not conserved at the order level. The significantly negative aggregation efficiency in *P. porticola* and *S. marina* co-cultures was in fact indicative of an increase in suspended phytoplankton biomass, suggesting that these bacteria led to an enhancement of growth of the phytoplankton relative to control conditions. This is consistent with observations that, while generally exhibiting ecological associations with phytoplankton, members of this group can have vastly different effects on phytoplankton growth. For instance, some members of the *Rhodobacterales* are the dominant providers of B₁₂ (cobalamin) to phytoplankton (Sañudo-Wilhelmy et al. 2014), where, for example, *Ruegeria pomeroyi* substantially restored the growth rate of B₁₂ limited *Thalassiosira pseudonana* (Durham et al. 2015). Additionally, *Sulfitobacter* strains, also within the *Rhodobacterales*, have been shown to enhance the growth rate of *T. pseudonana* by 18–35%, but closely related strains of *Phaeobacter* had no effect on diatom growth (Amin et al. 2015). The variation observed among phytoplankton-bacteria dynamics within the same order highlights the complexity of microbial interactions and might be explained by differences in the exchanges of signalling molecules between the phytoplankton and bacteria (Amin et al. 2012).

Of the bacterial isolates from within the *Sphingomonadales* order examined here, both strains of *Erythrobacter* were responsible for invoking the highest levels of aggregation of *N. oceanica*, with similar effects observed on *T. weissflogii*. Previous studies have shown that members of *Sphingomonadales* are known producers of TEP (Ashtaputre and Shah 1995; Yamazaki et al. 1996; Cydzik-Kwiatkowska 2015), are abundant members of particle-attached bacterial communities during phytoplankton blooms (Li et al. 2011; Louati et al. 2015) and represent a dominant group in aquatic biofilms (Niederdorfer et al. 2016) and within flocs in activated sludge (Neef et al. 1999). Given that both bacteria from the *Erythrobacter* genus induced high levels of *N. oceanica*

aggregation and produced some of the highest amounts of TEP, we propose that their role in aggregate formation may have important ecological and biotechnological implications.

Members of the *Flavobacteriales* have been widely demonstrated to often have a preference for attached lifestyles (Sapp et al. 2007; Zheng et al. 2017) and to occur in association with phytoplankton (Buchan et al. 2014). All of the isolates in the order *Flavobacteriales* caused significantly high aggregation efficiency compared with the control culture. Furthermore, *M. lutimaris* produced a significant amount of TEP when in co-culture with *T. weissflogii* and *N. oceanica*. In contrast, despite causing aggregation, *Aquaticitalea lipolytica*, *Maribacter dokdonensis* and *Winogradskyella poriferorum* did not produce significant TEP when co-cultured with either *T. weissflogii* or *N. oceanica*. Members of the *Cytophaga-Flavobacterium-Bacteroides* phylum seem to play an important role in the degradation of algal blooms, with high levels of attachment to phytoplankton cells during bloom decline observed (Fernández-Gómez et al. 2013). As dominant colonisers of marine snow (Bidle and Azam 2001; Simon et al. 2002), all *Flavobacteriales* appear to be able to hydrolyse polymers and complex polysaccharides (Reichenbach 1992). In fact, when co-cultured with *N. oceanica*, the presence of *M. dokdonensis* led to negative TEP values, suggesting bacterial consumption of TEP produced under baseline conditions. As well-known colonisers and degraders of phytoplankton-derived TEP (Teeling et al. 2012; Buchan et al. 2014; Milici et al. 2017), this species of *Flavobacteriales* may therefore have initiated aggregation of the phytoplankton in order to consume the resulting TEP.

In general, the three key phylogenetic groups of bacteria implicated in aggregation, namely *Sphingomonadales*, *Flavobacteriales* and *Rhodobacterales*, are major groups in the class *Alphaproteobacteria* and *Flavobacteria*, which are known degraders of dissolved and particulate organic matter. In this study, these bacteria have been shown to effectively aggregate *T. weissflogii* and *N. oceanica*, as well as stimulate the production of significant amounts of TEP, though whether TEP production originates from phytoplankton or bacteria remains unclear. All of these groups of bacteria have elsewhere been shown to be associated with phytoplankton (Schäfer et al. 2002; Sapp et al. 2007; Goecke et al. 2013; Ramanan et al. 2015) and to exhibit different roles (from symbiotic to parasitic) in bacteria-phytoplankton associations (Mayali and Azam 2004; Seyedsayamdost et al. 2011; Amin et al. 2015). The results of this study demonstrate that members of these groups also play a role in the way that phytoplankton biomass is aggregated and potentially subsequently transferred to depth. Therefore, we propose that members of these groups likely play important roles in ocean carbon cycling, while also having great potential for contributing to biotechnological harvesting of algal biomass.

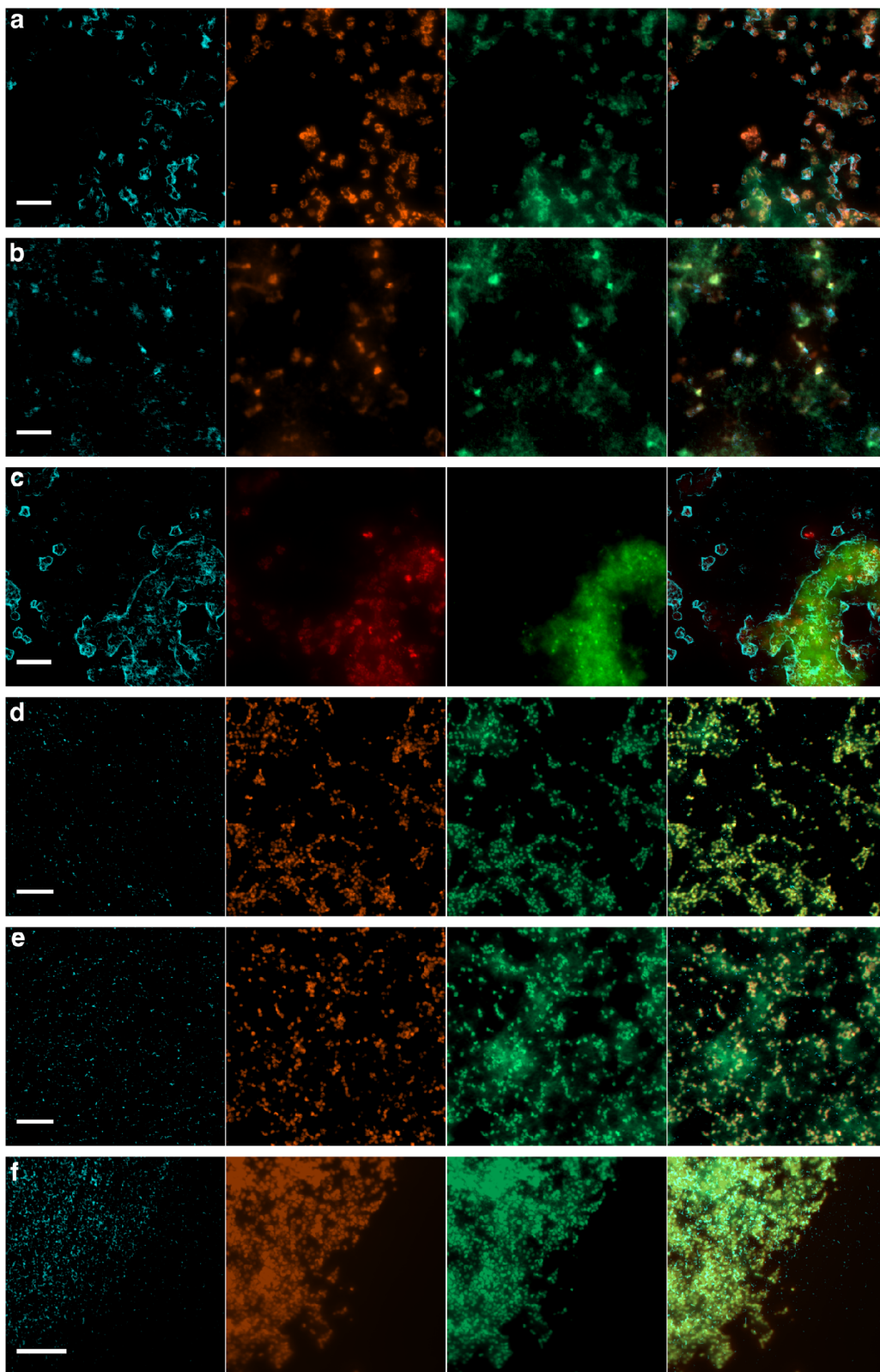


Fig. 4 Inverted brightfield and epifluorescence images of *T. weissflogii* in **a** the control culture, **b** non-aggregating co-culture with *S. marina* and **c** aggregating co-culture with *E. citreus*; and of *N. oceanica* in **d** the control culture, **e** non-aggregating co-culture with *S. marina* and **f** aggregating co-culture of *E. citreus*. The mosaic from the left to right represents

transparent exopolymer particles (TEP, blue) stained with Alcian Blue, chlorophyll autofluorescence of *N. oceanica* and *T. weissflogii* cells (red), bacterial and algal nuclei stained with SYBR-Green (green) and an overlay of all three channels. Scale bar = 10 μ m

Contribution of TEP to aggregation

The influence of TEP in phytoplankton aggregation has been demonstrated in previous studies (Decho 1990; Heissenberger and Herndl 1994). For example, bacterial colonisation of marine diatoms coincides with the production of large amounts of exopolymer substances, which increase phytoplankton cell stickiness and consequently aggregation (Smetacek 1985). From the forty bacteria-phytoplankton co-cultures tested in this study, TEP production was found to account for 34.1% and 27.7% of the aggregation observed in *T. weissflogii* and *N. oceanica*, respectively. It however remains unclear whether TEP was produced by the bacteria in the presence of the phytoplankton or whether the bacteria induce TEP production in the phytoplankton, and current methods make it impossible to distinguish between the sources of TEP production. Overall, there was a positive relationship between TEP concentration and aggregation efficiency. This was supported by epifluorescence microscopy, which revealed that, in cases where aggregation occurred, both bacteria and phytoplankton cells became embedded in a matrix of TEP.

Overall, higher levels of TEP production were measured in *T. weissflogii* than *N. oceanica* co-cultures, suggesting that the phytoplankton cell plays a key role in determining TEP production during interactions with bacteria. This is consistent with previous studies that have observed significant differences in TEP production between different phytoplankton species in cultures (Passow and Alldredge 1994) and in the environment (Grossart and Simon 1997, Grossart 1998). A study by Kiørboe and Hansen (1993) investigated the stickiness of five diatom species and two flagellates, and found that four of the five diatom species were sticky and neither flagellate species was. Vast differences in TEP production have been reported in *T. weissflogii* cultures, ranging from little TEP and no observable aggregations of cells (Crocker and Passow, 1995) to copious amounts of TEP (Passow 2002b). Since the phytoplankton cultures used in this experiment were xenic, a possible explanation for these discrepancies is differences in the bacterial consortia associated with the phytoplankton. There is the possibility of synergistic effects with the introduction of new bacteria, which may have triggered TEP production in those bacteria already associated with the phytoplankton or co-aggregation between bacteria as previously observed in biofilms (Rickard et al. 2003; Burmølle et al. 2006). Furthermore, it was not possible to accurately measure the final cell ratio of phytoplankton to bacteria due to aggregate formation. These factors warrant further consideration for better insight into phytoplankton-bacteria aggregation dynamics. Therefore, identifying the bacteria in the phycosphere and understanding their potential interaction in co-cultures could be the basis for future work.

Diverse groups of marine bacteria have been shown to produce significant amounts of exopolymers (Decho 1990;

Costerton et al. 1995; Sugimoto et al. 2007; Decho and Gutierrez 2017). When *T. weissflogii* and *N. oceanica* were co-cultured with *M. lutimaris*, TEP concentration was at its highest. This is consistent with the observation that marine *Bacteroidetes* possess 2–3 times more glycosyltransferases (proteins that generate polysaccharides) per mega base pair than *Alphaproteobacteria* and *Gammaproteobacteria*, which has been proposed to aid attachment mechanisms via the production of TEP (Fernández-Gómez et al. 2013). Observations of large variations in TEP concentration among phytoplankton-bacteria co-cultures are perhaps not surprising due to the widespread evidence that marine bacteria not only lead to enhanced production of TEP (Costerton et al. 1995; Mykkestad 1995; Passow 2002b; Sugimoto et al. 2007) but can also metabolise it (Kiørboe and Hansen 1993; Grossart and Ploug 2001; Kirchman 2002). A widespread characteristic among different marine bacterial groups (*Cytophagales*, *Pseudomonadales* and *Vibrionales*) is their ability to degrade polymers, which may hinder aggregation (Smith et al. 1992; Smith et al. 1995). Ultimately, aggregation will occur when the production of phytoplankton exudates and TEP are favoured over the hydrolytic capacity of the bacteria (Smith et al. 1995).

TEP is certainly implicated in aggregation formation and sinking; therefore, interactions between phytoplankton and bacteria may have profound implications on the biological carbon pump. The dynamic interactions between bacteria and TEP include production, degradation and modification, and these interactions are complex (Passow 2002a). From a biotechnological perspective, applying a bacterium with aggregation capacity in co-culture with phytoplankton is a potentially attractive option for enhancing phytoplankton aggregation, because many bacteria naturally co-exist and thrive in association with phytoplankton. However, the risk of bacterial contamination and the associated costs for bacterial cultivation must also be considered.

Our data indicate that TEP production is only partially responsible for bacteria-mediated phytoplankton aggregation and that other bacterial phenotypes may play a key role. For example, some marine bacteria have specific flagellin genes (Winstanley & Morgan 1997), and adhesion genes in marine *Flavobacteria* (Fernández-Gómez et al. 2013) might also play a role in the aggregation of phytoplankton cells. Quorum sensing (QS) has been documented in eukaryotic host-associated gram negative bacteria (Gram et al. 2002; Rolland et al. 2016; Zhou et al. 2017), and mutations in the signalling molecule acyl-homoserine lactone (acyl-HSL) synthase in the bacterium *Rhodobacter sphaeroides* have led to hyper aggregation of cells in liquid culture (Puskas et al. 1997). A similar QS phenotype was observed in the *Gammaproteobacteria*, *Yersinia pseudotuberculosis* where expression of a surface protein with homology to flagellin was thought to mediate aggregation (Atkinson et al. 1999). This evidence highlights that cell signalling may be crucial to the aggregation phenotype in

bacteria, but there remains a lack of mechanistic understanding of the molecular markers contributing to aggregate formation.

In conclusion, our results indicate that TEP production is an important factor contributing to aggregation efficiency in the marine phytoplankton *T. weissflogii* and *N. oceanica*. The presence of bacteria is an important contributor to aggregation efficiency and TEP production. The large variations observed among even closely related taxonomic groups highlight the inherent complexities of species-specific interactions and strain-specific differences in aggregation efficiency were clear. In general, however, bacteria in the order *Rhodobacterales*, *Flavobacteriales* and *Sphingomonadales* caused the highest levels of aggregation, which also often coincided with the greatest amount of TEP production. We point out that TEP production is just one, of potentially many, mechanism that influence the aggregation of phytoplankton, and future experiments using omics techniques to elucidate other phenotypes and mechanisms involved in bacteria-mediated phytoplankton aggregation will be valuable. However, our results demonstrate both the ubiquity and diversity of bacterial-mediated aggregation of phytoplankton, providing further support to the concept that this form of phytoplankton-bacteria interaction might have widespread importance within both the context of ocean carbon flux and as a means to enhance the efficiency of harvesting of phytoplankton biomass for biotechnological purposes.

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Data availability The datasets generated and analysed during the current study are available as [Supplementary Material](#).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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