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Impact of *Phaeobacter inhibens* on marine eukaryote-associated microbial communities

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Originality-Significance Statement: This study demonstrates that the marine, host-associated and conspicuous secondary metabolite producer *Phaeobacter inhibens* can shape the microbiomes of eukaryotic host organisms. It also offers insight into how the abundance of specific members, such as vibrios, of the microbial communities of the coccolithophorid microalga *Emiliana huxleyi* and the European flat oyster *Ostrea edulis* is affected by increasing abundance of *P. inhibens*.

Summary

Bacteria-host interactions are universal in nature and have significant effects on host functionality.

Bacterial secondary metabolites are believed to play key roles in such interactions as well as in interactions within the host-associated microbial community. Hence, prominent secondary

metabolite-producing bacteria may be strong drivers of microbial community composition in natural host-associated microbiomes. This has however not been rigorously tested, and the purpose of this study was to investigate how the secondary metabolite producer *Phaeobacter inhibens* affects the diversity and composition of microbiomes associated with the microalga *Emiliana huxleyi* and the European flat oyster, *Ostrea edulis*. Roseobacters were indigenous to both communities exhibiting relative abundances between 2.8 % and 7.0 %. Addition of *P. inhibens* caused substantial changes in the overall structure of the low-complexity microbiome of *E. huxleyi*, but did not shape microbial community structure to the same degree in the more complex oyster microbiomes. Species-specific interactions occurred in both microbiomes and specifically the abundances of other putative secondary metabolite-producers such as vibrios and pseudoalteromonads were reduced. Thus, the impact of a bioactive strain like *P. inhibens* on host-associated microbiomes depends on the complexity and composition of the existing microbiome.

Introduction

In nature, microorganisms live and interact as part of complex multispecies communities. These interspecies interactions may be of a synergistic, amensal, or commensal nature and can be facilitated by the exchange of metabolites in syntrophic cooperation, or by the production of bioactive secondary metabolites (Cole, 1982). In parallel to the use of secondary metabolites as defense against microbial infections by plants (Pusztahelyi *et al.*, 2015), bacterial secondary metabolites with antimicrobial properties are currently believed to facilitate the success of the compound-producer by killing competitors. Not all bacteria are equally proficient in secondary metabolite production, and whereas some groups appear to produce few or no metabolites, others such as filamentous soil bacteria and marine vibrios, roseobacters, and *Pseudoalteromonas* spp. produce an array of different bioactive compounds (Brinkhoff *et al.*, 2004; Murphy *et al.*, 2012; Rasmussen *et al.*, 2014; Maansson *et al.*, 2016; Sonnenschein, Stierhof, *et al.*, 2017). Hence, if the role of these compounds is to eliminate competing microorganisms, proficient secondary metabolite producers should be strong drivers of microbial community composition in natural environments.

The *Roseobacter* group represents one of the most abundant groups of marine bacteria, constituting on average 3 – 5 % of microbial communities in the upper mixed layer (Wietz *et al.*, 2010). On a global scale, the group exhibits a positive abundance-chlorophyll *a* correlation (Wietz *et al.*, 2010) and may exhibit relative abundances of up to 20 – 30 % during algal blooms (González and Moran, 1997; González *et al.*, 2000; Zubkov *et al.*, 2001; West *et al.*, 2008), suggesting association with microalgae. The paraphyletic *Roseobacter* group comprises multiple deeply branching clades (Newton *et al.*, 2010; Simon *et al.*, 2017), of which especially clade 1 includes prominent producers of bioactive secondary metabolites, such as the antimicrobial compounds tropodithietic acid (TDA) (Brinkhoff *et al.*, 2004; Bruhn *et al.*, 2005; Sonnenschein, Nielsen, *et al.*, 2017), indigoidine (Cude

et al., 2012; Gromek *et al.*, 2016), and likely multiple other small molecules (Machado *et al.*, 2015; Bentzon-Tilia and Gram, 2017; Sonnenschein *et al.*, 2018).

One conspicuous genus of *Roseobacter* clade 1 is *Phaeobacter*, which is often found in microbial communities associated with a wide variety of marine eukaryotes including micro- and macroalgae (Rao *et al.*, 2005; Segev, Wyche, *et al.*, 2016), mesozooplankton (Freese *et al.*, 2017), and larger animals such as bivalve molluscs (Ruiz-Ponte *et al.*, 1998; Prado *et al.*, 2009; Wegner *et al.*, 2013). Recently, it was shown that the species *Phaeobacter inhibens* produces small bioactive molecules, e.g. indole-3-acetic acid, which affect the metabolism of the coccolithophorid microalga *Emiliana huxleyi* (Segev, Castañeda, *et al.*, 2016; Segev, Wyche, *et al.*, 2016). It has been proposed that the interaction between *E. huxleyi* and *P. inhibens* exhibits a biphasic pattern where a mutualistic symbiosis gives way for a parasitic interaction where the bacteria accelerates algal lysis in response to algal break-down products (Seyedsayamdost *et al.*, 2011). Mutualistic mechanisms have also been suggested for the symbiosis between bioactive *Roseobacter* clade 1 organisms living in association with the Hawaiian bobtail squid (*Euprymna scolopes*), specifically on the outer surface of the eggs and in the accessory nidamental gland (Collins *et al.*, 2012; Gromek *et al.*, 2016), where they supposedly ward off potential pathogens through the production of antimicrobials. Hence, proficient secondary metabolite-producing species, such as members of the *Phaeobacter* genus might be strong modulators of both the behavior and the microbiome composition of their eukaryotic hosts.

For *P. inhibens*, TDA is the most studied secondary metabolite and its antimicrobial property is likely a result of the ability of TDA to act as a proton antiporter at the cytoplasmic membrane (Wilson *et al.*, 2016). Hence, TDA is a broad-spectrum antibiotic affecting a wide range of both Gram-positive and Gram-negative bacteria (Porsby *et al.*, 2011). Despite the fact that resistance towards TDA does not arise easily (Porsby *et al.*, 2011; Rasmussen *et al.*, 2016), a large fraction of

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bacterial isolates from eukaryote-associated microbiomes similar to those harboring TDA-producers are tolerant towards TDA (Harrington *et al.*, 2014). Such microbiomes may hence be resilient to perturbations caused by compounds such as TDA. Recently, Geng *et al.* (2016) showed that additions of the pure TDA compound had pronounced dose-dependent effects on community structure and composition of the microalgal *Nannochloropsis salina* microbiome at relatively low concentrations (31 – 500 nM). At these concentrations, TDA may act as an inter-microbial signaling molecule rather than an antibiotic (Beyersmann *et al.*, 2017). However, the concentrations, at which secondary metabolites are produced in natural communities are currently unknown, and to what extent the presence of secondary metabolite-producing organisms directly affect the microbial communities has not been addressed. Hence, considering the scarcity of *in vivo* models exploring the effects of prominent secondary metabolite producers on natural eukaryote-associated microbiomes, the purpose of the present study was to establish co-culture model systems to investigate how TDA-producing *P. inhibens* shapes the *E. huxleyi* and *Ostrea edulis* microbiomes over time. One of our key goals was to investigate how different concentrations of TDA-producing *P. inhibens* would alter the community structure due to a gradual increase in roseobacters (dose-response) mimicking the increase in algal blooms. The algal model system was chosen as there is a marked increase in roseobacters in the natural environment during algal blooms and we used levels of *P. inhibens* DSM 17395 reflecting the *in situ* abundances (Amin *et al.*, 2015; Segev, Wyche, *et al.*, 2016; Sonnenschein *et al.*, 2018). The oyster system was chosen as a model for another trophic layer, which would be affected by algal blooms and potentially be exposed to high densities of roseobacters given its feeding mechanism.

Results

The impact of *P. inhibens* strain DSM 17395 on marine eukaryote-associated microbiomes was assessed by sequencing 16S rRNA gene V4 region amplicons from the two different hosts; *E. huxleyi* (microalga) and *O. edulis* (European flat oyster).

***Emiliana huxleyi*-associated microbial community composition.**

To investigate if *P. inhibens* can affect the microbiome composition and diversity of microalgae, co-cultures of *E. huxleyi* and *P. inhibens* were set up in three groups; i) untreated controls, ii) low density (10^4 CFU mL⁻¹) of *P. inhibens*, and iii) high density (10^6 CFU mL⁻¹) of *P. inhibens*. The added densities were equivalent to 0.4 and 40 bacterial cells per algal cell, and 0.09 and 9 *Phaeobacter* cells per bacterium of the initial microbiome, mimicking the ratios observed during algal blooms. The initial algal microbiome was sampled at time point 0 h prior to treatment and the total bacterial count was $5.48 \pm 0.58 \log_{10}$ CFU/mL (using a qPCR-based quantification). Each co-culture was sampled three times: at 24 h, 48 h and 96 h. The most abundant bacterial OTU in the co-cultures (EH_OTU 4) was identified as a *Phaeobacter* sp. (SILVA annotation) and the representative sequence was 100 % identical to *P. inhibens* strain DSM 17395 (accession no. CP002976.1). However, it is important to note that the V4 region does not allow for the discrimination between *P. inhibens* and closely related roseobacters (Table S1). In the amplicon sequencing data, EH_OTU 4 was observed in all samples at relative abundances of 4.0 % to 4.2 % in the initial microbiome, 4.2 % to 8.2 % in the untreated controls (24 h to 96 h), 8.3 % to 11.2 % in the microbiomes treated with the low density of *P. inhibens*, and finally 53.8 % to 79.9 % in the microbiomes of algae treated with the high density. However, using qPCR, *P. inhibens* was below the detection limit ($3.06 \log_{10}$ CFU/mL) in the initial microbiome samples. Accordingly, EH_OTU 4 was excluded from subsequent analyses. Taxonomy and relative abundance of the ten most

abundant OTUs (excluding EH_OTU 4) across all samples can be found in Supplementary material (Table S2, Table S3).

The community was dominated by orders of the Proteobacteria and Bacteroidetes phyla regardless of treatment or incubation time (Figure 1) and neither parameter influenced the composition at class or order level notably. Gammaproteobacteria, Flavobacteria, and Alphaproteobacteria were equally dominating across samples. Co-culturing with high levels of *P. inhibens* altered the relative abundance of other Rhodobacterales members from 18 % to 23 % in controls, to 16 % to 24 % in low *P. inhibens* density co-cultures, and 7.1 % to 9.7 % in high *P. inhibens* density co-cultures except for one high-density, 96 h co-culture (19 %; Figure 1). However, the relative abundance of the order Flavobacteriales and an unidentified Gammaproteobacteria order remained stable across treatments with relative abundances of 23.1 % to 50.1 % and 22.0 % to 34.8 %, respectively.

Oyster-associated microbial community composition

Oysters were divided into two groups: i) untreated controls, and ii) high density (10^7 CFU mL⁻¹) of *P. inhibens*. Two to three oysters were sacrificed before (0 h) and 48 h after treatment to assess potential changes occurring in the microbiome. The total bacterial count of the initial microbiome was $6.99 \pm 0.91 \log_{10}$ CFU/mL (using qPCR-based quantification), hence, the density of *P. inhibens* is equivalent to 0.3 *Phaeobacter* cells to 1 indigenous bacterium. The most abundant OTU in the more complex oyster microbiomes (OE_OTU 1) was identified as an unclassified member of the Rhodobacteraceae family (SILVA annotation). The OE_OTU 1 representative sequence was 100 % identical to *P. inhibens* strain DSM 17395 (accession no. CP002976.1) (Table S4). OE_OTU 1 was observed in all samples; the relative abundance accounted for 2.8 % – 7.0 % of the initial microbiome, decreased to 0.5 % – 0.6 % upon 48 hour incubation of the untreated control oysters but was increased to 33.1 % – 46.7 % in the microbiomes treated with *P. inhibens*. However, *P.*

inhibens was below the qPCR detection limit ($3.06 \log_{10}$ CFU/mL) in the initial microbiome samples. Accordingly, OE_OTU1 was excluded from subsequent analyses. Taxonomy and relative abundance of the ten most abundant OTUs (excluding OE_OTU 1) across all samples can be found in Supplementary material (Table S5, Table S6).

Five different phyla (Bacteroidetes, Firmicutes, Fusobacteria, Proteobacteria, and Tenericutes) with relative abundances above 2 % were observed across the samples of the oyster microbiomes.

Proteobacteria was the dominant phylum in all samples and it increased in relative abundance from 0 to 48 hours. At class level, Gammaproteobacteria was the major contributor, which was mainly due to the orders Alteromonadales and Vibrionales (Figure 2). The relative abundance of Vibrionales decreased in oysters treated with *P. inhibens* (3.1 % – 4.4 %) compared to the 48 h control oysters (16 % – 29 %). In contrast, Alteromonadales were more abundant in the *P. inhibens* treated samples (56 % – 70 %) in comparison to the control (31 % – 47 %). Tenericutes were dominant in the untreated controls after 48 h, but not in the samples treated with *P. inhibens*. At order level, this phylum consisted mainly of Mycoplasmatales.

Impact on richness and diversity of host-associated microbiomes

Microbiomes associated with the algae were less complex than the oyster microbiomes exhibiting estimated OTU richness values (Chao1) in the range of 140 to 493 OTUs (Figure 3A) compared to 810 to 5,746 OTUs for oysters (Figure 3B). Some variation was observed in the estimated richness of the *E. huxleyi* microbiomes, but no clear temporal patterns or treatment effects were apparent. In contrast, the *P. inhibens* treatment of oysters resulted in a significantly increased richness index in comparison to the initial and the control populations (t-test, $P < 0.05$), while the latter, untreated microbiomes (0 h vs. 48 h) did not significantly change during the time of incubation (t-test, $P > 0.05$). As indicated by the estimated richness of the microbiomes, the diversity of oyster

microbiomes (Figure S2B) were higher than that of the alga (Figure S2A), but in contrast to the effect on species richness in the oyster microbiome, the introduction of *P. inhibens* did not affect overall diversity in any of the microbiomes (Figure S2). Hence, the addition of *P. inhibens* had a significant positive effect on species richness in the oyster microbiomes, but the abundances of these species were not evenly distributed and did not affect overall microbiome diversity.

Impact on community structure of host-associated microbiomes

Community structure analyses of both *E. huxleyi* and *O. edulis* microbiomes were based on Bray-Curtis dissimilarity indexes. For *E. huxleyi*, treatment-dependent clustering was observed for the microbiomes receiving the high density of *P. inhibens*, and as indicated by order-level community composition (Figure 1), the untreated communities clustered independently of sampling time point (Figure 4), and hence the effect of *P. inhibens* on the community was immediate (within 0 – 24 h).

In agreement with the negligible effects on the abundance of EH_OTU 4 in communities receiving the low density, these communities were interspersed between the other treatment groups.

Furthermore, samples from replicate cultures of the same treatment were found in separate, individual clusters regardless of time point, thus indicating a strong “bottle” effect in the low density co-cultures (Figure 4). Similarly, for *O. edulis*, the microbiomes co-cultured with the high *P. inhibens* densities clustered separately from the untreated controls (Figure 5) and the untreated microbiomes were dispersed independent of time point, suggesting that high levels of *P. inhibens* altered community structure for both microbiomes. However, there was substantial variation between some biological replicates.

Analysis of Molecular Variance (AMOVA; Bray Curtis distances; 10,000 iterations) on the *O. edulis* microbiomes indicated a significant variance among the three treatment groups (T₀, 48 h control, 48 h *P. inhibens* treatment; $P = 0.0044$), but none of the pairwise variance comparisons

were significantly different from each other. AMOVA was not performed on the *E. huxleyi* microbiome given the low replication level. However, the analyses of the community structures indicate that the less complex microbiome associated with *E. huxleyi* was likely influenced by the introduction of high concentrations of *P. inhibens*, while the differences observed in the more complex oyster microbiome were not significant under the conditions tested.

Impact on individual OTUs

At the order level, the effect of *P. inhibens* on community composition in the *E. huxleyi* microbiome was subtle (Figure 1), and hence we investigated how the differences observed in community structure came across at the species level (OTU clustering at 97 % sequence similarity). Analyzing the 40 most abundant OTUs from both host-associated microbiomes confirmed some variation among replicates. For the *E. huxleyi* microbiome, the relative abundances of the seven most abundant OTUs (2, 3, 5, 6, 7, 10, 8; Table S2) were unaffected by the presence of *P. inhibens* (Figure 4). EH_OTU 9 (*Loktanella* sp.) and EH_OTU 12 (*Marivita* sp.) decreased only in communities treated with the high density of *P. inhibens*. Two gammaproteobacterial OTUs of the *Vibrio* and *Pseudoalteromonas* genera (EH_OTU 20 and 18) were present in the initial microbiomes and increased in relative abundance over time in control microbiomes. However, their abundance decreased in microbiomes supplemented with *P. inhibens*, irrespective of treatment density. In contrast, two OTUs, EH_OTU 33 (*Colwellia* sp.) and 56 (*Neptuniibacter* sp.), were abundant in the microbiomes co-cultured with *P. inhibens* while absent in the controls throughout the experiment. Thus, trends of co-occurring changes in relative abundance in the presence or absence of *P. inhibens* seems to be species if not strain specific in the *E. huxleyi* microbiome.

In contrast to observations from the *E. huxleyi* microbiome, OTUs related to *Colwellia* (OE_OTU 2 and 6; Table S5) were unaffected by the presence of *P. inhibens* in the oyster microbiome (Figure

5). However, as for the *E. huxleyi* microbiome, species of the *Vibrio* and *Pseudoalteromonas* genera (OE_OTU 3 and 4) decreased in relative abundance in samples treated with *P. inhibens*.

Furthermore, OE_OTUs 7, 9, 26 (*Mycoplasma* sp.), 5 (*Shewanella* sp.), and 77 (unclassified proteobacterial OTU) also decreased in the presence of *P. inhibens*. Finally, one *Mycoplasma*-related OTU (OE_OTU 729) increased in relative abundance in the presence of *P. inhibens*.

Henceforth, strain rather than species-specific changes in relative abundances of *Mycoplasma* species occur in the presence of *P. inhibens* in the oyster microbiome.

To investigate a potentially specific, amensal interaction between *P. inhibens* and vibrios, given the potential pathogenicity of certain species, we assessed *Vibrio* spp. abundances in oyster microbiomes quantitatively using quantitative real-time PCR (qPCR). As suggested by the community composition analysis (Figure 2), the introduction of *P. inhibens* did not remove vibrios in the oyster microbiomes completely (Figure S3). However, it resulted in a reduction of *Vibrio* spp. 16S rRNA genes to $5.61 \pm 0.23 \log_{10} \text{ copies g}^{-1}$ as compared to the initial abundance of $5.91 \pm 0.40 \log_{10} \text{ copies g}^{-1}$, and it further reduced *Vibrio* abundances significantly with more than one order of magnitude compared to control oysters (6.89 ± 0.18 ; $P = 0.0328$), thus corroborating the results of the sequence analyses.

Discussion

Bacterial communities associated with eukaryotes have a significant impact on the health and function of their hosts, and investigating how microbiomes of higher organisms are formed and affected by external and internal factors, has become an area of broad and current interest (e.g. Ley *et al.*, 2008; Lebeis *et al.*, 2015). Some members of the host-associated microbiomes carry the capacity to produce bioactive secondary metabolites that may act in the competition with other

members of the microbiomes, yet, it is currently unknown to what extent these bacteria can directly influence and shape the structure of host-associated microbial communities. Our results suggest that the TDA-producing *P. inhibens* has the capacity to influence and shape marine eukaryote-associated microbiomes, yet the effects are variable, dependent on the abundance of *P. inhibens*, and on the complexity and species composition of the host microbiome. Furthermore, the imposed changes occur within a short temporal scale (≤ 24 h) and are otherwise independent of time (within five days).

The two eukaryote model systems were used to investigate how a TDA-producing *P. inhibens* would shape eukaryote-associated microbiomes; during algal blooms vs. non-bloom conditions for the microalga, and how potentially high densities of roseobacters would impact the microbiome of a filter-feeder at another trophic layer during algal blooms. Several studies have been conducted on bacteria associated with *E. huxleyi* and oysters (Zabeti *et al.*, 2010; Carella *et al.*, 2013; Farto Seguín *et al.*, 2014; Green *et al.*, 2015), but the amount of comprehensive culture-independent, diversity studies is limited. The oyster microbiomes of *Crassostrea* spp. have been characterized (King *et al.*, 2012; Wegner *et al.*, 2013; Ossai *et al.*, 2017; Vezzulli *et al.*, 2018) due to their importance in aquaculture, but the present study is to the best of our knowledge, the first culture-independent study investigating the bacterial population of *O. edulis*. Roseobacters were indigenous to both of the investigated microbiomes in agreement with previous findings of molluscan species (Ruiz-Ponte *et al.*, 1998; Grigioni *et al.*, 2000; Barbieri *et al.*, 2001; Martens *et al.*, 2006; Prado *et al.*, 2009) and algae (González *et al.*, 2000; Green *et al.*, 2015; Segev, Wyche, *et al.*, 2016). The occurrence of roseobacters in both native microbiomes supports our choice of *P. inhibens* DSM 17395 as a model organism for a secondary metabolite producer in the natural environment of the eukaryotes in the event of an algal bloom.

The estimated OTU richness of the *E. huxleyi* microbiome was four to seven times lower than the richness of the complex *O. edulis* microbiome. Increasing the *P. inhibens* abundance in the low complexity microbiome of *E. huxleyi* had little to no effect on the total species richness and diversity, whereas the richness increased dramatically in oysters. If *P. inhibens* uses its bioactive compounds to kill competitors, i.e. as antibiotics, a decrease in bacterial richness and diversity could be expected, but the addition of *P. inhibens* had the opposite effect in the oyster system, indicating that the bioactive compounds, such as TDA, are either not produced or serve another function. The expression of TDA-encoding genes have been shown in algal co-culture systems (D'Alvise *et al.*, 2012). Furthermore, TDA is likely produced in our model systems as *P. inhibens* DSM 17395 was pre-grown at conditions known to induce TDA production, i.e. nutrient/iron rich broth. While nutrient-rich medium, such as marine broth, differs from the natural environment, some heterotrophs thrive at high nutrient levels (Alonso and Pernthaler, 2006; Pohlner *et al.*, 2017), which are comparable to the dense, nutrient-rich surroundings of algal cells or in oysters. Henceforth, it is most likely that TDA has another function than being a broad-spectrum defense compound, and that it is probably highly dependent on the investigated conditions. Species-rich microbiomes are typical of healthy, marine invertebrates including oysters (King *et al.*, 2012; Wegner *et al.*, 2013), whereas ill and diseased oyster microbiomes are characterized by a decrease in complexity, loss of rare bacterial strains (Wegner *et al.*, 2013), disruption of the community structure (Lokmer and Mathias Wegner, 2015), and increased abundance of few, specialist OTUs (Wegner *et al.*, 2013; Lokmer and Mathias Wegner, 2015). Thus, it could be speculated that an increase in *P. inhibens* might be beneficial for the host by decreasing the load of potential opportunistic pathogens and allowing rare taxa to proliferate, which has been suggested as the role of various *Roseobacter* group members associated with other molluscs (Collins *et al.*, 2012; Gromek *et al.*, 2016) and microalgae (Seyedsayamdost *et al.*, 2011).

P. inhibens influenced the bacterial community structure in a dose dependent manner, independently of temporal space. The high density of *P. inhibens* caused a shift in the community structure of the *E. huxleyi* microbiome, but no evident clustering patterns were observed for the algal microbiome exposed to the low density. While due to the low sample number these results still require further verification, similar patterns were observed in the microbiome structure of *N. salina* after the addition of pure TDA (Geng *et al.*, 2016).

The oyster microbiome was richer than the algal microbiome indicating that the microalga represented a narrower niche in comparison to the larger, more differentiated bivalve. As filter feeders, oysters accumulate detritus and hence also bacteria. In combination with the higher degree of tissue differentiation, it is not surprising that the oyster microbiome comprises a broader taxonomic assortment of bacteria than that of the microalga. Furthermore, the alga has been kept under continuous laboratory cultivation since its isolation, which may have reduced the richness and diversity of its associated microbiome. Proteobacteria dominated the bacterial community of both *E. huxleyi* and *O. edulis* in agreement with previous observations in coccolithophorid microalgae and other oyster species (Wegner *et al.*, 2013; Green *et al.*, 2015; García Bernal *et al.*, 2017). Green *et al.*, 2015 used a culture-dependent approach and found that Alphaproteobacteria were dominating in their coccolithophorid cultures. Similarly, it has been reported that Gamma- and Alphaproteobacteria of the orders Alteromonadales and Rhodobacterales dominated *E. huxleyi* blooms in the North Atlantic Ocean (Segev, Wyche, *et al.*, 2016). In our culture-independent approach, we found both Gamma- and Alphaproteobacteria as well as Flavobacteria (Bacteroidetes) dominated in the *E. huxleyi* microbiomes.

In *O. edulis*, Tenericutes and Bacteroidetes were prominent phyla although less dominating than Proteobacteria. Wegner *et al.* (Wegner *et al.*, 2013) similarly found Proteobacteria as the dominant phyla followed by Flavobacteria and Bacteroidetes in pacific oysters (*Crassostrea gigas*). The

community composition and dominance of individual taxa depends on local environment, tissue (different organs vs. whole organism), and individual oyster genetics and physiology (King *et al.*, 2012; Wegner *et al.*, 2013; García Bernal *et al.*, 2017; Vezzulli *et al.*, 2018). The Vibrionales and Mycoplasmatales orders decreased in abundance in the presence of *P. inhibens* while Alteromonadales increased in abundance. In the microbiome of the microalga *N. salina*, the abundance of Alteromonadales also increased in the presence of pure TDA while Rhodobacteraceae decreased (Geng *et al.*, 2016). We did not find that *P. inhibens* decreased other Rhodobacterales in the oyster microbiome, however, any change in relative abundance of this order would not be observed since it was below the 2 % cutoff in all microbiomes. Although, the relative abundance of the Rhodobacterales order did decrease in the *E. huxleyi* microbiome when exposed to the high density of *P. inhibens* while the abundance of *Alteromonadales* was unaffected. Hence, the impact of the TDA-producer *P. inhibens* on overall community composition is likely dependent on the eukaryotic host and/or the indigenous community composition.

Given the subtle alterations at higher taxonomic levels in some microbiomes, we assessed changes at the OTU-level (species level) to identify the underlying causes. Indeed, we observed some differences in the OTUs affected by different densities of *P. inhibens*, though with noticeable variability between replicate cultures. Some species (*Colwellia* and *Sulfurospirillum*) were either unaffected or increased by the presence of *P. inhibens*, whereas others including *Vibrio* spp. and *Pseudoalteromonas* spp. were reduced in both eukaryote microbiomes. Interestingly, vibrios and pseudalteromonads are also considered proficient secondary metabolite producers and hence it is plausible that *P. inhibens* specifically and efficiently antagonizes other microorganisms occupying similar niches. The efficient inhibition of vibrios by TDA-producing roseobacters have been repeatedly demonstrated in studies targeting the fish pathogenic *Vibrio* spp. in marine eukaryotes (Porsby *et al.*, 2008; Prado *et al.*, 2009; D'Alvise *et al.*, 2012; Grotkjær, Bentzon-Tilia, Paul W

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D’Alvise, *et al.*, 2016; Grotkjær, Bentzon-Tilia, Paul W. D’Alvise, *et al.*, 2016; Porsby and Gram, 2016; Rasmussen *et al.*, 2018). The high degree of target organism specificity is however somewhat surprising considering the spectrum of bioactivity of TDA (Porsby *et al.*, 2011). Multiple *Mycoplasma* spp. decreased in abundance while a single OTU classified to the same genus increased in abundance further substantiating that the effects of *P. inhibens* are very specific (species level or below). Corroborating the observation that the majority of the species in the microbiomes exhibited minor changes in abundance due to the presence of *P. inhibens*, is the findings by Harrington *et al.* (2014) who observed a high degree of TDA tolerance in non-TDA producing bacterial isolates from marine eukaryote-associated microbiomes. Thus, the impact of increased abundances of a particular secondary metabolite producing microorganism such as *P. inhibens* seems to be highly selective and likely dependent on its specific secondary metabolite profile.

In conclusion, TDA-producing *P. inhibens* can shape host-associated microbiomes of marine eukaryotes. These alterations are subtle at the broader taxonomic levels, but seems to be highly selective and consistent at the OTU level (97 % similarity) across eukaryote host systems. The effects of *P. inhibens* on the species richness and microbiome structure are multifaceted; the richness in the complex microbiome of oysters increased while it remained constant in the microalgal model and the microbiome structure shifted only due to high densities of *P. inhibens*. Thus, the impact likely relies on the composition and complexity of the indigenous bacterial communities. This suggests that a highly diverse microbiome is more stable, though future work across more, varied eukaryote-microbiome associations with larger sample sizes would provide more knowledge to substantiate this hypothesis.

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Figure legends.

Figure 1. The composition of bacterial communities associated with *Emiliania huxleyi* in response to the addition of *Phaeobacter inhibens* DSM 17395 at 0, 24, 48 and 96 hours in duplicates. The compositions of individual microbiomes are illustrated as relative abundances of all the bacterial orders observed in co-cultures of the microalga and different densities of *Phaeobacter inhibens* DSM 17395 over time. Only orders with abundance above 2 % were included (the remaining low abundance orders are represented by the distance up to 1.00). EH_OTU 4 containing the added *P. inhibens* was removed from the dataset prior to plotting. T₀: untreated time zero control, C: untreated control, LD: low density (10⁴ CFU mL⁻¹), HD: high density (10⁶ CFU mL⁻¹).

Figure 2. The composition of bacterial communities associated with European flat oysters (*Ostrea edulis*) in response to the addition of *Phaeobacter inhibens* DSM 17395 at 0 and 48 hours in triplicates (control) and duplicates (treated). The compositions of individual microbiomes are illustrated as relative abundances of bacterial orders observed in oysters over a time course of 48 h. Only orders with abundance above 2 % were included (the remaining low abundance orders are represented by the distance up to 1.00). OE_OTU 1 containing the added *P. inhibens* was removed from the dataset prior to plotting. Some oyster received a high density (HD) of *Phaeobacter inhibens* DSM 17395 (10⁷ CFU mL⁻¹) while others were untreated (controls; C).

Figure 3. Richness of bacterial microbiomes observed in A) *Emiliania huxleyi* (microalga) and B) *Ostrea edulis* (European flat oysters) in response to the addition of *Phaeobacter inhibens* DSM

17395. The richness is depicted as the average Chao1 richness estimate, error bars represent the standard deviation of the average. OE_OTU 1 and EH_OTU 4 containing the added *P. inhibens* were removed from the datasets prior to plotting. T₀: untreated time zero control, C: untreated control, LD: low density, HD: high density. Note the difference in the y-axis. Statistical significance of the change in the oyster microbiomes ($P \leq 0.05$) is indicated by an asterisk.

Figure 4. Heatmap indicating the $\log_{10}(x+1)$ transformed relative abundances of sequences of the 40 most abundant OTUs of the *Emiliana huxleyi* microbiome in response to the addition of *Phaeobacter inhibens* DSM 17395. Individual microbiomes are arranged according to the Bray-Curtis distances between samples as indicated by the tree above the heatmaps (tree not drawn to scale). The SILVA annotation with identity scores (%) are listed next to the individual OTU. OTUs, which were unclassified at genus level, were listed with their nearest classified level (family, order or class level).

Figure 5. Heatmap indicating the $\log_{10}(x+1)$ transformed relative abundances of sequences of the 40 most abundant OTUs of the *Ostrea edulis* microbiome in response to the addition of *Phaeobacter inhibens* DSM 17395. Individual microbiomes are arranged according to the Bray-Curtis distances between samples as indicated by the tree above the heatmaps (tree not drawn to scale). The SILVA annotation with identity scores (%) are listed next to the individual OTU. OTUs, which were unclassified at genus level, were listed with their nearest classified level (family, order or class level). OTUs 7, 29, and 12 were only classified as “Bacteria” according to the SILVA database and thus, the most significant alignment from NCBI’s BLAST has been used instead (GenBank accession number and % identity is listed).