Brief Report

Consistency in microbiomes in cultures of *Alexandrium* species isolated from brackish and marine waters

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Summary

Phytoplankton and bacteria interactions have a significant role in aquatic ecosystem functioning. Associations can range from mutualistic to parasitic, shaping biogeochemical cycles and having a direct influence on phytoplankton growth. How variations in phenotype and sampling location, affect the phytoplankton microbiome is largely unknown. A high-resolution characterization of the bacterial community in cultures of the dinoflagellate *Alexandrium* was performed on strains isolated from different geographical locations and at varying anthropogenic impact levels. Microbiomes of *Alexandrium ostenfeldii* isolates were dominated by Betaproteobacteria and were consistent over strain variation, phylogenetic and genotypic *Alexandrium* strain variation, resulting in identification of an *A. ostenfeldii* core microbiome. Comparisons with *in situ* bacterial communities showed that taxa found in this *A. ostenfeldii* core were specifically associated to dinoflagellate dynamics in the Baltic Sea. Microbiomes of *Alexandrium tamarense* and *A. minutum*, isolated from the Mediterranean Sea, differed from those of *A. ostenfeldii* in bacterial diversity and composition but displayed high consistency, and a core set of bacterial taxa was identified. This indicates that *Alexandrium* isolates with diverse phenotypes host predictable, species-specific, core microbiomes reflecting the abiotic conditions from which they were isolated. These findings enable in-depth studies of potential interactions occurring between *Alexandrium* and specific bacterial taxa.

Introduction

Interactions between phytoplankton and bacteria in aquatic environments are intricate and have large impacts on ecosystem functioning (Azam and Malfatti, 2007; Buchan et al., 2014). Specifically, phytoplankton bacteria interactions, ranging from mutualism (Amin et al., 2009) to resource competition (Risgaard-Petersen et al., 2004), have effects on the whole ecosystem in terms of the amount of carbon that is recycled or stored (Cole, 1982; Jiao et al., 2010), the level of regenerated nitrogen (Eppley and Peterson, 1979) and utilization of captured energy (Buchan et al., 2014). The diversity, functioning and efficiency of bacteria associating with phytoplankton cells (the microbiome) are thought to be determined by the biochemical composition of phytoplankton and their released organic matter (Azam and Malfatti, 2007; Buchan et al., 2014; Seymour et al., 2017). The composition of the microbiome has been shown to be host specific in association with, for example, diatoms and dinoflagellates (Schäfer et al., 2002; Guannel et al., 2011; Lawson et al., 2017; Behringer et al., 2018).

Dinoflagellates release organic matter continuously during their life cycle (Villacorte et al., 2015) affecting both composition and function of their microbiome (Sarmento et al., 2013). The role of bacteria-dinoflagellate interactions may include growth stimuli (Sakami et al., 1999; Ferrier et al., 2002; Bolch et al., 2011), life cycle cues (Adachi et al., 2003) and regulation of toxin production (Doucette and Powell, 1997; Gallacher and Smith, 1999). Lawson et al. (2017) showed the existence of a consistent core microbiome among dinoflagellate *Symbiodinium* clades, pointing at taxonomically consistent interactions with significance to dinoflagellate functions. Few studies have characterized these complex interactions (Hattenrath-Lehmann...
and Gobler, 2017), which is a prerequisite for understanding consequences for large scale biogeochemical processes.

Dinoflagellates of the genus *Alexandrium* are reported from many geographical areas in the world’s oceans (Anderson et al., 2012) and are known to release bioactive compounds, potent toxins (Anderson et al., 2012) and allelochemicals that are poorly chemically characterized (Ma et al., 2009), which can interact with microbial food web dynamics (Weissbach et al., 2011). *Alexandrium* populations display a large intraspecific phenotypic diversity, in terms of growth rate (Suikkanen et al., 2013; Brandenburg et al., 2018), toxin production (Tillmann et al., 2009; Suikkanen et al., 2013; Martens et al., 2017; Brandenburg et al., 2018), allelochemical activity (Hakanen et al., 2014; Brandenburg et al., 2018), nutritional strategies (Gilbert and Legrand, 2006) and ability to bioluminesce (Valiati et al., 2012). This has led to the development of a large number of isolation methods at conditions mimicking those of their respective origins, brackish (salinity 6.5 or 7) for *A. ostenfeldii* and marine (salinity 31) for *A. minutum/tamarense* (Supporting Information Table S1). Microbial isolates observed using 16S amplicon sequencing, which resulted in a total of 9,776,193 sequences and 3,649 operational taxonomic units (OTUs; 97% similarity clustering; Supporting Information Table S2). In this study, we define microbiome as consisting of the whole bacterial assemblage found in the *Alexandrium* cultures. Our aim was to explore how host specificity, intraspecies variations and sampling location influence the diversity and consistency of the microbiome of *A. ostenfeldii*, *A. minutum* and *A. tamarense* isolates, and investigate the existence of a species-specific core microbiome. In total 28 strains were included, of which 20 belong to *A. ostenfeldii*, isolated from four distinct locations in the brackish Baltic Sea Proper. These strains have been shown to have phenotypic (Suikkanen et al., 2013; Hakanen et al., 2014) and genotypic (Tahvanainen et al., 2012) differences. The remaining strains belonged to *A. minutum* (*n = 7*) and *A. tamarense* (*n = 1*), isolated from eight locations in the north-west Mediterranean Sea (marine conditions) with different anthropogenic impact levels, classified using the Land Uses Simplified Index, LUSI (E. Flo, unpubl.). Two in situ datasets from the Baltic Sea were used to investigate if members from the identified core microbiome of *A. ostenfeldii* in culture were linked to natural dinoflagellate dynamics.

**Results and discussion**

**Identification of dinoflagellates and diversity of microbiomes**

The dinoflagellates were taxonomically identified by amplification and sequencing of their ITS-regions which corroborated identifications previously made using microscopy (S. Fraga and I. Bravo, unpubl.; Kremp et al., 2009) except for the strain AL10C, which was previously identified as *A. minutum*, but showed a high level of identity (99%) to an *A. tamarense* strain (AJ005048.1; Supporting Information Fig. S1). The *Alexandrium* strains were cultured using sterile methods at conditions mimicking those of their respective origins, brackish (salinity 6.5 or 7) for *A. ostenfeldii* and marine (salinity 31) for *A. minutum/tamarense* (Supporting Information Table S1). Nucleic acid samples were obtained by filtering cultures (density of *A. ostenfeldii*: 0.03–0.15 x 10^5 cells/ml and *A. minutum/tamarense*: 2.10–3.50 x 10^5 cells/ml; Supporting Information Table S1) using 0.2 μm filters, capturing the whole bacterial assemblage of the cultures at late exponential growth phase. The microbiomes were characterized using high-throughput 16S rRNA gene amplicon sequencing, which resulted in a total of 9,776,193 sequences and 3,649 operational taxonomic units (OTUs; 97% similarity clustering; Supporting Information Table S2). In this study, we define microbiome as consisting of the whole bacterial assemblage found in the *Alexandrium* cultures. Our aim was to explore how host specificity, intraspecies variations and sampling location influence the diversity and consistency of the microbiome of *A. ostenfeldii*, *A. minutum* and *A. tamarense* isolates, and investigate the existence of a species-specific core microbiome. In total 28 strains were included, of which 20 belong to *A. ostenfeldii*, isolated from four distinct locations in the brackish Baltic Sea Proper. These strains have been shown to have phenotypic (Suikkanen et al., 2013; Hakanen et al., 2014) and genotypic (Tahvanainen et al., 2012) differences. The remaining strains belonged to *A. minutum* (*n = 7*) and *A. tamarense* (*n = 1*), isolated from eight locations in the north-west Mediterranean Sea (marine conditions) with different anthropogenic impact levels, classified using the Land Uses Simplified Index, LUSI (E. Flo, unpubl.). Two in situ datasets from the Baltic Sea were used to investigate if members from the identified core microbiome of *A. ostenfeldii* in culture were linked to natural dinoflagellate dynamics.
Similarity of microbiome composition

To study if the composition of the microbiomes associated with *Alexandrium* could be related to intraspecies variations of *Alexandrium* strains or specific conditions at the location of isolation an nMDS analysis, using Bray–Curtis dissimilarity, was performed. This analysis showed that there was a clear separation between *A. ostenfeldii* and the *A. minutum/tamarense* strains regarding frequently occurring taxa (Fig. 1 and Supporting Information Fig. S3). The *A. ostenfeldii* microbiomes were very homogeneous (permAnova $R^2 = 0.13$, $P$ value: 0.73; Fig. 1) considering that the strains were isolated from four geographically distant locations of the Baltic Sea and the inherent intraspecific variations previously demonstrated for *A. ostenfeldii* (Kremp et al., 2016; Martens et al., 2017; Supporting Information Fig. S4). Consequently, our results indicate that intraspecific variations or sampling location in the Baltic Sea did not have a significant impact on the composition of the microbiomes. These findings indicate that the interactions that occur between the dinoflagellate and their microbiomes, are consistent within a species-specific functional range of *Alexandrium*, disregarding phenotypic variations of individual dinoflagellate strains. In the nMDS, the *A. minutum/tamarense* associated microbiomes were dispersed with a spread similar to that of the *A. ostenfeldii* microbiomes (Fig. 1), and they did not cluster according to the anthropogenic impact level at each location (estimated using LUSI; E. Flo, unpubl.; Supporting Information Fig. S4). The results indicate a high microbiome similarity also across the *A. minutum/tamarense* strains. Taken together, our results show that both the environment at the sampling location, brackish or marine, and the species of *Alexandrium*, had important impacts on the composition of the microbiomes.

Microbiome of *A. ostenfeldii* isolates

The microbiomes of the *A. ostenfeldii* isolates were highly similar, forming four clusters (based on Bray–Curtis dissimilarity) of 3–7 samples each, originating from different locations (Supporting Information Fig. S3). These microbiomes included sequences affiliated with a broad range of bacterial classes: Beta- (58% of total sequences), Alpha- (14% of total sequences), Gamma- (3% of total sequences) and Deltaproteobacteria (6% of total sequences), Flavobacteriia (9% of total sequences) and Cytophagia (1.5% of total sequences). Represented, at family level, by *Methylphilaceae* (28% of total sequences), *Hydrogenophilaceae* (10% of total sequences), *Flavobacteriaceae* (7% of total sequences), *Comamonadaceae* (4% of total sequences), *Desulfovulbaceae* (4% of total sequences) and *Rhodobacteraceae* (3% of total sequences; Fig. 2). The high frequency of Betaproteobacteria in association with *A. ostenfeldii*, may be a reflection of the *in situ*
brackish bacterial community composition of the Baltic Sea (Herlemann et al., 2011), which hosts more Betaproteobacteria compared to environments with higher salinity. The betaproteobacterial sequences, were primarily affiliated with Methylphilaceae, Burkholderiaceae/Limnobacter and Comamonadaceae (Supporting Information Fig. S5). Although Betaproteobacteria are not frequently found to dominate bacterial communities associated with phytoplankton, both Methylphilaceae and Limnobacter have previously been found in association with diatoms and coccolithophores (Amin et al., 2012; Green et al., 2015). Members of the family Methylphilaceae, are known to produce the growth promoting hormone indole-3-acetic acid (IAA) in association with plants (Doronina et al., 2014). Bacterial IAA has been observed to be exchanged for tryptophan in diatom cultures (Amin et al., 2015). Thus, a similar interaction may also occur between A. ostenfeldii and Methylphilaceae. Methylphilaceae prefer C1-compounds, methanol or methylamine, as a source of carbon (Doronina et al., 2002) and they are known to degrade dimethylsulfide (DMS) (Eyice et al., 2015). The consistency of the dominating groups of the microbiome suggest that associations between these groups and A. ostenfeldii may be of importance for dinoflagellate metabolism, at brackish conditions, and are not an artefact of culturing.

Microbiome of A. minutum and A. tamarense isolates

The microbiomes of A. minutum/tamarense isolates were clustered by similarity into two groups, of 2 and 3 samples, respectively, mixing samples from different environmental conditions (estimated using LUSI; E. Flo, unpubl.; Supporting Information Figs S3 and S4). These microbiomes were dominated by sequences affiliated with Alphaproteobacteria (56% of total sequences), Flavobacteria (21% of total sequences) and Gammaproteobacteria (13% of total sequences). At family-level these were represented by Rhodobacteraceae (46% of total sequences), Cryomorphaceae

**Fig. 2.** Composition of microbiomes of Alexandrium isolates at (A) Class and (B) Family level. Samples are grouped by dissimilarity (Supporting Information Fig. S3); AOA-D: A. ostenfeldii group A–D, AMA-B: A. minutum/tamarense group A–B. The cultures were harvested during late exponential phase using 0.2 μm, 47 mm filters and DNA was extracted (Boström et al., 2004). The V3-V4 region of the 16S rRNA gene was amplified using primers 341F (CCTACGGGNGGCWGCAG) and 805R (GACTACHVGGGTATCTAATCC) and sequenced using Illumina MiSeq (Herlemann et al., 2011; Hugerth et al., 2014). The read-pairs were clustered into OTUs using Usearch v8.1 (radius 1.5) (Edgar, 2013) corresponding to ~97% sequence identity and singletons were removed (Supporting Information Table S2). OTUs were classified using SILVA db 123 SSURef NR99; (Quast et al., 2013) using SINA v. 1.2.13 (Pruesse et al., 2012). The graph was constructed using ggplot2 (v. 2.2.1) (Wickham, 2009). The numbers for each sample specify the ID of each strain given in Supporting Information Fig. S4. The sequence data have been submitted to the European Nucleotide Archive (ENA) database under accession numbers ERS1617530-ERS1617557.

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Of the core microbiomes indicates that associations between Alexandrium and bacteria in culture are stable and predictable. These findings enable further identification and characterization of specific Alexandrium-bacterial interactions.

Culture conditions

There are several considerations that should be made before drawing conclusions about in situ associations from in vitro conditions (Sala et al., 2005; García et al., 2007; Hatlenrath-Lehmann and Gobler, 2017; Behringer et al., 2018). For example, culture conditions and time in culture can have significant impacts as culturing can alter the bacterial community composition within hours (Sapp et al., 2007; Weissbach et al., 2010). To investigate the effect of long term culturing on the associated bacterial communities, A. ostenfeldii (strains 13–20) and A. minutum/tamarense (strains 21–28) isolates were monitored after 6 and 4 months of culturing respectively. Similar to studies of diatoms in culture (Schäfer et al., 2002; Behringer et al., 2018), the bacterial communities of the Alexandrium isolates is this study were shown to be consistent over time (Supporting Information Table S1 and Fig. S8). However, in the high-resolution sequencing data, shifts in the relative abundance among some of the most frequent betaproteobacterial OTUs were observed in A. ostenfeldii strains 1–12, compared to strains 13–20 (Supporting Information Fig. S5). Strains 13–20 had previously been kept at the same conditions as 1–12, but were moved to another lab and sampled after 6–9 weeks of exposure to slightly different conditions with regards to light, salinity and temperature (Supporting Information Table S1). Taken together these results show that, given stable conditions, the members of the core microbiome of Alexandrium are consistent, but shifts in the relative abundance of the microbiome, including members of the core, can be expected as a reflection of changed environmental conditions.

Connections with in situ dinoflagellate populations in the Baltic Sea

In contrast to the Mediterranean Sea isolates, which were isolated as vegetative cells, the A. ostenfeldii isolates were isolated as cysts (Tahvanainen et al., 2012). To investigate if OTUs from the A. ostenfeldii core microbiome were present in the pelagic zone, and if their frequency could be related to natural dinoflagellate dynamics, two Baltic Sea microbial datasets were explored (Fig. 3A). The Prodi versa cruise took place during April 2013, representing a transect of the Baltic Sea Proper, capturing a mixed dinoflagellate spring bloom (Bunse et al., 2016; Godhe et al., 2016). The Planfis dataset was sampled during the productive season (April–October) of 2010–2011, covering a smaller geographic area west of the island of Gotland.
capturing both spring and summer blooms of different dinoflagellate species (Legrand et al., 2015) (Fig. 3B). OTUs corresponding to members of the core microbiome (OTU_000043 and OTU_000260), were found to follow the biomass dynamics of the dinoflagellate spring bloom, captured by the Prodiversa cruise (Fig. 3A). These OTUs were affiliated to Betaproteobacteria (Oxalobacteraceae and Comamonadaceae; Supporting Information Table S4A). In the Planfish dataset, one of the same core OTUs (OTU_000260), increased in relative abundance during spring and summer dinoflagellate blooms (Fig. 3B). These results indicate that members of the core microbiome of A. ostenfeldii in culture followed in situ dinoflagellate occurrences, suggesting that they may be closely associated with Baltic Sea dinoflagellates, regardless of species.

Conclusions

This study, the first high resolution molecular characterization of the microbiome of A. ostenfeldii isolates, showed that neither intraspecific variations nor the location of sampling in the brackish Baltic Sea Proper had a significant impact on the isolate microbiome composition. Similarly, the level of anthropogenic impact at the different sites of isolation of A. minutum/tamarense strains could not be linked to the composition of the different microbiomes. Instead, the microbiomes were highly consistent among strains of A. ostenfeldii and A. minutum/tamarense, respectively, and core microbiomes were identified. These core microbiomes were likely shaped by abiotic conditions, like salinity, light and temperature as shifts in relative abundances could be seen upon changed culture conditions, and selected by the respective dinoflagellate host in a species-specific manner. The identification of an Alexandrium core microbiome makes this model system ideal for in-depth studies of specific interactions likely occurring between the phytoplankton and key bacterial taxa – principal for understanding the impact of interactions on the ecosystem. In future studies, core microbiomes could be identified from natural phytoplankton blooms using culture independent methods such as single cell genomics.
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Conflict of interest

The authors declare no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Appendix S1**: Supporting Information

**Table S3A** – Core microbiome of *Alexandrium ostenfeldii*, with an OTU-abundance > 0.0001% (Lawson *et al.*). Specified by the Class and Family/Genus of the closest relative in GenBank (as given in Table S4A), the number of OTUs per Family and the relative abundance of those OTUs per group. Groups are specified in Fig. S4; AO – all *A. ostenfeldii* strains; AOA – strains 1–4, 6, 8, 10; AOB – strains 5, 11, 12; AOC – strains 7, 14, 16; AOD – strains 13, 15, 17, 19. Note that samples 18 and 20 are excluded (<162 000 reads) and samples 9 is considered an outgroup of the *A. ostenfeldii* cluster (Fig. S4) and are therefore not included in any group.

**Table S3B** – Core microbiome of *Alexandrium minutum/tamarense*, with an OTU-abundance > 0.0001% (Lawson *et al.* 2017). Specified by the Class and Family/Genus of the closest relative in GenBank (as given in Table S4B), the number of OTUs per Family and the relative abundance of those OTUs per group. Groups are specified in Figure S4; AM – all *A. minutum/tamarense* strains (excluding samples 25, 26 (<162 000 reads); AMA – strains 21, 23, 28; AMB – strains 22, 27. Note that strain 24 is considered an outgroup of the *A. minutum/tamarense* cluster in Figure S4 and therefore not included in any group.

**Table S4A** Core microbiome (55 OTUs) of *Alexandrium ostenfeldii*, specifying: OTU, accession number of closest relative in GenBank, % identity, genus and family of that strain and the sequence of the OTU.

**Table S4B** Core microbiome (52 OTUs) of *Alexandrium minutum/tamarense*, specifying: OTU, accession number of closest relative in GenBank, % identity, genus and family of that strain and the sequence of the OTU.