Zooming in on dynamics of marine microbial communities in the phycosphere of *Akashiwo sanguinea* (Dinophyta) blooms

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Abstract

Background: Characterising ecological relationships between viruses, bacteria, and phytoplankton in the ocean are critical to understanding the ecosystem, yet these relationships are infrequently investigated together. To understand the dynamics of microbial communities and environmental factors in harmful algal blooms (HABs), we examined the environmental factors and microbial communities during *Akashiwo sanguinea* HABs in the Jangmok coastal waters of South Korea by metagenomics.

Results: Specific bacterial communities showed synergistic and antagonistic relationships with *A. sanguinea* bloom. Endoparasitic dinoflagellate *Amoebophrya* sp. 1 controlled the bloom dynamics, as an increase in their abundance was correlated with HAB decline. In the nucleocytoplasmic large DNA viruses, abundance of Pandoraviridae increased following an increase in HAB. Operational taxonomic units and environmental factors associated with *A. sanguinea* were also visualized by network analysis: *A. sanguinea*-*Amoebophrya* sp. 1 ($r=0.81$, Time-lag: 2 day) and *A. sanguinea*-Pandoravirus dulcis (0.64, 0 day) relationships showed close association. *A. sanguinea*-dissolved organic carbon and -dissolved inorganic phosphorus relationships were also very closely correlated (each 0 day time-lag, respectively).

Conclusions: Microbial communities and the environment dynamically and complexly changed in *A. sanguinea* bloom, and a rapid turnover of microorganisms could respond to ecological interactions. *A. sanguinea* bloom dramatically changes the environments through their exudation of dissolved carbohydrates by autotrophic processes, followed by changes in microbial communities involving host-specific viruses, bacteria, and parasitoids. Thus, microbial communities in HAB ecology are composed of various organisms and they interact in a complex way. Therefore, to interpret their ecosystem, the complex reactions among various microorganisms should be studied rather than studying a simple 1:1 reaction, such as a prey-predator interaction.

Background

In ecology, phytoplankton are considered a double-edged sword[1]. Although phytoplankton is an essential component of the marine ecosystem, with multiple roles in matter cycling[2], a few phytoplankton taxa form harmful algal blooms (HABs), which can adversely impact marine ecosystems and human health[3]. A vast majority of the known HABs are dinoflagellates[4], among which, *Akashiwo sanguinea* causes frequent blooms world-wide[5,6]. *A. sanguinea* produces surfactants that saturate the feathers of marine birds with water and cause severe hypothermia[7] and have also been related to fish kills and marine mammal strandings[8]. However, the environmental changes caused by these strategies in dissolved organic matters and specific nutrient sources of this bloom are still poorly understood.

Marine microbial communities are diverse and support other marine organisms. Microbial communities, including viruses, bacteria, fungi, and some parasitic algae, have the potential to impact population dynamics of HAB organisms[9]. Viruses are the most common biological entities in the marine environment, which contribute significantly to the flux of energy and matter, and influence

biogeochemical cycling[10]. Nucleocytoplasmic large DNA viruses (NCLDV) infect both animals and unicellular eukaryotes[11]. Members of the Phycodnaviridae family are large icosahedral NCLDV that are mostly known to infect eukaryotic algae[12]. However, each viral family has not yet been assigned a species-specific host group. For example, Mimiviridae infect *Acanthamoeba* and other protists serve as natural hosts[13], but members of this group have also been found to infect various phytoplankton species, recently. Thus, role of each group of the NCLDV in host-specific infection is yet to be elucidated[13,14].

Interactions between phytoplankton and bacteria are important in shaping their environment, and consequently, the biogeochemical cycles[15]. Phytoplankton relies on bacteria to remineralise organic matter back to its inorganic substituents[16]. Recently, specific bacterial phylotypes have been detected in association with different microalgae. Yang *et al.*[17] reported species-specific relationships between bacterial communities and *A. sanguinea* bloom. In eukaryotic parasitoids, *Amoebophrya* sp. kills its host and controls the dinoflagellate bloom[18]. *Amoebophrya* sp. has relatively short generation time and high prevalence in nature[19,20]. Studies on the interaction between *Amoebophrya* sp. and *A. sanguinea* bloom as host are in the laboratory[19]; however, ecological species-specific host-parasitoid interactions are yet to be elucidated.

Interactions among microbial communities in an ecosystem are very complex. Therefore, assessment of the changes in environmental characteristics and their interaction with the microorganisms in *A. sanguinea* bloom can increase our understanding of the microbial communities. With the advancement of metagenomic next-generation sequencing (mNGS) technology, a large volume of sequencing data have been analysed and baseline information regarding genetic traits has been developed. In addition, many ecological studies have used mNGS to estimate changes in population dynamics and communities[21]. Also, the new technologies for studying aquatic microbial diversity require smaller volume and nanograms of DNA[22]. To explore changes in environmental characteristics and microbial communities in the phycosphere of *A. sanguinea* bloom and to estimate the potential control mechanisms for *A. sanguinea* bloom, we investigated the ecological phenomena when *A. sanguinea* was bloomed in the Jangmok Bay Time-series Monitoring Site (JBTMS). In particular, we conducted an intensive monitoring plan (i.e., daily sampling) for understanding dynamics of microbial communities and environments and established an 100 L-scale in-door microcosm to mimic a real ecosystem. Microcosm and/or mesocosm studies can clarify the effect of variation in environmental and biological factors on the system, and provide an important link between laboratory and field data[23].

Results

Environmental characteristics during *Akashiwo sanguinea* bloom

Variations were observed in the environmental characteristics of JBTMS from June 2016 to June 2017 (Supplementary Fig. 1). *A. sanguinea* bloom sustained for 44 days; it developed on October 31st and declined on December 13th, 2016 (Fig. 1). During this blooming period, the mean abundance of *A.*

sanguinea was 542 cells mL⁻¹, with a maximum abundance of 2,935 cells mL⁻¹ on November 18th; the water temperature gradually decreased, and *A. sanguinea* bloom rapidly declined below 16 °C (after November 21st). Dissolved silica (DSi) concentrations remained between 21.18 and 30.67 µM, and did not show a significant correlation with the abundance of *A. sanguinea*. Dissolved inorganic nitrogen (DIN) concentrations rapidly decreased at the beginning of *A. sanguinea* bloom and remained between 1.19 and 2.87 µM. Dissolved inorganic phosphorus (DIP), dissolved organic carbon (DOC), and chlorophyll *a* concentrations showed similar changes following *A. sanguinea* bloom and were significantly correlated with change in *A. sanguinea* abundance.

Daily monitoring of the JBTMS from November 14th to December 26th, 2017, showed that the dominant phytoplankton was *Bathycoccus prasinus* (Chlorophyta) (Supplementary Fig. 2). We also observed that the water temperature was lower in 2017 than in 2016, rising over 16 °C for only two days in 2017. pH and dissolved oxygen (DO) concentration were also lower in 2017 than in 2016. DIN, DIP, and DOC concentrations did not change with *B. prasinus* abundance. Changes in the DIP concentration showed no significant correlation with *B. prasinus*. The in-door microcosm experiment showed environmental characteristics similar to those of the JBTMS in 2016 (Supplementary Fig. 3). *A. sanguinea* abundance decreased gradually with decreasing water temperature. Ammonia, DIP, and DOC concentrations and total bacterial abundance decreased with decreasing *A. sanguinea* abundance, and increased again because of the substance release caused by the destruction of *A. sanguinea* cells in a closed environment.

Species-specific bacterial community during *A. sanguinea* bloom

mNGS results for bacterial community in JBTMS are summarised in Supplementary Table 1. In 2016, the bacterial community was classified into four groups at 73 % similarity by non-metric multidimensional scaling (nMDS) analysis (Fig. 2a). Group I was associated with “before *A. sanguinea* bloom” (4th and 31st October). This group comprised communities of Alpha-proteobacteria (73 %), Flavobacteriia (13 %), Gamma-proteobacteria (8 %), and other bacteria. Group II and III were associated with “during *A. sanguinea* bloom” (November 7th to 28th), wherein Flavobacteriia increased rapidly to 39.94 %. Group IV was associated with “after *A. sanguinea* bloom” (29th November to 26th December). In this group, abundance of Gamma-proteobacteria (13 %) increased from that of Group III. In 2017, bacterial community was divided into two groups at 70 % similarity by nMDS analysis (Fig. 2b). Group I was associated with “dominance of *B. prasinus*” (14th November to 13th December), and the group also comprised Alpha-proteobacteria (44 %), Flavobacteriia (20 %), Gamma-proteobacteria (7 %), and others (28 %). In group II (After decrease of *B. prasinus* abundance, December 19th and 26th), Alpha-proteobacteria rapidly increased to a proportion of 80 %.

The number of operational taxonomic units (OTUs) and alpha diversity showed a trend similar to that in the read counts and varied according to the period (Supplementary Table 1). The most abundant bacterial OTUs in 2016 belonged to Alpha-proteobacteria (9 OTUs), Gamma-proteobacteria (7), Flavobacteriia (11), and other bacterial species, including Beta-proteobacteria (1) and Actinobacteria (2) (Fig. 3, Supplementary Fig. 4). Before *A. sanguinea* bloom (Group I), eight bacterial OTUs were common

species, and *Cribrihabitans marinus* (Alpha-proteobacteria) was dominant at 53.91 % (relative abundance). During *A. sanguinea* bloom (Group II and III), 18 bacterial OTUs were common species, and uncultured Alpha-proteobacterium (OTU #2) and *C. marinus* (Alpha-proteobacteria; 21.01% and 13.83%, respectively), and *Tenacibaculum aiptasiae* and *Polaribacter marinivivus* (Flavobacteriia; 10.95% and 10.41%, respectively) were dominant with an accumulated proportion of 56.2 %. In particular, the changes of uncultured bacterium (OTU #2) and *A. sanguinea* cells were significantly correlated ($r = 0.90$, $p < 0.001$). After *A. sanguinea* bloom (Group IV), 22 bacterial OTUs were common species, including *C. marinus*, and *Amylibacter ulvae* (Alpha-proteobacteria; 7.98 % and 15.94 %, respectively), *Mesonina algae* (Flavobacteriia; 16.61 %), and *Methylophilus methylotrophus* (Beta-proteobacteria; 3.14 %). In 2017, the most abundant bacterial OTUs belonged mainly to Alpha-proteobacteria (7), Gamma-proteobacteria (6), Flavobacteriia (11), and other bacteria including Beta-proteobacteria (1) and Actinobacteria (1) (Fig. 3, Supplementary Fig. 4). During the dominance of *B. prasinos* (Group I), *C. marinus* (20.77 %), *A. ulvae* (11.71 %), and *Euzebya tangerine* (25.83%, Actinobacteria) were the pre-dominant species in 16 common bacterial OTUs. After the decrease in abundance of *B. prasinos* (Group II), *A. ulvae*, *Lentibacter algarum*, and *Planktomarina temperata* (Alpha-proteobacteria; 40.71 %, 11.49 %, and 21.37 %, respectively), and *E. tangerine* (5.52 %) were the dominant species in the nine common bacterial OTUs.

Potential NCLDV infection of *A. sanguinea* bloom

The mNGS results for NCLDV of both JBTMS (2016 and 2017) and in-door microcosm are summarised in Supplementary Table 2 and Supplementary Table 3, respectively. In 2016, the most abundant NCLDV OTUs belonged mainly to Phycodnaviridae (9 OTUs), Pandoraviridae (3), Poxviridae (6), and Iridoviridae (1). According to nMDS analysis of NCLDV composition in 2016, NCLDV were clustered in four groups at 79 % similarity (Fig. 2c). Before *A. sanguinea* bloom (Group I), the major NCLDV groups at family level were Poxviridae (55 %), Phycodnaviridae (25 %), Pandoraviridae (20 %), and others (including Mimi- and Irido-viridae) (<1 %). During *A. sanguinea* bloom (Group II and III), Pandoraviridae increased rapidly to 61 %. In particular, Pandoravirus salinus and Pandoravirus dulcis were positively correlated with *A. sanguinea* abundance ($r=0.67$ and $r=0.66$, $p<0.01$, respectively) (Supplementary Fig. 5). After *A. sanguinea* bloom (Group IV), Pandoraviridae decreased to 31 %, while Phycodnaviridae and Poxviridae relatively increased to 32 % and 36 %, respectively; however, Pandoraviridae increased again when *Heterocapsa triquetra* (Dinophyta) appeared (Fig. 4a). In 2017, the most abundant NCLDV OTUs mainly belonged to Phycodnaviridae (11 OTUs), Pandoraviridae (4), Poxviridae (6), Iridoviridae (1) and Mimiviridae (1). NCLDV were clustered in three groups (Fig. 2d). During the dominance of *B. prasinos* (Group I), Phycodnaviridae and Pandoraviridae were dominant, adding up to 89.59 % of the total relative abundance. In Phycodnaviridae, *H. akashiwo* virus 1 and *P. globosa* virus were strongly and positively correlated to *B. prasinos* abundance, while *Ostreococcus lucimarinus* virus 2, *O. lucimarinus* virus OIV5, and *O. tauri* virus 2 were negatively correlated to *B. prasinos* abundance (Supplementary Fig. 5). In Pandoraviridae, Pandoravirus salinus, Pandoravirus dulcis, and an unidentified Pandoravirus were positively correlated with changes in *B. prasinos* abundance (Supplementary Fig. 5). However, after the decrease in abundance of *B. prasinos* (Group II and III), Phycodnaviridae rapidly increased to 96 % (Fig. 4b). In the in-door microcosm, NCLDV showed patterns similar to those of JBTMS in 2016. In particular,

Pandoraviridae (mainly, Pandoravirus salinus and Pandoravirus dulcis) decreased as *A. sanguinea* cells decreased (Fig. 4c and Supplementary Fig. 5). Phycodnaviridae (mainly, *Heterosigma akashiwo* virus1 and *Phaeocystis globosa* virus) showed a pattern opposite to that of *A. sanguinea* cells.

Endoparasitic dinoflagellate dynamics during *A. sanguinea* bloom

To explore co-occurrence patterns, focusing primarily on potential parasitic interactions between endoparasitic dinoflagellate *Amoebophrya* sp. ex. *A. sanguinea*, we assessed the relationship between *Amoebophrya* sp. (Syndiniales) and *A. sanguinea* in JBTMS and the indoor-microcosm. mNGS results of eukaryotic (18S rDNA) communities in JBTMS and the microcosm are summarised in Supplementary Table 4 and Supplementary Table 5, respectively. In 2016, *Amoebophrya* sp. 1 trends were strongly associated with those of *A. sanguinea* bloom in the JBTMS (Fig. 5a). Moreover, dinospores of *Amoebophrya* sp. 1 changed similarly to *A. sanguinea* cells (Fig. 5a). After *A. sanguinea* disappeared, *Heterocapsa triquetra* emerged, and another OTU (*Amoebophrya* sp. 2) was detected (December 26th, Fig. 5a). Other Syndiniales seldom appeared during *A. sanguinea* bloom. In 2017, *Amoebophrya* sp. 1 was barely detected in seawater, but *Amoebophrya* sp. 2 increased rapidly when *H. triquetra* emerged (Fig. 5b). In the in-door microcosm, *Amoebophrya* sp. 1 (same sequences of *Amoebophrya* sp.1 as that in JBTMS) in seawater increased for 3 days, but rapidly decreased after 4 days and was barely detected after 6 days (Fig. 5c). Dinospores of *Amoebophrya* sp. 1 also showed similar patterns to those of *A. sanguinea*.

Network analysis during *A. sanguinea* bloom in 2016 and no-bloom in 2017

Network analyses of microbial communities were focused on *A. sanguinea* in 2016 and *B. prasinos* in 2017 and exhibited distinct associated interaction with specific microbial communities and environmental factors (Supplementary Tables 6-8, Fig. 6). The 2016 network had significantly correlated biological and environmental factors with 67 nodes and 577 edges (Fig. 6a). *A. sanguinea* association networks identified factors that were highly correlated to specific OTUs, such as bacteria (19 OTUs), NCLDV (6), and parasitic dinoflagellates (2), and environmental factors (5) (Fig. 6b). Our association network supports the paradigm that *A. sanguinea* bloom is regulated by both parasitic dinoflagellate (*Amoebophrya* sp. 1, $r=0.81$, and time-lag: 2 day) and NCLDV (Pandoravirus dulcis and Pandoravirus salinus, $r=0.64$ and 0.61 , 0 day delay time, respectively). In networks with bacterial communities, uncultured Alpha-proteobacterium (OUT #2) was highly and positively correlated (0.93) with 0 day time-lag, but other specific bacterial species in Gamma-proteobacteria and Beta-proteobacteria were negatively linked. In network with environmental factors, strong positive connectivity of DOC (0.86, 0 day) and DIP (0.98, 0 day) may reflect *A. sanguinea*-selective interactions.

The 2017 network showed biological and environmental factors with 80 nodes and 824 correlations (Fig. 6c), and this network was more diversely correlated than the 2016 network. Association networks of *B. prasinos* were correlated with 74 specific OTUs (11 phytoplankton OTUs, 6 ciliates, 2 parasitic dinoflagellates, 27 bacteria, and 28 NCLDV) and 9 environmental factors (Fig. 6d). In networks with bacterial communities, Alpha-proteobacteria (-0.78, 0 day), Gamma-proteobacteria (-0.60, 0 day), Beta-

proteobacteria (-0.76, 2 day), and Flavobacteriia (-0.63, 2 day) were negatively correlated at different time lags with *B. prasinos*, and Actinobacteria were only positively correlated (0.71) at 0 day time-lag. The *B. prasinos* network indicated connections of diverse microbial communities with interconnected niches. The network of *B. prasinos* was not correlated with *Amoebophrya* sp. 1 and mildly correlated with Pandoravirus dulcis, while *B. prasinos* was strongly and positively correlated with Cowpox virus OTU (0.86) at 0 day time lag, and this interaction may reflect *B. prasinos*-selective infection. Moreover, *B. prasinos* showed strong positive connectivity with *Strombidium* ciliate, which may reflect competition and feeding pressure. In the network with environmental factors, *B. prasinos* negatively correlated with DOC (-0.74, 2 day) and showed relatively low correlation with DIP (0.61, 2 day) as compared with the *A. sanguinea* network in 2016.

Discussion

HABs are a common ecological issue in coastal waters globally[1,3]. We show that *A. sanguinea* abundance initially increased by taking up DIN from the surrounding waters, and DIP and DOC concentrations strongly and immediately increased with the development of *A. sanguinea* bloom. This could be because of the dissolved carbohydrate (DCHO) being released by *A. sanguinea* cells. In marine systems, evidence for strong correlations between DCHO concentrations and phytoplankton biomass were found in oceanic surface waters[24,25,26]. DCHO production by marine phytoplankton depends on the species, growth stage, and environmental conditions[27,28,29]. Urbani et al.[30] reported biodegradability of DCHO released by *Thalassiosira pseudonana* and *Skeletonema costatum* (centric diatoms). Thus, *A. sanguinea* bloom markedly increases biological carbon export into the surrounding waters. *A. sanguinea* bloom is dominant worldwide in cold seasons[5,6]. In this study, *A. sanguinea* bloom rapidly decreased when water temperature was below 16 °C (Fig. 1). Our microcosm experiment results proved that *A. sanguinea* growth was inhibited at water temperature below approximately 15 °C. Matsubara et al.[31] reported that the temperature range for growth of *A. sanguinea* is very wide (10–30 °C), while Du et al.[6] mentioned that the temperature range for growth of *A. sanguinea* is very narrow (11–12 °C). Therefore, in our study, temperature below 16 °C could be a limiting factor for the growth of the bloom.

According to Yang et al.[5], bacterial abundance greatly increased in the *A. sanguinea* bloom area during a bloom in the Xiamen sea, and DO concentration dropped as a result of bacterial decomposition of *A. sanguinea*. However, bacterial abundance was not significantly associated with *A. sanguinea* bloom and did not affect DO levels in our study (Fig. 1). Nevertheless, certain bacterial communities were closely related to *A. sanguinea* bloom (Fig. 3, Supplementary Fig. 4). Specific bacteria, such as *P. marinivivus* and uncultured Alpha-proteobacterium (OUT #2) may have a symbiotic association in *A. sanguinea* bloom, while *A. ulvae*, *M. algae*, and *L. syltensis* may be inhibited in the HABs. Hence, it is important to elucidate the ecological role of specific bacteria associated with HABs[32,33]. Our results are in accordance with results from previous reports[34], which state that phytoplankton harbour (habitat “phycosphere”) specific bacterial communities. We found that bacterial species composition varied at different growth stages of *A. sanguinea* (Fig. 3). Antibacterial metabolites are produced by some phytoplankton[33], which possibly

inhibit certain bacterial species. These antibacterial substances are particularly associated with DCHO excreted from a phytoplankton cell[27]; thus, the physiological flexibility of bacteria may support their colonization. In previous studies, algicidal bacteria have shown a sudden increase in the presence of HABs[35]. Mayali and Azam[36] suggested that algicidal bacteria may affect HAB dynamics, as their abundance increases with the decline of algal blooms. In our study, the most common algicidal bacteria belonged to Gamma-proteobacteria and Bacteroidetes (Supplementary Table 9), and no sign of HAB control by algicidal bacteria was observed: *Alteromonas* and *Pseudoalteromonas* in Gamma-proteobacteria as well as *Saprospira* and *Cytophaga* in Bacteroidetes (i.e., common algicidal bacteria) had low abundance (or not detected), and their abundance did not increase after the decline of *A. sanguinea* bloom. Therefore, no specific bacteria in this study showed the potential to control the *A. sanguinea* bloom.

Our results revealed that Pandoraviruses were positively correlated to *A. sanguinea* bloom, indicating that Pandoraviruses can potentially infect *A. sanguinea*. For further studies, similar interactions between species-specific NCLDV and *A. sanguinea* blooms could be investigated. In the in-door microcosm, Pandoraviruses were closely related to *A. sanguinea* bloom based on their similarity with the pattern of Pandoraviruses in the field. Pandoraviruses are mainly known to infect amoebas[37], but sufficient studies have not been performed on different hosts; thus, there is no substantial evidence to determine whether Pandoraviruses have the potential to infect *A. sanguinea* or whether this behaviour is normal for the viruses in lower water temperature periods in any host. It has been previously proposed[38] that Pandoraviruses are highly evolved Phycodnaviruses[39]. Phycodnaviridae showed low relative abundance during *A. sanguinea* bloom, which increased after the bloom disappearance. Thus, our results revealed that the distribution of Pandoraviruses is closely related to the distribution of *A. sanguinea* bloom, which is the key to understanding the reason underlying termination of *A. sanguinea* bloom. Recently, unlike the “killing the winner hypothesis”[40], Silveira and Rohwer[41] founded the “piggyback-winner hypothesis”, which states that viruses potentially infect hosts, until they liquefy in time to the outside environment. Therefore, further study is required to identify specific infection mechanisms of Pandoraviruses against *A. sanguinea* and to estimate the ecological role of these viruses in nature.

An endoparasitic dinoflagellate, *Amoebophrya* sp. has the capacity to efficiently control populations of their dinoflagellate hosts, and infection, as this parasitoid spreads rapidly through dense dinoflagellate populations, facilitating the decline of the dinoflagellate bloom[42]. Many marine phycologists[9,19,20,43] have reported that *Amoebophrya* sp. can easily infect *A. sanguinea*. mNGS has revealed the enormous genetic diversity of *Amoebophrya*-like organisms within the marine alveolate group II[43,44]. In this study also, there were genetic divergences among several *Amoebophrya* spp.[i.e., *Amoebophrya* sp. 1 (OTU #24) and *Amoebophrya* sp. 2 (OTU #14)]. OTUs of *Amoebophrya* sp. 1 in sea water (free living *Amoebophrya* sp. 1), in *A. sanguinea* (infected dinospores of *Amoebophrya* sp.) in the JBTMS, and in the in-door microcosm verified the evident control of *A. sanguinea* blooms by *Amoebophrya* sp. 1 (Fig. 5). This can be explained by the short generation time of *Amoebophrya*[20]. The ecological role of host-specific *Amoebophrya* infection might have a greater impact on the population dynamics of toxic bloom-forming dinoflagellates than by the microzooplankton grazing (Supplementary Fig. 6); *Amoebophrya* can

potentially eliminate an entire host population within a few days[45]. This study has revealed a natural phenomenon wherein an endoparasitic dinoflagellate controls their host. However, the study did not consider *Amoebophrya* sp. killing mechanisms. Thus, further experiments are needed on host-parasitoid interactions to estimate the ecological role of *Amoebophrya*, and to study how various *Amoebophrya* species coexist in a nature.

The network analysis revealed that the *A. sanguinea* bloom in 2016 was associated with specific microbial communities and some environmental factors and showed differentiation of network results of 2017. A major strength of this study is the high-resolution sampling approach. Day-to-day sampling of an *A. sanguinea* bloom spanning is a great resource for understanding the fine scale dynamics of the life-cycle of the bloom. In 2016, connected partners with *A. sanguinea* blooms included taxa (OTUs) from all trophic positions (i.e., infections, parasites, phototrophs, and heterotrophs). Environmental factors, such as water temperature, salinity, DO, DIP, and DOC can immediately respond to *A. sanguinea* bloom (0-day time-lag). Thus, network analyses showed changes in DIP and DOC concentration derived from DCHO released by *A. sanguinea* bloom. Moreover, specific NCLDV (including Pandoraviruses) and endoparasitic dinoflagellate (*Amoebophrya* sp. 1) with positive associations have been found to regulate *A. sanguinea* bloom. In previous studies *A. sanguinea* bloom has been negatively associated with competition, niche partitioning, and grazing[46,47]. Thus, the high connectivity displayed by some microbial communities and environmental factors with *A. sanguinea* bloom suggest similarity in their ecological properties (symbiosis and inhibition as well as infection etc.). Our study shows the value of frequent sampling to evaluate community responses and microbial interactions among protists by reinforcing recent ideas about rapid dynamics and the importance of parasites.

On the basis of these results, we propose three stages of interactions between environmental characteristics and microbial communities in *A. sanguinea* bloom via a conceptual diagram (Fig. 7): (i) “before *A. sanguinea* bloom”: diatoms and Alpha-proteobacteria were common phytoplankton and bacterial groups, respectively. Low abundance of *Amoebophrya* sp. 1 was observed in this stage. The predominant NCLDV group was Poxviridae. In environmental characteristics, most parameters (particularly DIN) were detected to be higher than those in the other stages; (ii) “during *A. sanguinea* bloom”: *A. sanguinea* bloom showed marked changes in the environmental characteristics (which were exported into the surrounding waters), followed by changes involving species-specific virus, bacteria, and parasitoids. *A. sanguinea* abundance initially increased by taking up DIN and DIP from the surrounding waters, but changes in DIP and DOC concentrations were strongly positive correlated with changes in HABs. *A. sanguinea* bloom harboured and promoted specific bacterial populations. In particular, host-specific bacterial group (Flavobacteriia increased rapidly) that remineralise extracellular products from *A. sanguinea* participates in biogeochemical cycling and play an important part in the dynamics of microbial communities. Specific NCLDVs, Pandoraviridae, increased following an increase in *A. sanguinea* abundance, particularly during bloom peaks. Endoparasitic dinoflagellate, *Amoebophrya* sp. 1, has attracted attention regarding its roles in trophic interactions; (iii) “After *A. sanguinea* bloom”: when *A. sanguinea* bloom was terminated, water temperature was below 16 °C, and most environmental characteristics showed little changes. Succession of common phytoplankton groups occurred from *A.*

sanguinea to diatoms (*Pseudo-nitzschia delicatissima* and *Skeletonema marinoi-dorhnii* complex species) and *Heterocapsa triquetra* (dinoflagellate). Variations in bacteria and NCLDV groups were observed, such as relative increase in Gamma-proteobacteria and Phycodnaviridae, respectively. *Amoebophrya* sp. 1 rapidly decreased with the termination of *A. sanguinea* bloom.

Conclusions

In the present study, the following new evidence to support the interaction between environmental characteristics and microbial communities in *A. sanguinea* bloom was obtained: (i) DIP and DOC concentrations rapidly and strongly shifted with *A. sanguinea* bloom; (ii) a specific bacterial community associated with *A. sanguinea* bloom showed ecological symbiosis, inhibitory role; they might provide a suitable environment for a diverse subset of bacteria from a “Phycosphere” perspective; (iii) Pandoraviridae may have an important role in controlling *A. sanguinea* population, despite being known to infect mainly amoeba; (iv) *Amoebophrya* sp. may control *A. sanguinea* bloom dynamics with low water temperature, as the increase in its abundance is correlated with HAB decline. Consequently, microbial communities and the environment dynamically and complexly changed in *A. sanguinea* bloom, and a rapid turnover of microorganisms could respond to ecological interactions. Microbial communities in HAB ecology are composed of various organisms and they interact in a complex way. Therefore, to interpret their ecosystem, the complex reactions among various microorganisms should be studied rather than studying a simple 1:1 reaction, such as a prey-predator interaction.

Methods

Sample collection

The sampling site (Jangmok Bay Time-series Monitoring Site, JBTMS: 34° 59′ 37″ N and 128° 40′ 27″ E) is a semi-closed bay on the southern coast of South Korea (Supplementary Fig. 7). The JBTMS is a eutrophic system that is subjected to strong mixing between the surface and bottom layers. Its maximum tidal range is approximately 2.2 m, and the mean water depth at the sampling station is approximately 8.5 m. A total of 90 sub-samples during June 2016 and June 2017 were obtained from surface water (sampling depth: 1 m under sea surface). In particular, when *A. sanguinea* bloomed (November 11th to December 13th 2016), sampling was performed daily. We also explored the differences between *A. sanguinea* bloom and no-bloom condition daily between November 14th to December 13th 2017. We drew 20-L samples of seawater from the surface layer and stored them in a cooler until arrival (5 min) at the laboratory of the South Sea Institute of Korea Institute of Ocean Science & Technology (KIOST, Geoje, South Korea), where the seawater was prepared immediately.

Monitoring of environmental factors

Temperature, salinity, pH, and dissolved oxygen were evaluated using a portable YSI environmental multi-parameter (YSI 6920 Inc., Yellow Springs, OH, USA). A 100-mL aliquot of each sub-sample was filtered

through a 47-mm glass fibre filter (GF/F, Whatman, Clifton, NJ, USA), and the filtered seawater was stored in an acid-cleaned polyethylene bottle in a deep freezer (at -80 °C). Subsequently, concentrations of dissolved inorganic nutrients, such as inorganic nitrogen (DIN; $\text{NO}_2^- + \text{NO}_3^- + \text{NH}_4^+$), inorganic phosphorus (DIP), and silica (DSi) were determined in each sample using an automatic nutrient analyser (Quaatro39; Seal analytical instrument, UK). To analyse the dissolved organic carbon (DOC) concentration, a 10-mL aliquot of each water sample was filtered through the GF/F filter (pre-combusted at 450 °C overnight) under gravity pressure, and the DOC concentration was determined using a high-temperature catalytic combustion instrument (TOC-V_{CPH}; Shimadzu, Kyoto, Japan). To determine chlorophyll *a* concentration, 500 mL of each sample was filtered through the GF/F filter under low vacuum pressure. Each filter was soaked in 15 mL of cold 90 % acetone-distilled water (v/v) and sonicated to break the cell walls. Then, chlorophyll *a* was extracted for 24 h at 4 °C in the dark, and its concentration was measured using a fluorometer (10-AU; Turner Designs, Inc., San Jose, CA, USA).

Microscopic observation

To count total heterotrophic bacteria, a 10-mL aliquot was collected from each sub-sample in a 15-mL sterilised polyethylene bottle and fixed immediately with glutaraldehyde (at a final concentration of 2 %). The sample was stored in the dark at 4 °C prior to analysis. The fixed bacterial cells were filtered through a black isopore membrane filter (GTBP 02500; Millipore, Bedford, MA, USA) and stained with 1 $\mu\text{g mL}^{-1}$ of 4',6-diamidino-2-phenylindole solution[48]. At least 600 stained bacterial cells per sample were counted using an epifluorescence microscope (Axioplan, Zeiss, Oberkochen, Germany) at a magnification of 1,000×. To count and identify phytoplanktonic communities, at least 500 phytoplankton cells per sub-sample were identified and counted using a phytoplankton (or Sedgwick–Rafter) counting chamber under a light microscope (Axioplan) at a magnification of 400–1,000×.

Preparation for DNA extraction of microbial communities

The microbial communities are multi-phylogroup communities, ranging from the numerically dominant viruses to the phylogenetically diverse eukaryotic plankton. For mNGS, Flaviani et al.[22] concluded that 250 mL of seawater is sufficient to analyse microbial diversity (from double-stranded DNA virome to phytoplankton). Therefore, we analysed NCLDV, bacteria, and eukaryotic planktonic organisms, including the endoparasitic dinoflagellate *Amoebophrya* spp., from 1 L surface seawater. Moreover, for analyses of various microbial communities, we harvested the microbes in three steps according to their size fraction; 1), a 10 μm polycarbonate filter (TCTP04700, Millipore) was used, which focused on eukaryotic plankton and dinospores of *Amoebophrya* sp. in *A. sanguinea* cells at cell size of > 10 μm . To remove organisms < 10 μm in size and particles attached to *A. sanguinea* cell surfaces, cells collected on the 10 μm filter were washed three times with approximately 50 mL distilled water at approximately 50-60 °C. 2), a 3 μm polycarbonate filter (TSTP04700) was used, which focused on free-living *Amoebophrya* spp. and nano-sized phytoplankton at cell size of 10-3 μm , and 3), a 0.22 μm polycarbonate filter (GTTP04700) was used, which focused on bacteria and NCLDVs at cell size of 3-0.22 μm . The filters were stored at -80 °C until DNA extraction.

mNGS analyses of bacteria and eukaryotic plankton

The filters at each size fraction were cut into several pieces for genomic DNA (gDNA) extraction. To analyse the bacterial community in the water samples, gDNA was extracted using the DNeasy Powersoil Kit (Qiagen, Valencia, CA, USA) from the 3–0.22 μm size fraction; the DNA was diluted to a final concentration of 20 $\text{ng } \mu\text{L}^{-1}$. The quantity and quality of the total gDNA was determined using Nano-drop (Nano-MD-NS, SCINCO Ltd., South Korea). The V3-V4 hyper variable regions of bacterial 16S rDNA genes were amplified using the universal Illumina tagged forward (341F) and reverse (800R) primers (Supplementary Table 10). To analyse eukaryotic plankton, gDNA was extracted from the > 10 μm and 10–3 μm size fractions using a DNeasy PowerSoil kit; DNA was diluted to each final concentration of 30 $\text{ng } \mu\text{L}^{-1}$ and 20 $\text{ng } \mu\text{L}^{-1}$, respectively. The V4-V5 region of the 18S rDNA gene was targeted using the Illumina tagged forward (TAREuk454FWD1) and reverse (TAREukREV3) primers (Supplementary Table 11). Although we did not perform replicate experiments, we attempted to overcome the experimental bias and obtain more accurate results as follows; 1), intensive daily continuous monitoring in JBTMS, 2), indoor microcosm experiments to verify changes in endoparasitic dinoflagellates and NCLDVs in the field, and 3) three PCRs performed in distinct tubes, and then mixing of the PCR reaction products to obtain more accurate mNGS results[49]. The amplified products from the first PCR were individually purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany). The second PCR lasted 10 cycles using tags of Nextera XT 96 index kit v2 (Illumina). DNA concentration was measured in a Bio-analyzer 2100 (Agilent Technologies, Palo Alto, CA, USA). Equal concentrations of the PCR products for each sample were pooled, and the merged samples were analysed using a Mi-Seq platform (Illumina, San Diego, CA, USA).

After each sequencing procedure was completed, the data were pre-processed using Mi-Seq Control Software (MCS) v2.4.1. Raw sequences were first analysed using FastQC[50] to check basic statistics, such as GC %. Furthermore, quality score distribution per base, and poor-quality sequences were flagged. Additionally, ambiguous and chimeric reads were removed, and the noised sequences (denoising), which involved OTUs with 1, 2, and 3 reads, were removed at a cut-off of 97%. The processed pair-end reads were then merged using the fast length adjustment of short reads (FLASH) software tool[51]. After each sequencing procedure, a quality check was performed to remove short sequence reads (<150 bp), low-quality sequences (score < 25 in analysis of 16s rDNA; score < 33 in analysis of 18s rDNA), singletons, and non-target sequences. Using Basic Local Alignment Search Tool (BLAST)[52], all the sequence reads were compared with those from the National Center for Biotechnology Information (NCBI) database. Sequence reads with an E-value smaller than 0.01 were considered for further analysis. A pairwise global alignment was performed on the selected candidate hits to identify the most similar sequences. The taxonomy of the sequence with the highest similarity was assigned to the sequence read (species or genus levels with >97 % or >94% similarity, respectively). To analyse operational taxonomic units (OTUs), the CD-HIT-OTU software[53] was used for clustering and metagenomic functional information. To calculate alpha-diversity, including Shannon-Weaver diversity, Chao richness, and Simpson evenness, we used the closed-reference protocol published by Mothur[54] and QIIME[55] based on the OTU table.

mNGS analysis of Nucleo Cytoplasmic Large DNA Viruses

To analyse NCLDV, gDNA was used of extracted it for analysis of bacterial metagenomics, and sequencing library of NCLDV was generated using NEBNext Ultra DNA Library Prep Kit (Illumina, San Diego, USA) following the manufacturer's instructions. The library was prepared by random fragmentation of the DNA sample, followed by 5' and 3' adapter ligation. "Tagmentation", which combines the fragmentation and ligation reactions into a single step and greatly increases the efficiency of library preparation, was used. Adapter-ligated fragments were then amplified using 12 PCR cycles and purified by gel electrophoresis and a gel extraction kit (Qiagen, Hilden, Germany). Libraries were analysed for size distribution by using a Bio-analyser 2100 model (Agilent Technologies, Palo Alto, CA, USA), which indicated that the final library contained inserts between 35 and 1,000 base pairs[56]. Clustering of the index-coded samples was performed on a cBot Cluster Generation System according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina Hi-Seq 2500 platform (Illumina, San Diego, CA, USA).

FASTAQ files were imported into the CLC Genomics Workbench v. 11.0.1 (Qiagen, Hilden, Germany). Reads below 0.05 quality score cut-off and adapter trimming were removed from subsequent analyses. The remaining reads were trimmed of any ambiguous and low quality 5' bases, and only reads at the full length were retained for assembly. Quality controlled reads were then *de novo* assembled using the assemble function (minimum >300 bp) of the CLC Genomics Workbench for ambiguous nucleotide trimming and bad reads filtered at the limit of Q20. To identify viruses, the assembled contigs were subjected to a BLAST search against viral reference genome sequences, using the NCBI virus genome database (<http://www.ncbi.nlm.nih.gov/genome/viruses/>) for taxonomic assignment using BLASTX (E-value < 10^{-5}).

Indoor microcosm experiment

To investigate the changes in environmental factors, and the potential biological control mechanisms of *A. sanguinea* bloom, we conducted 100-L in-door microcosm experiments (Supplementary Fig. 8)[23]. The experiment included three enclosures (triplicate experiments), each containing 80 L of seawater when natural *A. sanguinea* bloomed at a mean density of 920 cells mL⁻¹. Each microcosm was a 100 L enclosure within a cylinder of transparent acrylic material. To observe the survival and growth of *A. sanguinea* cells in terms of changes in the water temperature, the room temperature was maintained at an average of 18 °C for the first three days and sustained at 15 °C for the last period of the experiment. Light intensity was 50 E m⁻² s⁻¹ under a 16 h : 8 h (light : dark) cycle. Water in each microcosm was mixed by three impellers (each 15 cm long and 6 cm wide) at a speed of 10 rpm on a 15 min : 15 min automatically controlled run : stop cycle. The experiment lasted 22 days. During this time, we collected 17 sub-samples (daily collection for 12 days and bi-daily collection the last 10 days) for analysis of environmental factors and microbial communities.

Statistical interpretation of the data

Pearson's correlation analysis was performed to examine the relationships between the measured parameters using SPSS v.12 (SAS Institute Inc., Cary, NC, USA). Cluster analysis was performed using group average clustering by the Bray-Curtis similarity method on the most abundant OTUs of bacteria and NCLDV (over 1 % in at least one sample). Using the ranked similarity matrix, an ordination plot was produced by non-metric multidimensional scaling (nMDS) using PRIMER 6 (version 6.1.13). Hierarchical agglomerative clustering using the group average method was carried out on the most abundant OTUs based on groups selected from nMDS analysis. To test the null hypothesis (no significant difference between the groups discriminated according to the agglomerative clustering analysis), similarities were analysed with ANOSIM in PRIMER[57].

Extended local similarity Analysis (eLSA)[58] was used for data of 2016 and 2017 with 33 and 29 days, respectively, (a time interval of two days) to analyse covariation between the most abundant OTUs, each displaying a relative abundance >1 % in at least one sample, resulting in 63 and 76 OTUs of microbial communities and 9 environmental parameters each in 2016 and 2017, respectively. P-value was determined using statistical approximation followed by permutation testing to reduce computing time, and ensuring accuracy and Q-value (false discovery rate) was calculated to estimate the likelihood of false positives[59]. eLSA network of delay-shifted Spearman correlation coefficients (SCC) between variables was visualized using Cytoscape v3.7.2[60] with $P < 0.01$ and $Q < 0.05$. Because the sampling was evenly spaced for two-day intervals, maximum time-lags were considered to be 10 days. The networks were selected by *A. sanguinea* identification or edge type (or example, correlations between specific OTUs). Random undirected networks of equal size by number of nodes and edges were calculated by the Erdős-Rényi model using the Random Network plugin in Cytoscape. Network statistics were calculated with the network analyzer as undirected networks using the defaults[61].

Declarations

Availability of data and materials

The raw sequencing data (Fastaq files) of 16s rDNA and 18s rDNA genes obtained from the Mi-Seq platform were deposited in the Sequence Read Archive database at NCBI under accession numbers: SRR11123089-11123163 (PRJNA 607609), SRR11136919-11136959 (PRJNA 607810), and SRR11131500-11131531 (PRJNA 607814). The raw sequencing files of NCLDV (over 1 % in at least one sample) obtained from the Hi-Seq platform were deposited in the Sequence Read Archive database at NCBI under accession number SRR11172603-11172644 (PRJNA 608210).

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Author Information

Contributions

Jung S.W., Park P., and Lee T.K. designed the research plan; Jung S.W., Park J., Kang J., Kim H.-J., Joo H.M., Seo H., Kim S., and Jang M.C. performed the experiments; Jung S.W., Park J., Kang J., Joo H.M., Kang D., Jang M.C., Lee K., Oh S.J., Lee S., and Lee T.K. discussed the results; Jung S.W., Park J., Kang J., Kim H.-J., Joo H.M., Kang D., Lee K., Oh S.J., and Lee T.K. wrote the paper.

Ethics declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

There are no conflicts of interests to declare.

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Figures

Figure 1

Daily changes in environmental factors before, during, and after *Akashiwo sanguinea* bloom periods in 2016. a, water temperature; b, salinity; c, pH; d, dissolved oxygen; e, dissolved inorganic nitrogen; f, dissolved silica; g, dissolved inorganic phosphorus; h, dissolved organic carbon; i, chlorophyll *a*; j, total bacterial abundance. Coloured areas in the figure correspond to the common phytoplankton groups in 2016. *r* value in each figure (upper right) indicates Pearson correlation coefficient between each environmental factor and *A. sanguinea* abundance.

Figure 2

Non-metric multidimensional scaling (nMDS) plot by the Bray–Curtis dissimilarity method of bacterial community operational taxonomic units (OTUs) in 2016 (a, similarity: 73 %) and 2017 (b, similarity: 70 %). nMDS plot of Nucleo Cytoplasmic Large DNA Viruses (NCLDVs) OTUs in 2016 (c, similarity: 79 %) and 2017 (d, similarity: 80 %). The coloured areas indicate the statistically supported groups by nMDS analysis, to facilitate the rapid identification of samples. The pie chart plots indicate high-ranking taxonomy distribution of the class level of bacteria community and family level of NCLDVs.

Figure 3

Time series circle plot showing the most abundant bacterial operational taxonomic units (OTUs) (each displaying a relative abundance >1 % in at least one sample) in 2016 and 2017. The colours in circle plots correspond to the common bacterial groups. The coloured areas correspond to the common phytoplankton groups in 2016 and 2017. To show the differences in relative abundance for the displayed OTUs, the circle is on a 0–100 scale representing relative abundance (%).

Figure 4

Daily changes in the most abundant Nucleo-Cytoplasmic Large DNA Viruses (NCLDVs) at family level (displaying a relative mean abundance >1 %) during *Akashiwo sanguinea* bloom in 2016 (a), pico-sized green alga dominance, *Bathycoccus prasinos* (no *A. sanguinea* bloom), in 2017 (b) and in-door microcosm experiment. The coloured areas in the figure 4a and 4b correspond to the common phytoplankton groups in 2016 and 2017, respectively. The red-coloured bar in the figure 4c is represented

experiment (c). *Amoebophrya* spp. are mostly divided in *Amoebophrya* sp. 1, sp. 2, and other Syndiniales species. The coloured areas in the figure 4a and 4b correspond to the common phytoplankton groups in 2016 and 2017, respectively. The red-coloured bar in the figure 4c is represented as a mean value (triplicate experiments) of *A. sanguinea* abundance and error bar indicates standard deviation.

Figure 6

Network analysis derived from the most abundant operational taxonomic units (OTUs) and environmental factors showing only statistically significant correlations between OTUs and environmental factors (Spearman correlation coefficient ≥ 0.5 ; $p < 0.01$; false discovery $q < 0.05$) in 2016 (a), sub-network associated with *Akashiwo sanguinea* (b) and in 2017 (c), and sub-network associated with *Bathycoccus prasinus* (d).

Figure 7

Conceptual schematic diagram of the changes in environmental factors and specific microbial communities in phycosphere of *Akashiwo sanguinea* (Dinophyta) blooms. Three stages of interactions between environmental factors and microbial communities in *A. sanguinea* bloom. The first step is “before *A. sanguinea* bloom”, the second is “during *A. sanguinea* bloom”, and the last is “after *A. sanguinea* bloom”. Each step is introduced under different ecosystem scenarios. In particular, key

changes in specific environmental factors and microbial communities in *A. sanguinea* bloom are introduced in the second step.

Supplementary Files

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