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Learning from mistakes: challenges in finding holobiont factors from environmental samples and the importance of methodological consistency

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The cause of harmful algal blooms has been a mystery, but research to elucidate its mechanism has progressed over the years thanks to genetic technologies. We have monitored toxic algae and its associated bacteria as a community, the so-called 'holobiont' in Chilean coastal waters for years from the perspective of bacteria as an algal bloom driver. This review describes the challenges of holobiont monitoring, specifically with respect to standardizing and compliance with the monitoring protocols to collect reliable and sustainable data. Further, we suggest adopting the high-throughput sequencing (HTS) standard operating procedure (SOP) by the International Human Microbiome to improve the quality and consistency of holobiont monitoring in the harmful algal world.

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Harmful algal blooms

Harmful algal blooms (HABs), commonly known as red tides, are a phenomenon of phytoplankton overgrowth observed on the coast of every continent [1]. Some phytoplankton release endogenous toxins that cause fatal paralysis, diarrhea, and neurotoxicity syndrome in shellfish, fish, birds, and mammals [1–3]. Even phytoplankton that do not produce toxins can damage the marine environment by increasing biomass, causing hypoxia/anoxia, and altering native species [4]. These negative events caused by HABs lead to coastal closures, suspension of the tourism and recreation industries, and further economic damages. During the 2015/2016 west coast Dungeness crab season in the United States, fisheries were restricted for five months because of a high domoic acid concentration in crabs from a *Pseudo-nitzschia australis* bloom. The direct revenue loss by this event was estimated to be US$26.1 million, which did not include damages to other fisheries and aquaculture industries, the cost of treating associated illnesses, and loss of tourism [5,6].

The earliest-recorded human fatality by a HAB occurred in Poison Cove, British Columbia in 1793 when Captain George Vancouver and his crew ate local shellfish, and one person was allegedly killed by what is now referred to as paralytic shellfish poisoning [7]. Since then, HABs have been reported worldwide, and new HAB species are constantly being discovered [8,9]. For example, 22 species of toxic dinoflagellates were recognized in 1984, which increased to 59 species a decade later [10,11]. Improved tools and advanced sampling and enumeration technologies for monitoring HABs, such as Imaging FlowCytobot and FlowCam, contributed to the increased detection of HAB species. These instruments use a combination of flow cytometric and video technologies and can capture high-resolution images of hundreds of suspended phytoplankton in real time. Yet, it is apparent that HAB frequency has increased in the last few decades. Nevertheless, the complete mechanism of HAB is not fully understood as of today and increasing coastal monitoring and research have been implemented to develop successful models and adequate means to predict and mitigate HAB occurrences.

Causes of algal blooms

It is difficult to elucidate the causes of HABs because HABs are not caused by a single factor but a combination of multiple factors originating from human activity and natural events [12]. The changes in physicochemical factors (i.e. salinity, water temperature, oxygen content, nitrate, and silicate) are often discussed as HAB drivers.

Among all, global warming seems to be the most influential HAB factor because climate regulates water temperature, nutrients, and light conditions, and global warming presumably provides warmer water with higher nutrients that are optimal conditions for algal growth [13–16]. Other well-discussed HAB factors include nutrient loading on the marine environment from agriculture and aquaculture practices [17,18]. The usage of synthetic fertilizers since the beginning of the industrial era has caused more phosphorus, nitrates, and other nutrients (e.g. potassium) to flow into coastal areas, and the correlation between increased fertilizer use and increased HAB occurrences has been reported [17,19]. For instance, the increased fertilizer uses in China from 5×10^6 tons in 1970 to 70×10^6 tons in 2000 coincided with the increased HAB occurrence in Chinese coastal waters from a few in 1970–80 in 2000 [17]. HAB studies are further complicated because the causes of HABs vary among regions based on HAB species-specific conditions, geographic conditions and physicochemical oceanographic parameters unique to each region [1,19]. Therefore, it is necessary to separately investigate each affected area to develop strategies to protect the ecosystem from HAB-induced damages.

Symbiotic relationships between many algae–bacteria pairs have been recently reported, in which they exchange nutrients such as vitamins and carbon sources. Therefore, in the HAB world, it is becoming an attractive topic that certain bacteria may control paring phytoplankton growth and its blooms. Bell and Mitchell introduced the idea that phytoplankton-rich areas were surrounded by organic mucus called 'phycosphere' formed from the extracellular waste of phytoplankton, and microbial activities were altered in the phycosphere; this indicated that bacteria habitat fed on these nutrients [20]. Since then, research has gradually expanded to understand the role of bacteria as a HAB driver. Numerous studies suggested that there are specific interactions between phytoplankton and bacteria, and their mutualistic association is promoted by nutrient exchange [21–23].

Holobionts

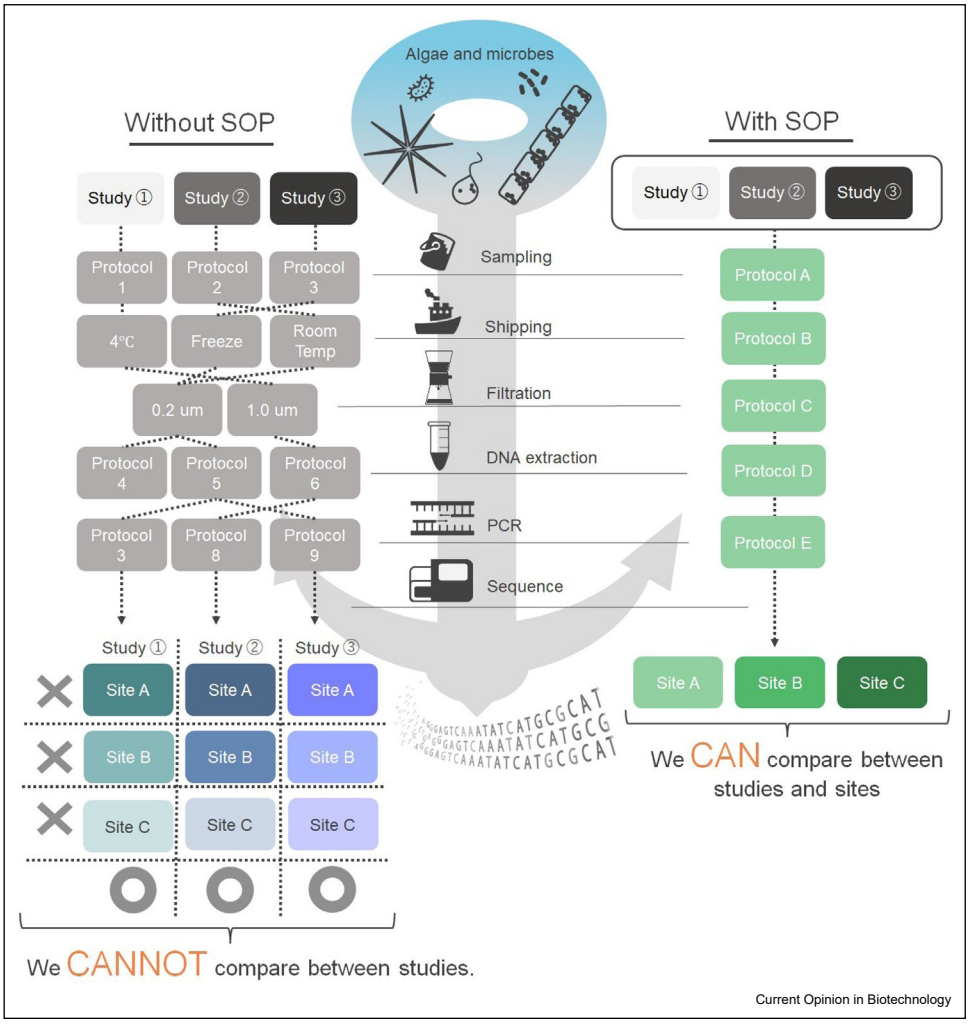
Community analysis of holobionts is based on the interaction of mutualistic species and environmental conditions using molecular biology and modeling techniques. Development of omics technologies and advances in statistical methods have facilitated the use of holobionts for HAB research in the last decade [24,25]. In addition to the traditional microscopic HAB species identification and toxin assays, an increasing number of HAB programs have implemented holobiomics using nucleic acid analyses such as massive parallel sequencing and metabarcoding analysis [26–28]. These technologies have helped HAB researchers understand the species of so-called holobionts involved in the target algal community. Consequently, increasing information obtained by holobiomics on marine bacteria as a possible HAB driver is being reported every year [29–32]. However, the available holobiont information from HAB monitoring has been obtained using different methodologies; thus, it is difficult to determine whether the reported data are comparable (Figure 1).

Coastal monitoring for harmful algal blooms and holobiont identification by high-throughput sequencing

High-throughput DNA sequencing, often called high-throughput sequencing (HTS), is a powerful and revolutionary tool to study HAB dynamics from the perspective of bacterial communities. It provides, in a single experiment, millions of sequence reads that correspond to hundreds of operational taxonomic units/amplicon sequence variants of bacteria and eukaryotes from each seawater sample, even when the population is complex and its density is low [33]. Additionally, coastal monitoring using HTS can provide precise taxonomic identification at the species level based on genetic information, even for species that cannot be distinguished by conventional methods [33] or species that are rare (less abundant and/or unknown).

Coastal monitoring of HABs and holobionts using HTS comprises six stages: sampling, filtration, DNA extraction, sequencing, bioinformatic analysis, and statistics and visualization. The obtained data can also be integrated with environmental and climatic information. By comparing several available coastal monitoring datasets for HABs, it was apparent that each report used a different method at every stage (Table 1). Such inconsistent methodologies at each stage lead to the inability to compare results between studies and sometimes to erroneous conclusions [34]. Comparison of studies listed in Table 1 revealed that the methodological variability among research groups was relatively small for the later stages of the HTS-based coastal monitoring process: bioinformatics and statistics. Moreover, even if a different method is used at these stages, they are 'reversible,' which means that reanalyzing the data using another method is possible, although cumbersome. However, the methodologies used in the earlier stages, which include sampling, filtration, and DNA extraction, have higher variability among research groups. Furthermore, once samples are processed at these stages, they are 'non-reversible,' which means that it is impossible to start over by collecting the same samples from the same place on the same date. These nonreversible stages can result in incomparable datasets among studies obtained by different methodologies, although it should be emphasized that each dataset is still informative. The coastal monitoring of HABs and associated holobionts

Figure 1



Coastal monitoring of HAB and holobionts with HTS that consists of six stages: sampling, filtration, DNA extraction, sequencing, bioinformatic analysis, and statistical analysis.

with HTS become more meaningful when a thoroughly standardized protocol, which mainly focuses on the beginning stages, is used among research groups.

Standardization of coastal monitoring protocols

In recent years, HABs in Chile have caused severe pollution in the marine environment and shellfish and salmon industries [34,46–48]. Fish and shellfish production is a pillar that supports the Chilean economy because they are exported throughout the world, including to the United States, Europe, and Japan [49,50]. Therefore, the frequent HABs have significantly impacted the Chilean economy. We have monitored 14 stations along the 4300 km of Chilean coast from north and south since 2019 under the MACH program (https://www.mach-satreps.org/en/). This program aims not only to construct baseline coast water information such as phytoplankton count, water temperature, salinity, oxygen concentration, and nutrient concentrations but also to investigate the cause of HABs from an algal–bacterial interaction perspective using molecular biological techniques.

Aware of the above-listed issues that could arise during the coastal monitoring of HABs and holobionts using HTS, we created a standard operating procedure (SOP) before starting the program [35,36]. The SOP provides detailed guidance for each stage, from sampling to statistical analysis. For example, it states that the filtration must be performed from 1-L seawater and completed within 12 h from sampling, noting that less water volume can be filtered if the water is dense, and recording the filtered volume is required to normalize the results. Such

Table 1

Comparison of coastal monitoring methodologies.

STEP 1: Sampling				
Substep	Factor	Advantage	Limitation	Ref.
Depth	0–2300 m	Can compare vertical columns	Need a special device to access depth	[26]
	6 m	Can collect data from subsurface	Need a boat/ship	[30]
	2 m	Easy access	Only surface water data collection	[31]
	0–10 m	Can collect data from subsurface	Need a boat/ship	[35]
Collection method	Bleach-cleaned bucket connected to hose	Easy to prepare	Good only for surface to subsurface	[27]
	Hose	Can collect water from surface to ~50 m	Time-consuming cleaning to avoid contamination	[35
	Niskin bottle	Easy to prepare	Expensive	[29,31]
	Sterile screw-capped bottle	Easy to prepare	Good only for surface water	[30]
Sample transfer	Freezer	May maintain species composition	Damages cells by freeze–thaw; requires freezer	[36,37]
	4°C	May maintain species composition and diversity	Requires refrigerator	
	Ambient	Easy maintenance	Good only for a short time	
STEP 2: Filtration				
Substep	Factor	Advantage	Limitation	Ref.
Volume	100 mL and 500 mL	Easy to operate	Sample is selective	[31]
	1 L	Easy to operate	May take time to filter	[27]
	2 L	Easy to operate	May take time to filter	[29]
	75 000 L	Can collect information over a large area	Labor-intensive; requires a device	[28]
Time from sampling	Within 12 h	Fresh sample	N/A	[35]
	Most literature did not state	N/A	Sample quality is not guaranteed	
Pore size	0.22 μm	Captures most microorganisms	Free-living and attached particles are not separated	[31]
	0.45 μm	Captures bacteria or larger microorganisms	Does not capture free-living particles; no community separation	[27]
	Tandem 0.22 μm, 1 μm	Separates two communities	No free-living particle separation	[35]
	Tandem 200 μm, 0.2 μm	Separates two communities	Does not capture free-living particles	[26]
	Tandem 200 μm, 65 μm, 0.2 μm	Separates three communities	Does not capture free-living particles	[28]
	Tandem 3 μm, 0.2 μm	Separates two communities	Does not capture free-living particles	[29]
	Tandem 1.6 μm, 0.7 μm	Separates two communities	Does not capture free-living particles	[31]
Filter material	Polycarbonate	Pore size is well defined; suitable for fluorescence microscopy counts	Expansive; clogs easily	[26]
	Cellulose acetate	Low cost, hard to clog, low protein adhesion	Not suitable for fluorescence microscopy because of its thickness	[27]
	GF/F	Hard to clog, DNA adsorption	Not suitable for fluorescence microscopy because of its thickness	[31]
Filter storage	Liquid nitrogen and −80℃ freezer	Long-term preservation	Requires expensive facilities or equipment for preservation	[26,29-,31]
	−20℃ freezer	Relatively inexpensive	Not suitable for long-term storage	[28,35]
	Longmire buffer	No special facilities or equipment required for preservation	Sample use is limited for certain analysis purposes	[27]
STEP 3: DNA extraction				
Substep	Factor	Advantage	Limitation	Ref.
Extraction	Chelex buffer and boil	Easy, low cost	May not work for some DNA	[35]
	Alkali buffer and boil	Easy, low cost	May not work for some DNA	
	Masher	Easy	Need masher and sample volume	
	Bead beating	Easy	Need beads-beater	
	Phenol:chloroform:isoamyl alcohol	Traditional method; can be compared to prior studies	Labor-intensive, no quality assurance, use hazardous chemicals	[27]
	HP Plant DNA Kit (Omega)	Quality-controlled reagent, standardized procedure	Not available if discontinued, may not work for some DNA	[26]

Table 1 (*continued*)

	PowerWater DNA Isolation Kit (Mobio)	Quality-controlled reagent, standardized procedure	Not available if discontinued, may not work for some DNA	[29]
	PowerSoil DNA isolation Kit (Qiagen)	Quality-controlled reagent, standardized procedure	Not available if discontinued, may not work for some DNA	[28,32]
	GFX genomic DNA purification kit (Amersham Bioscience)	Quality-controlled reagent; standardized procedure	Not available if discontinued, may not work for some DNA	[30]
STEP 4: Sequencing				
Substep	Considerations			Ref.
Amplicon target	[*Eukaryotes*] • In general, it is recommended to study both V4 and V9 regions of 18S rRNA gene, but the research group found that only V9 is sufficient. • If you want algae-focused community structure data, ITS and COI are also available [*Prokaryotes*] 16S rRNA gene V1–V3, V1–V9, V3–V4, V4, and V4–V5 regions *Recommendation*: Eukaryotes: 18S rRNA gene V9 region because of the primer universality and its extensive database Prokaryotes: 16S rRNA gene V3–V4 region because of its extensive database			[38,39]
PCR	• DNA polymerase depends on the type and quality of the template DNA sample and amplicon length • Works without an inhibitory effect of the template DNA sample • Always prepare a negative control with sterile water in place of template DNA sample, PCR, and electrophoresis to make sure the negative control is not amplified • The higher the number of cycles, the greater the bias; minimize the number of cycles as much as possible *Recommendation*: DNA polymerase for amplifying even low-purity DNA samples: MightyAmp DNA Polymerase Ver. 3 (TAKARA); KAPA3G Plant PCR Kit (Kapa Biosystems) Amount of template DNA: 5–50 ng PCR cycles: ≦25			[40]
Sequence	• Popular HTS approaches are NovaSeq, Next Seq, MiSeq from Illumina; PromethION, GridION, and MinION from Oxford Nanopore Technologies; RS II and Sequel from PacBIO • Long-read sequences can provide high-resolution taxonomic identification but a relatively high error rate • Short-read sequences can generate a good quality result but limited taxonomic resolution *Recommendation*: For community analysis, Illumina Miseq is recommended because of the high quality of reads and its extensive database. To learn more about the specific functions of each device, see https://genohub.com/ngs-instrument-guide/.			[41]
STEP 5: Bioinformatics				
Substep	*Consideration*			Ref.
Bioinformatic quality control and analyses	Examples: DADA2 (https://benjjneb.github.io/dada2/); Mothur (https://mothur.org/); QIIME2 (https://qiime2.org/); Qiagen CLC Workbench (https://digitalinsights.qiagen.com/) *Recommendation*: New tools for community analysis are frequently coming out; therefore, reading the latest reviews, such as those listed in Reference column, and choosing better tools is always recommended.			[42,43] [44]
Database	*Examples*: SILVA (https://www.arb-silva.de); PR2 (https://pr2-database.org); PhytoREF (http://phytoref.sb-roscoff.fr); R-Syst::diatom (https://www6.inrae.fr/r-syst_eng/Databases/R-Syst-diatom); UPA/LSU (https://scholarspace.manoa.hawaii.edu/items/3fa155e5–8f6c-4759-bd7d-caf508b97b40); RDP (http://rdp.cme.msu.edu); Greengenes (https://greengenes.secondgenome.com); NCBI (https://www.ncbi.nlm.nih.gov/taxonomy); µgreen-db (http://microgreen-23sdatabase.ea.inra.fr); metaPR2 (https://shiny.metapr2.org/metapr2/) *Recommendation*: For general use, SILVA or Greengenes is recommended; for fine-scale assignment, PR2 or metaPR2 is recommended. There is no standard for species-level analysis and the appropriate approach needs to be determined based on research purpose because the databases are still being developed.			[45]
STEP 6: Statistics and visualization				
Tools	Examples			Ref.
	R package: vegan; phyloseq			[26,35]
	MEGA7			[32]

Table 2

Recommendations to improve consistency in algal holobiont monitoring.

1	Preparation	• Determine sampling sites • Determine the number and frequency of sampling events • Construct SOP and provide training to the team
2	Sampling	• Collect samples under a controlled methodology (strictly follow the SOP) • Collect all data at each timepoint (try not to miss any data points) • Collect positive and negative controls during sampling if available
3	DNA extraction	• Include negative and positive controls; positive controls can be a complex environmental sample or mock sample consisting of both prokaryotic and eukaryotic microorganisms • Record details of the DNA extraction process in a laboratory notebook each time, include information on positive and negative control in the notebook • Have a second analyst review the laboratory notebook for consistency and reproducibility • Use the same DNA extraction protocol and devices across studies and institutes involved in the project
4	Sequencing	• Confirm existence of life in low biomass samples using methods other than sequencing, such as microbial culture and fluorescent *in situ* hybridization, to verify that the sequence results are not a false positive

information is essential to reduce biases in the results. In addition to the SOP, this program focused on educating the sampling team, warning of on-site precautions, and training sample collection and processing procedures. Despite these attempts to standardize protocols, the coastal monitoring for HABs and holobionts using HTS has additional issues, which are described below.

Challenges after methodology standardization

Committing to long-term, regular, and sustainable coastal monitoring is fundamental to understanding holobionts in algal communities, and teamwork is required. In reality, the involvement of multiple personnel produces variability in data quality even with an SOP and training. There are many occasions that one data point (e.g. water temperature) is not recorded at a given timepoint, and the whole dataset collected at that timepoint must be excluded from statistical analysis because of the lack of one piece of information. Furthermore, eliminating data points requires careful justification during statistical analysis so that it is not arbitrary.

Moreover, the passion for the research, understanding the importance of the data, and standard for quality work differed among designees involved in the project (i.e. researchers, students, technicians, and the public). Consequently, it was challenging to vary these gaps even with proper training and compensation. This is probably the main challenge in citizen science, which is becoming a popular method of the general public collecting data related to the natural world and professional scientists analyzing the data. In addition, personnel turnover is inevitable during a long-term monitoring program, and task transition from former to current designees often leaves gaps that result in unequal data quality, even with written and visual SOPs.

Because the coastal monitoring for HABs and holobionts with HTS requires processing a large sample set, a sample naming convention is crucial to analyze data efficiently. For example, naming samples in the order of YYYYMMDD before sequencing is highly recommended. However, DDMMYYYY has often been mistakenly used because of cultural customs, and it needs to be manually corrected during statistical analysis.

Practical considerations and conclusions

HABs have damaged the world's coastal waters; nevertheless, elucidating the cause of HABs has been a struggle due to complex mechanisms. Studying an algal community as a whole is a key to better understanding the HABs, and the advancement of omics technologies contributes to it. However, challenges still lay in such a holobiont monitoring, mainly due to the difficulty in standardizing and complying with the monitoring protocols to collect reliable and sustainable data. Here, we refer to the HTS protocol generated by the International Human Microbiome Standards group, which has been working for years to solve similar issues but in the bacteria world.

Research on the human microbiome has revealed that the DNA extraction process is the most crucial factor that influences HTS results [51,52], and the International Human Microbiome Standards group has made efforts to standardize the DNA extraction procedure [53]. Variability in DNA extraction results can be caused by a combination of many factors, such as sample/reagent or biomass-related contaminations, ease/difficulty of cell lysis depending on sample types, and operation by laboratory personnel/automation [51]. In addition, the lack of standard reference materials makes it difficult to compare studies that used different DNA extraction methods, and the inclusion of quality control samples is addressed [51], although it noted that selecting a standardized protocol for all studies is extremely difficult and using SOPs does not prevent other interlaboratory

differences. Yet, adapting the flow of HTS-based monitoring established by experiences in the International Human Microbiome group (Table 2) into the HAB world may facilitate higher precision in global-scale algal holobiont monitoring.

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CRediT authorship contribution statement

Kyoko Yarimizu: Conceptualization, Investigation, Visualization, Writing – original draft; **So Fujiyoshi:** Conceptualization, Investigation, Visualization, Writing – review & editing; **Ishara Perera:** Investigation, Writing – review & editing; **Michel Abanto:** Investigation, Writing – review & editing; **Jorquera Milko:** Investigation, Writing – review & editing; **Fumito Maruyama:** Conceptualization, Investigation, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Conflict of interest statement

All authors have participated in (a) conception and design, or analysis and interpretation of the data; (b) drafting the article or revising it critically for important intellectual content; and (c) approval of the final version. This manuscript has not been submitted to, nor is under review at, another journal or other publishing venue. The authors have no affiliation with any organization with a direct or indirect financial interest in the subject matter discussed in the manuscript. The following authors don't have affiliations with organizations with direct nor indirect financial interest in the subject matter discussed in the manuscript: Kyoko Yarimizu, So Fujiyoshi, Ishara Perera, Michel Abanto, Milko Jorquera, Fumito Maruyama.

Data availability

Data will be made available on request.

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- • of special interest
- •• of outstanding interest.

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