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## A standardized procedure for monitoring Harmful Algal Blooms in Chile by metabarcoding analysis

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**TITLE:**

A Standardized Procedure for Monitoring Harmful Algal Blooms in Chile by Metabarcoding Analysis

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**KEYWORDS:**

harmful algae blooms, Chile, HAB monitoring, metabarcoding analysis, algal-bacterial mutualism, phytoplankton, salmon aquaculture

## SUMMARY:

This protocol introduces steps of metabarcoding analysis, targeting 16S rRNA and 18S rRNA genes, for monitoring harmful algal blooms and their associated microbiome in seawater samples. It is a powerful molecular-based tool but requires several procedures, which are visually explained here step-by-step.

## ABSTRACT:

Harmful algae blooms (HABs) monitoring has been implemented worldwide, and Chile, a country famous for its fisheries and aquaculture, has intensively used microscopic and toxin analyses for decades for this purpose. Molecular biological methods, such as high-throughput DNA sequencing and bacterial assemblage-based approaches, are just beginning to be introduced in Chilean HAB monitoring, and the procedures have not yet been standardized. Here, 16S rRNA and 18S rRNA metabarcoding analyses for monitoring Chilean HABs are introduced stepwise. According to a recent hypothesis, algal-bacterial mutualistic association plays a critical synergetic or antagonistic relationship accounting for bloom initiation, maintenance, and regression. Thus, monitoring HAB from algal-bacterial perspectives may provide a broader understanding of HAB mechanisms and the basis for early warning. Metabarcoding analysis is one of the best suited molecular-based tools for this purpose because it can detect massive algal-bacterial taxonomic information in a sample. The visual procedures of sampling to metabarcoding analysis herein provide specific instructions, aiming to reduce errors and collection of reliable data.

## INTRODUCTION:

Many marine phytoplankton species are known to produce endogenous toxins, and when these species accumulate in sufficient numbers, they are harmful to the marine environment. Such Harmful Algal Blooms (HABs) are observed today on the coasts of most continents of the world<sup>1</sup>. Toxic phytoplankton first accumulate in bivalve tissues, leading to illness and death in higher trophic levels of organisms, including humans, upon digestion. Subsequently, these events bring severe implications to the local economy, socioeconomics, and public health<sup>2</sup>. The damage to the global economy caused by HABs is estimated to be millions to billions of dollars each year<sup>3</sup>. Chile is one of many countries suffering from frequent HABs.

Chile is a country of long land, stretching over 4,300 km north and south. The elongated western land faces the Pacific Ocean, which naturally increases the chances of Chile experiencing HABs. Especially in southern Chile, there are many world-famous salmon aquacultures, and every time a HAB occurs in the region, the algal-toxins result in massive farmed salmon falling ill and die<sup>4-6</sup>. In Chile, the year when the HAB hit the economy hardest was 2016, with an estimated annual loss of US \$ 800 million<sup>7,8</sup>. The causative toxic algal species vary by year and location. For the 2016 case, a complex of *Alexandrium catanella* and *Pseudochattonella verruclosa* caused a widespread HAB in most of the southern Chilean coast<sup>7,8</sup>. The most recent HAB in Chile occurred with the causative algal species of *Heterosigma Akashiwo* in March 2021 at Camanchaca, located in southern Chile, where the area has a large salmon farm.

Chile has been conducting coastal monitoring for many years using two main methods; observing seawater with microscopes to identify toxic algae species regularly and measuring toxin levels in

shellfish by biochemical assays<sup>9</sup>. Early detection of toxic algae and toxin levels in shellfish do not prevent HABs; however, these analyses can trigger immediate countermeasures and reduce damages to local communities. To further strengthen the effectiveness of this strategy, a molecular-based analytical method was recently added to our conventional Chilean HAB monitoring program to detect algae and related bacterial communities in seawater samples. Specifically, a Massive Parallel Sequencing method using metabarcoding that targets 16S rRNA and 18S rRNA genes was chosen. Although this technique requires complicated procedures and expensive machinery and reagents, it is an advanced technology that can detect thousands of algae and bacteria genera/species present in a seawater sample at once.

The causes of HAB are speculated to be various, such as temperature and season, but it is impossible to generalize them. This is because HAB species and frequencies depend on the region and spatiotemporal conditions, involving natural phenomena such as geographic uniqueness, upwelling nutrient mixing, and element runoff from land due to erosion<sup>2,10-12</sup>. Additionally, artificial factors such as eutrophication influence local HABs<sup>12,13</sup>. Due to the complex multifactor, it is not easy to make an accurate HAB prediction. In recent years, there is a view that specific bacterial populations may be related to the development of HABs as one of the factors, and research to support this hypothesis has been increasingly evident<sup>14-18</sup>. Molecular biology techniques are generally used to study bacterial assemblage; however, such a standardized method has not yet been established in Chilean HAB monitoring<sup>9</sup>. To study algal-bacterial association with HABs, it is imperative to concurrently perform metabarcoding analysis for the current Chilean coastal monitoring programs. Thus, this protocol visually introduces our Chilean HAB monitoring program, focusing on a stepwise procedure for analyzing algae and bacteria species detection in seawater samples using metabarcoding analysis.

The full protocol describing our Chilean HAB monitoring program is available in Yarimizu et al.<sup>9</sup>. It includes the procedures of seawater sampling, microscopic algal species detection, algal-bacterial gene detection, pigment analysis, meteorological data collection, and physical and chemical water property assays. The stepwise protocol of 16S rRNA and 18S rRNA metabarcoding analysis for algal and bacterial species detection is available as a preprint<sup>19</sup>. This protocol demonstrates particularly metabarcoding analysis steps since it is the most complicated part and the highlight of our HAB monitoring program. This protocol also includes an introduction of the program and microscopy detection of algal species. When analyzing algal species by metabarcoding, it is crucial to simultaneously perform microscopy to verify the results from the two methods. This protocol does not include how to use software for taxonomy assignment, but database recommendation is briefly stated at the end of the following section.

## **PROTOCOL:**

### **1. Sample collection and pretreatment**

**1.1. Collect approximately 3 L of water sample from the target spot.**

**1.2. Filter 1 L of water sample for 16S rRNA analysis through a tandem filtration (1 µm and 0.2**

µm pore-size membrane) to separate free-living and attached bacteria.

1.3. Filter another 1 L of water sample for 18S-rRNA analysis (phytoplankton detection) through a single filtration with a 0.2 µm membrane.

NOTE: Filtration of the water sample must be completed within 12 h of sampling.

1.4. Cut filtered membrane in half with sterile surgical scissors and wrap it with aluminum foil. Store at -20 °C for upto 1 month or proceed to the next step.

1.5. Extract DNA with Chelex method as described<sup>9</sup>. Store at -20 °C for upto 1 month.

## 2. Microscope analysis

2.1. Transfer 1 mL of the water sample by a pipette onto a 1 mL grid-slide.

2.2. Observe the sample under a microscope.

2.3. Record names and quantity of phytoplankton species.

## 3. 16S rRNA and 18S rRNA metabarcoding analysis

NOTE: This process consists of seven sections: preparation, first PCR amplicon generation, first amplicon cleanup, indexation by second PCR, second PCR amplicon verification and cleanup, DNA concentration adjustment, and DNA denaturation and sequencing. The entire process takes a minimum of 5 days (40 h) by an experienced lab personnel. See the **Table of Materials** for product numbers and manufactures.

### 3.1. Preparation

3.1.1. Clean pipettes and laminar hood cabinet with 70% ethanol followed by UV exposure for 30 min frequently. Sterilize materials to be used.

3.1.2. Thaw DNA samples at room temperature, centrifuge at 100 x g for 2 min, and transfer 100 µL of each sample supernatant to 8-tube strips.

### 3.2. First PCR amplicon generation

NOTE: Perform the following procedures in a laminar hood cabinet. Always dilute primers from 1 µM stock to the target concentration with PCR grade water to avoid primer contamination.

3.2.1. Prepare first PCR master mix in a sterile 1.5 mL tube for reactions (Table 1, Table 2).

3.2.2. Aliquot 22.5 µL of the master mix in an 8-tube strip and add 2.5 µL of DNA sample. Use

2.5  $\mu\text{L}$  of PCR grade water for negative control.

3.2.3. Run the first PCR cycle (**Table 3**).

3.2.4. Prepare 100 mL of 2% Agarose-TBE gel containing 10  $\mu\text{L}$  of 1x nucleic acid gel stain.

3.2.5. Load a mixture of 1.5  $\mu\text{L}$  of 1x DNA loading dye and 4  $\mu\text{L}$  of PCR product on the agarose gel. Also, load 100 bp DNA ladder on the gel.

3.2.6. Perform electrophoresis at 100 V for 30 min.

3.2.7. Ensure that there is a band at 500-600 bp range under a UV light image capture. Primer-dimer band is round 80 bp.

NOTE: Marine water samples contain PCR inhibitors. Missing amplicons can sometimes be solved by diluting samples 1:100 or 1:1000 with PCR grade water.

3.2.8. Store first PCR products at  $-20\text{ }^{\circ}\text{C}$  until the next step.

CAUTION: Do not exceed one week of storage.

### 3.3. First PCR amplicon cleanup

NOTE: This section can be performed outside a laminar hood cabinet.

3.3.1. Use magnetic beads DNA cleanup system to remove PCR reaction residues, including primer-dimer products.

3.3.2. Transfer 20  $\mu\text{L}$  of each cleaned first PCR product to a new 96 well plate. Seal the plate with a micro-seal film. Store at  $-20\text{ }^{\circ}\text{C}$  until the next step proceeds.

CAUTION: Do not exceed more than one week of storage.

### 3.4. Indexation by second PCR

NOTE: In this section, purified first PCR products will be amplified with various index primer combinations.

3.4.1. Dilute all Index 1 and Index 2 primers (**Table 4**) to 1  $\mu\text{M}$  with PCR grade water in 8 tube PCR strips placed in a laminar hood cabinet.

NOTE: The steps henceforward in this section can be performed outside of a laminar hood cabinet, as index primers are specific to the first PCR reactions overhang adapter.

3.4.2. Position Index 1 primer in a horizontal row Index 2 primers in a vertical row (**Table 5**).

3.4.3. In a new 96-well plate, add 12.5  $\mu$ L of hot-start-ready formulation to each well.

3.4.4. Add 2.5  $\mu$ L of each index primer (1  $\mu$ M) to each well as in **Table 4** using a multichannel pipette.

3.4.5. Add 7.5  $\mu$ L of purified first PCR product.

3.4.6. Mix gently by pipetting up and down 10 times. Cover the plate with a micro-seal film.

3.4.7. Run the second PCR cycle (**Table 3**).

3.4.8. Keep the plate at -20 °C.

CAUTION: Do not exceed one week of storage.

### 3.5. Second PCR amplicon verification and cleanup

3.5.1. Use a fragment analyzer and associated reagent. Vortex and spin down reagent before use.

3.5.2. Allow the sample buffer and DNA screen tape to equilibrate at room temp for 30 min. Then, place the DNA screen tape in a fragment analyzer.

3.5.3. Mix 2  $\mu$ L of sample buffer and 3  $\mu$ L of second PCR amplicon in new 8 tube strips. Insert the 8 tube strips into the fragment analyzer. Press **Run** to start.

CAUTION: The formation of air bubbles must be avoided.

3.5.4. Ensure that second PCR amplicons are approximately 613 bp (600 - 630 bp) for both 16S and 18S rRNA genes.

3.5.5. Purify second PCR products using a magnetic beads DNA cleanup system.

### 3.6. DNA concentration adjustment

3.6.1. Measure DNA concentration in the purified second PCR products using a nucleic acid quantification spectrophotometer.

3.6.2. Calculate target gene concentration in nM, accounting for the average final library size as 613 bp for both 16S and 18S rRNA genes:

$$16S \text{ or } 18S \text{ gene conc. (nM)} = 10^6 \times \frac{\text{measured DNA conc. } (\frac{ng}{\mu L})}{660/613}$$

3.6.3. Dilute each purified second PCR product with sterile PCR water to 4 nM in a new 0.2 mL 96 well plate.

NOTE: The plate can be stored at -20 °C here. Otherwise, the rest of procedures must be performed without halting.

3.6.4. Aliquot 3 µL of each 4 nM second PCR product and mix all in a new sterile 1.5 mL tube as a pooled library. Keep the tube at 4 °C or on ice bath at all times.

3.6.5. Measure the concentration of the pooled library for confirmation using a nucleic acid quantification spectrophotometer. Adjust the concentration to 4 nM if it is higher than 4 nM.

NOTE: Over-concentrated DNA produces overestimated read numbers, hampering analysis.

### 3.7. DNA denaturation and sequencing

NOTE: This section uses Illumina MiSeq system with specific reagents. See product numbers in **Table of Materials**.

NOTE: Strictly follow time. Thaw all reagents except cartridges on the day of sequencing.

3.7.1. A day before sequencing, remove a pre-filled ready-to-use reagent cartridge from -20 °C and store at 4 °C for thawing.

3.7.2. Set a heat block suitable for 1.5 mL centrifuge tubes to 96 °C.

3.7.3. Place the hybridization buffer on ice.

3.7.4. Dilute molecular grade NaOH from 1 N to 0.2 N in a new tube with PCR grade water.

3.7.5. Dilute a ready-to-use control library with TE buffer (pH 8.0) from 10 nM stock to 4 nM in a new tube (i.e., 2 µL of control library + 3 µL of TE buffer).

3.7.6. Mix 16 µL of 4nM pooled sample library with 4 µL of 4 nM control library in a new tube labeled "1".

3.7.7. Mix 10 µL of sample in tube "1" with 10 µL of 0.2 N NaOH in a new tube labeled "2".

3.7.8. Vortex the tube 2 for 5 s, spin down briefly, and incubate at room temperature for 5 min.

3.7.9. Add 980  $\mu$ L of hybridization buffer to tube 2.

3.7.10. In a new tube labeled “3”, mix 260  $\mu$ L the sample from tube 2 with 390  $\mu$ L of hybridization buffer. Mix by inverting the tube.

3.7.11. Incubate tube 3 at 96 °C for 2 min and immediately place it on ice for a maximum 2 min.

3.7.12. Remove the cartridge from 4 °C refrigerator.

3.7.13. Set up the sample-sheet for sequencing with each corresponding index 1 and index 2 adapters as in **Table 6**.

3.7.14. Remove the flowcell from the MiSeq v3 kit. Gently clean the flowcell with sterile molecular grade PCR water.

NOTE: Do not pour water on the capillary dots of the flowcell. Gently wipe off water from the flowcell with non-fibrous paper.

3.7.15. Load the full volume of tube 3 (650  $\mu$ L) into the cartridge.

3.7.16. In instrument operation software, select sequencing and follow the instructions. Insert flowcell, incorporation buffer, and cartridge. Load sample-sheet. Press **Run** to start the reaction.

NOTE: This sequence run will take 3–5 days. The data will be automatically uploaded to Basespace platform. Raw data will be stored in two folders in computer: Analysis folder containing fastq files and Output containing bcl files and jpeg pictures.

#### 4. Taxonomic assignment to sequencing data

##### 4.1. FASTQ file processing

NOTE: DADA2 package<sup>23</sup> or R<sup>24</sup> is one of recommended databases to process FASTQ files. The guideline is available at [[https://github.com/mickeykawai/exec\\_dada2](https://github.com/mickeykawai/exec_dada2)]. This guideline was prepared by adopting the recent version of DADA2 pipeline tutorial [<https://benjjneb.github.io/dada2/tutorial.html>].

4.1.1. Process raw FASTQ paired-end sequences for trimming, quality filtering, dereplication and counting of unique sequences, sample inference, merging into contigs and removing chimeric sequences.

4.1.2. Use following DADA2 specified parameters for both 16S rRNA and 18S rRNA gene fragments; trimLefts=0, 0, truncLens=[Check the sequence quality and set it to an appropriate length].

NOTE: Default values for the other parameters are maxN=0, maxEE=2,2, truncQ=2.

## 4.2. Taxonomy assignment

NOTE: SILVA rRNA database is recommended for taxonomy assignment for DADA2 [silva\_nr\_v132\_train\_set.fa.gz is set as a default].

4.2.1. Remove chloroplast and mitochondrial OTUs for 16S rRNA gene fragment analysis. Remove singletons from 16S rRNA and 18S rRNA analysis.

4.2.2. Ratify all samples to even sequencing depth based on the sample having the lowest sequencing depth.

4.2.3. Record the number of reads, filtered reads, OTUs assigned and at what level, and the number of singletons excluded.

## 4.3. Statistical analysis

4.3.1. Perform statistical analysis on microbial data using R packages of phyloseq<sup>25</sup> and vegan<sup>26</sup>.

## REPRESENTATIVE RESULTS:

This protocol uses 18S rRNA gene metabarcoding analysis to identify algal species in seawater samples. A representative result is shown in **Figure 1**, in which the seawater collected from Metri, Puerto Montt, Chile (−41.597; −72.7056) on February 19, 2019, was analyzed with this protocol. The result showed a total of 13,750 reads with over 30 algal species in the seawater sample. The dominant alga in this sample was *Navicula spp.* with a relative abundance of 70.77%. Also, sufficient abundance was observed for *Micromonas* (6.40%), *Chaetoceros spp.* (4.44%), *Scropsiella spp.* (2.44%), and *Prorocentrum spp.* (1.28%). *Pseudochattonella spp.*, one of the highest toxic algal causatives of Chilean HAB, was detected with 0.52% from this seawater sample.

To verify the data reliability, the algal species identified by 18S rRNA gene analysis were compared to those obtained by microscopy in the same seawater sample (**Figure 2**). Consistent with 18S rRNA gene analysis, the microscopy showed that the dominant species was *Navicula spp.* with a relative abundance of 74.1% and *Prorocentrum spp.* (0.60%) as a minor species. Conversely, *Heterocapsa spp.* (9.04%) documented from microscopic observation was not identified by 18S rRNA gene analysis in this sample. There was 12.6% of small around unidentifiable phytoplankton cells recorded by microscopy. This could be *Micromonas*, according to the 18S rRNA results.

The protocol uses 16S rRNA gene metabarcoding analysis to identify bacterial species in seawater samples. A representative result is shown in **Figure 3**, in which the same seawater used for 18S rRNA gene analysis was analyzed with 16S rRNA gene analysis. The result showed a total of 31,758 reads with over 30 bacterial species in the seawater sample. It should be outlined that this seawater sample was passed through tandem filter membranes (1 µm and 0.2 µm pore sizes) to

separate free-living bacteria from attached bacteria. Then, cells captured by each filter membrane were treated for DNA extraction, followed by the 16S rRNA gene analysis. The representative result in **Figure 3** shows bacteria species identified from 0.2 µm pore size membrane, which are defined as free-living bacteria. The dominant free-living bacteria was *Amylibacter spp.* with a relative abundance of 20.02%, followed by *Clade Ia* (13.53%) and *Aligiphilus spp.* (7.06%). The rest of the bacterial species detected from this seawater sample were relatively equally distributed. The same analysis can be done for cells captured by 1 µm pore-sized membrane as attach-bacteria species detection.

When these metabarcoding assays are performed at scheduled time-points over a certain study period, the results can be summarized as time-series analysis. One way to do it is to plot the relative abundance of particular algal and bacterial species as a function of time to find a unique growth pattern. **Figure 4** shows a representative time-series plot of *Alexandrium* and *Pseudochattonella* in Metri, Chile. Another way to summarize a time-series metabarcoding analysis is to plot all identified algal and bacterial as a function of time, representing the population change of certain groups of organisms. **Figure 5** and **Figure 6** summarizes the relative abundance of all the bacterial genus and order, respectively, which are detected from the seawater of Metri over five months.

#### FIGURE AND TABLE LEGENDS:

**Table 1: First PCR master mix content:** The table shows master mix content per reaction for 16S rRNA and 18S rRNA analyses. The primer sequences are listed in Table 3.

**Table 2: Primer sequences:** The primers for first PCR are listed for 16S rRNA and 18S rRNA analyses.

**Table 3: PCR cycles:** The thermal cycles for first PCR and second PCR are listed.

**Table 4: Index sequences:** Index 1 (i7) and Index 2 (i5) primers to be used for second PCR are listed.

**Table 5: Example of Index 1 and Index 2 positioning:** To reduce error, index primers must be placed in the position first, and aliquot of each is transferred to a 96-well plate using multichannel pipette.

**Table 6: Example of sample-sheet:** Prior to sequencing, a sample-sheet must be created corresponding to index 1 and index 2 adapters.

**Figure 1: Representative result of 18S rRNA metabarcoding analysis:** Algal species present in a seawater sample collected from Metri, Los Lagos, Chile on February 19, 2019, were identified by 18S rRNA metabarcoding assay. The sequences assigned “unknown” were eliminated from, and the relative abundance of each identified species was plotted.

**Figure 2: Representative result of microscopic analysis:** The algal species were identified from a water sample from Metri, Los Lagos, Chile on February 19, 2019, by microscopy. The quantity of each species was counted manually and plotted.

**Figure 3: Representative result of 16S rRNA metabarcoding analysis:** Bacterial species present in a water sample from Metri, Los Lagos, Chile on February 19, 2019, were identified by 16S rRNA metabarcoding assay. The relative abundance of each identified species was plotted.

**Figure 4: Representative time-series plot of *Alexandrium* spp. and *Pseudochattonella* spp. obtained from 18S rRNA metabarcoding in Metri, Chile:** Figure reprinted from Yarimizu et al<sup>9</sup>. The two toxic algal species, *Alexandrium* and *Pseudochattonella* were selectively monitored by 18S rRNA analysis over time, and the relative abundance were plotted as a function of time-point.

**Figure 5: Representative genus time-series plot obtained from 16S rRNA and 18S rRNA metabarcoding:** All bacterial genus identified in the water of Metri, Chile, monitored over five months.

**Figure 6: Representative order time-series plot obtained from 16S rRNA and 18S rRNA metabarcoding:** All bacterial orders identified in the water of Metri, Chile, monitored over five months.

**Table S1:** Raw data for Figure 5 and Figure 6.

**Table S2:** Result of QC check on raw data of Figure 5 and Figure 6.

## DISCUSSION:

This protocol successfully performed 18S rRNA and 16S rRNA gene metabarcoding analysis to identify algal and bacterial species in seawater samples for monitoring Chilean HABs. The dominant algae and some minor algae detected by this protocol were consistent with those obtained by microscopy, confirming the protocol's reliability. The highlight of the protocol is that the analysis detected *Alexandrium* spp. and *Pseudochattonella* spp., the two most problematic HAB causing species in southern Chile, from the seawater of Metri even at a low abundance. Especially, *Pseudochattonella* spp. are challenging species to identify under a microscope because the cells are small and easily ruptured under light or use of fixatives<sup>27,28</sup>. Metabarcoding can provide algal species information that is scarcely present in seawater samples that microscopy cannot identify. The protocol also detected over 30 bacterial species. Although how these bacteria are related to algal growth is unknown at this point, massive data collection of algal-bacterial species in the time-series analysis will possibly provide such important discoveries. Thus, adding metabarcoding analysis to the conventional Chilean HAB monitoring programs will undoubtedly strengthen the current HAB monitoring efficiency. In fact, this visual protocol for metabarcoding analysis can be beneficial not only for Chilean water's algal-bacterial monitoring but also for the coast monitoring programs in other HAB-affected areas worldwide.

Although this protocol provided the above-stated advantages, the drawbacks of the method

should be discussed. As seen in the visual protocol, the metabarcoding method is time-consuming and complex, requiring expensive machinery and reagents. The laboratory personnel must be specially trained, or it will waste material, labor, and time. In addition, algal detection by metabarcoding must be paired with microscopy to verify that the same dominant species are identified from the two orthogonal methods<sup>29</sup>. Microscopy is a non-invasive tool, for the most part, to identify algal species, meaning that it is harder to make mistakes when the algal species have unique and apparent shapes. Of course, human error can occur if the species have very similar shapes to one another. On the other hand, since metabarcoding analysis requires multiple steps, it naturally increases errors. It could be a sample mixed up, wrong reagent addition, or missing some procedures. Therefore, it is critical to compare the metabarcoding results with those obtained by microscopy. Lastly, the protocol is applicable only qualitatively, and the results must be calculated for relative abundance. As Lamb et al. stated in 2019, the current metabarcoding as the quantitative performance is still limited, and additional research is required before metabarcoding can be confidently utilized for quantitative applications<sup>30</sup>.

This protocol was optimized for 140 - 170 samples per run from the 16S Metagenomic Sequencing Library Preparation manual issued by Illumina Inc. The optimized protocol was tested many times and further modified to issue this final version. Therefore, it is highly recommended to follow each step precisely. The most critical part of the protocol is that extra care is required to avoid sample contamination. The pipettes and laminar flow hood must be cleaned with 70% alcohol and UV exposure at all times, and sterilizable materials should be autoclaved. The primers and reagents should be diluted directly from the stock at every new run, or reuse of reagents and exposure to several dilutions can be a sample contamination cause. The protocol specifies when the operation can be done outside of the laminar flow hood. Unless otherwise, samples should be treated in a cleaned laminar flow hood. When dealing with multiple 8-tube strips simultaneously, it is recommended to work on the first 8-tube strip and cap it before moving to the next 8-tube strip. Leaving the lid open for a long time can cause sample contamination as 16S and 18S genes are highly ubiquitous in surroundings. The stated time, such as mixing time and incubation time, should be followed as exactly described because the best duration for each step was selected based on the multiple test runs. Excess or insufficient time can, for example, reduce sample yield. The process should be halted only at the part that protocol says it can stop. It is crucial to have a negative control as it confirms that the positive outcomes are not artificial false positives. Lastly, it is highly recommended to plan the days because it requires at least five days to process all the steps, and some parts cannot be stopped until the next halt sign.

The limitation of taxonomic assignment for sequenced data are briefly noted here. Newly discovered nucleotide sequences for bacterial and algal species are updated in the databank daily. While well-studied algal and bacterial species are registered reliably, there are also many sequences for unknown species updated in the databank. It indicates that the resolution of registered nucleotide sequences is not always enough for species identification but only for genus identification. Thus, microscopic species identification must be orthogonally performed. Establishing an open-access pipeline for this work has a significant advantage in dealing with a large set of sequenced data at once and efficiently investigating when an error occurs. Additionally, the output of DADA2 is in a text or csv file, which makes it easy to further statistical

analysis. On the other hand, a large server is required to perform taxonomic assignments, especially when a dataset is large. To set up the pipeline, engineers are needed to set up the pipeline, and the professionals must understand parameters, operation, Linux, and bioinformatics.

Aside from the scope as a HAB monitoring tool, the use of this protocol can be expanded for investigative research purposes. For example, there are ongoing debates between the Chilean government and local fishermen along with environment peace associations regarding the increased number of local HAB<sup>31,32</sup>. The government claims that it is due to a natural phenomenon such as global warming and El Niño, while the later parties claim that salmon aquaculture is the cause. Salmon is not an indigenous species of Chile and was not in Chilean water a half-century ago. The Chilean government at that time aimed to grow the economy and create jobs for poor areas by developing a salmon business<sup>33</sup>. With the intervention from overseas for capital profit, Chile made great success in pen-style aquaculture development, and the number of salmon has increased remarkably in the last several decades<sup>31-33</sup>. Subsequently, a large amount of food for salmon has been thrown into the sea, leading local people to suspect that salmon aquaculture is the reason for the frequent local HABs<sup>31,32</sup>. The truth is unknown now, but it must be understood eventually so that strategies to protect the local marine environment, economy, and human health from HABs can be devised. The molecular-based analysis on the local algal-bacterial relationship may contribute to a step forward clarification for such a subject. For example, the technique can search the algal and bacterial species that were not present in the target oceanic area before but have increased dramatically in recent years, which is somewhat similar to the study done by Sakai et al. for the discovery of an unrecorded population of Yamato salamander (*Hynobius vandenburghi*) by GIS and eDNA analysis<sup>34</sup>. Therefore, beyond the HAB monitoring tool, this metabarcoding protocol has the potential usage for other prospects.

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#### DISCLOSURES:

The authors declare no conflict of interest. The funders had no role in the design of the study, in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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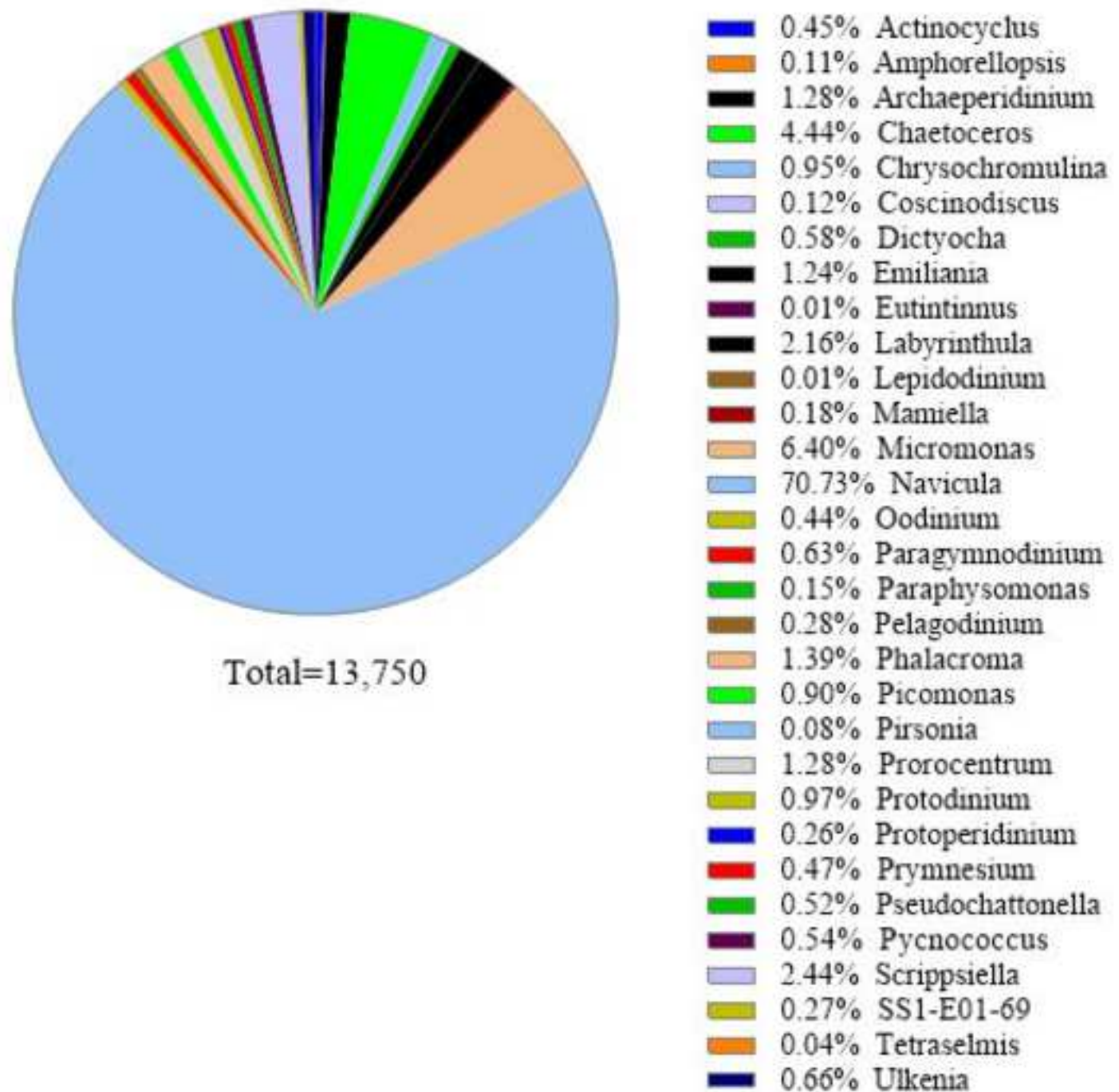


Figure 2

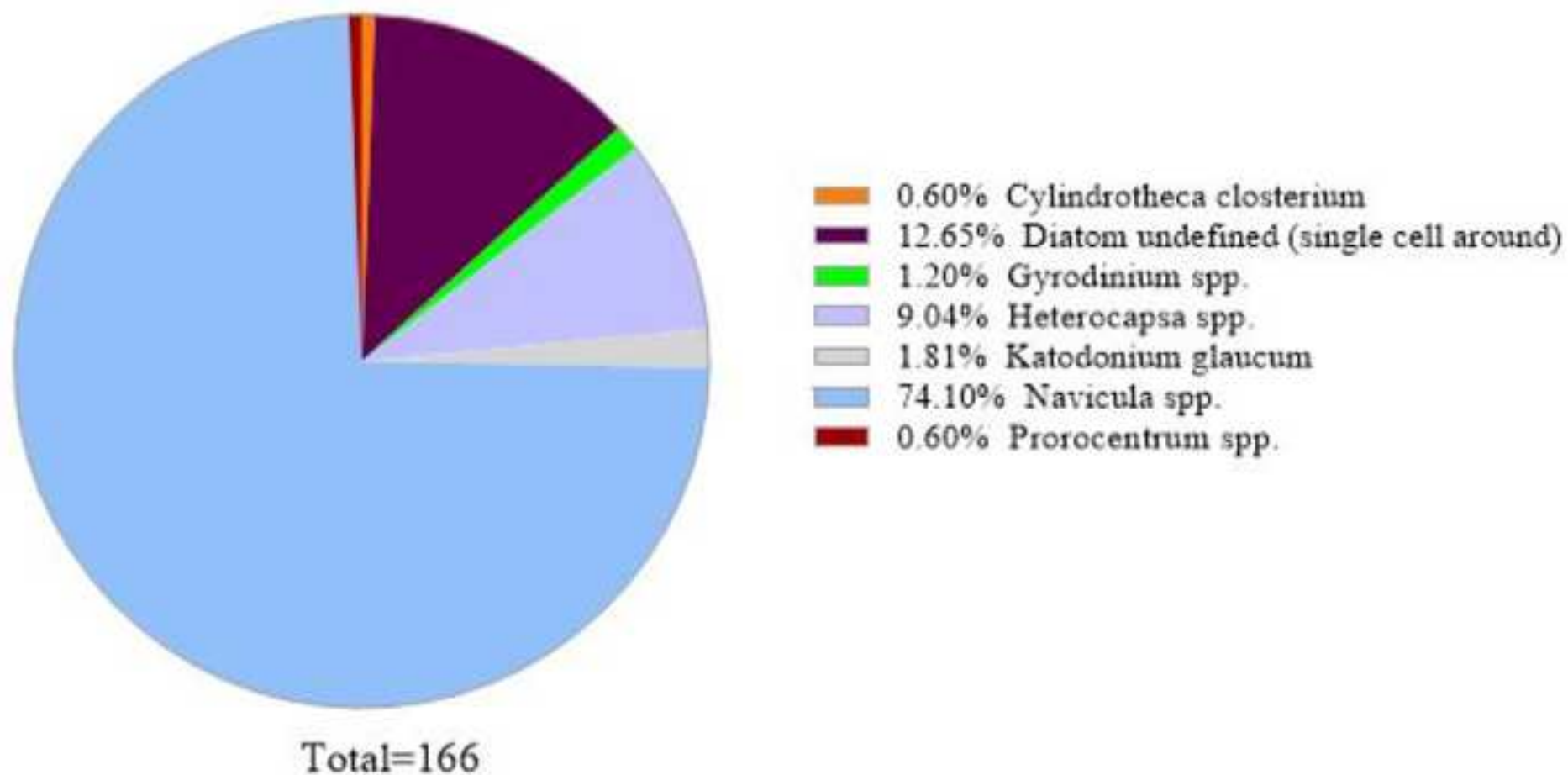
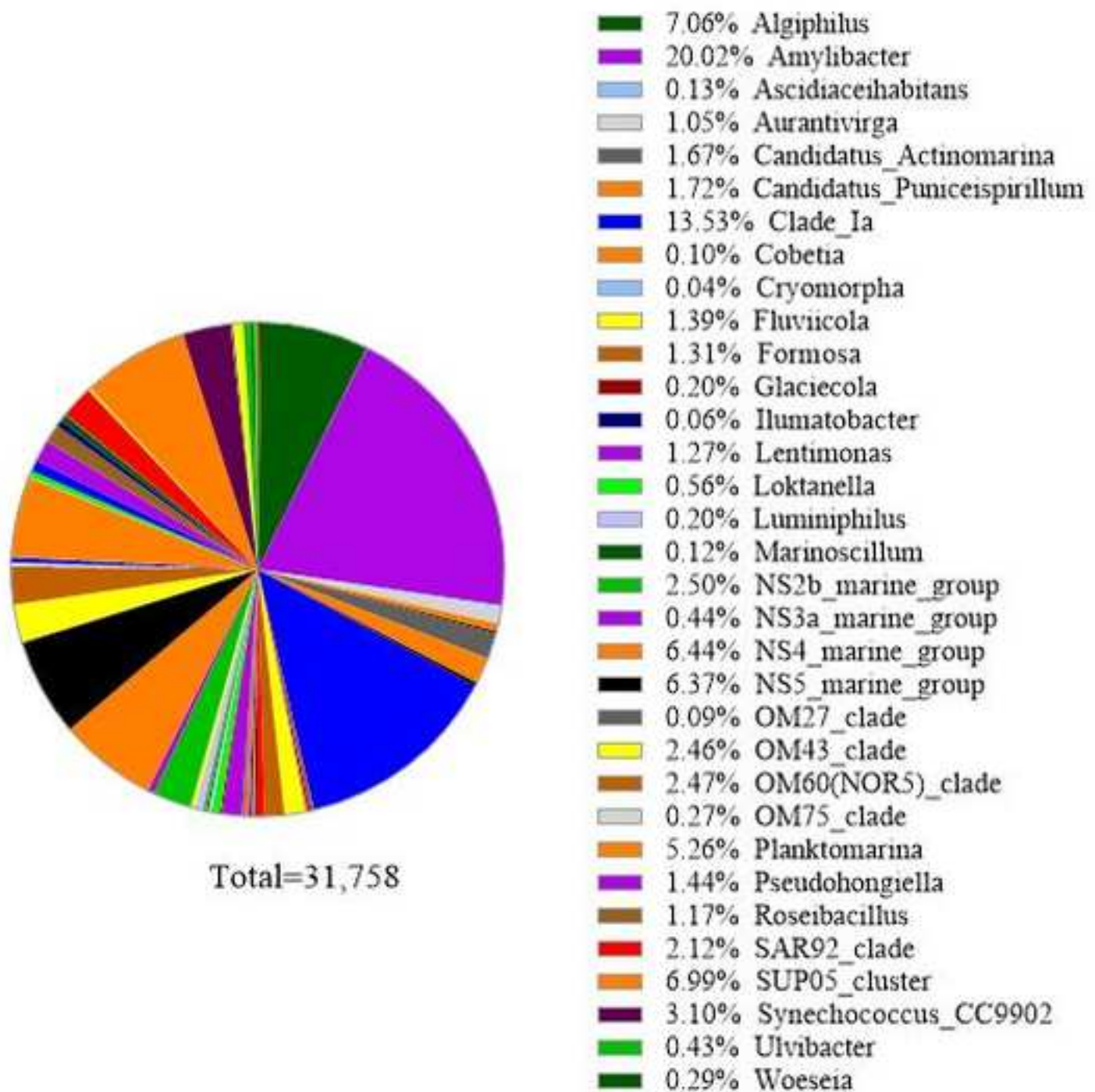
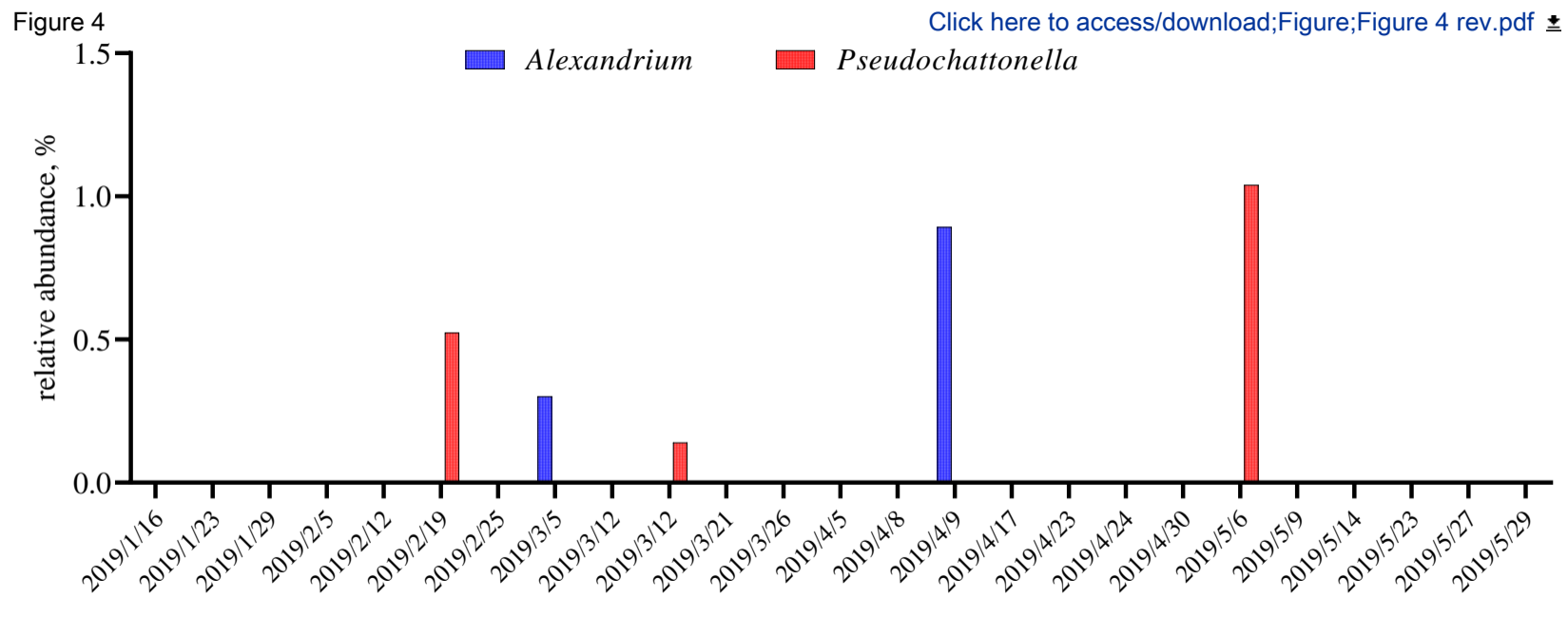
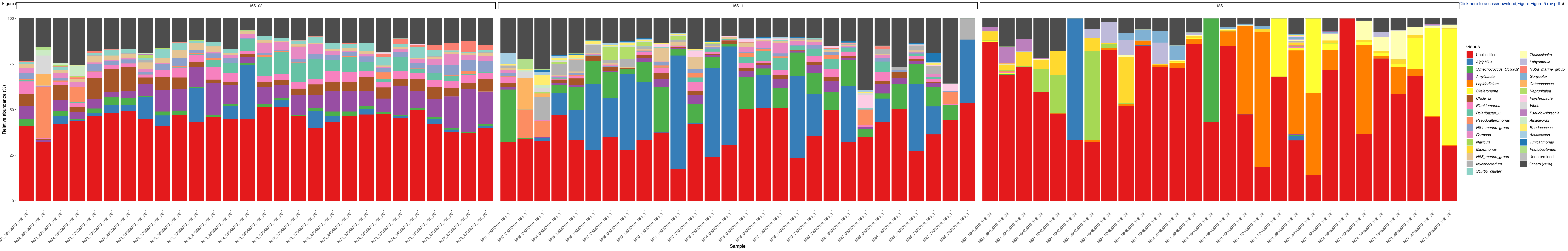
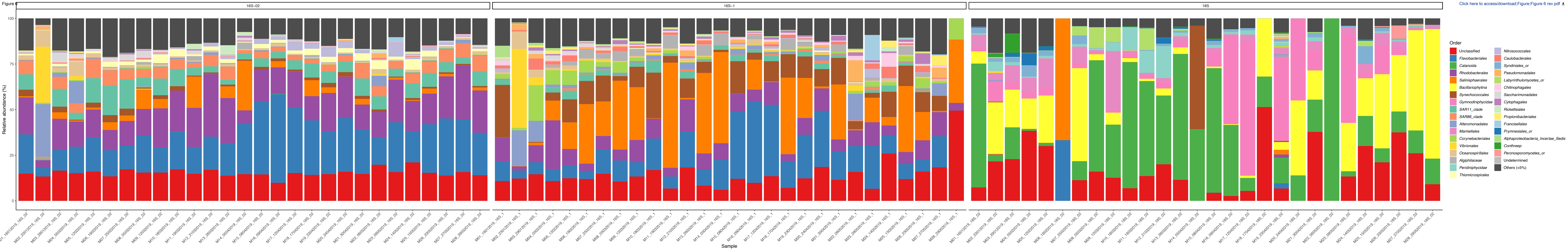


Figure 3









<b>16S rRNA master mix</b>	
16S-341F, 1 $\mu$ M	2.5 $\mu$ L
16S-805R, 1 $\mu$ M	2.5 $\mu$ L
2X PCR reaction buffer	12.5 $\mu$ L
Polymerase Mix, 1.25 U/ $\mu$ L	0.5 $\mu$ L
PCR grade water	5 $\mu$ L

<b>18S rRNA master mix</b>	
SSU-F1289, 1 $\mu$ M	2.5 $\mu$ L
SSU-R1772, 1 $\mu$ M	2.5 $\mu$ L
2X PCR reaction buffer	12.5 $\mu$ L
Polymerase Mix, 1.25 U/ $\mu$ L	0.5 $\mu$ L
PCR grade water	5 $\mu$ L

Primer Name	Overhang Adapter (5' → 3')	Region of Interest Specific Sequence (5' → 3')	Ref.
SSU-F1289	ACACTCTTCCCTACACGACGCTCTTCCGATCT	TGGAGYGATHGTCTGGTTDATTCCG	20, 21
SSU-R1772	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	TCACCTACGGAWACCTTGTTACG	
16S-341F	ACACTCTTCCCTACACGACGCTCTTCCGATCT	CCTACGGGNGGCWGCAG	22
16S-805R	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT	GACTACHVGGGTATCTAATCC	

First PCR cycle		
temperature	length	cycle
95 °C	3 min	x 1
95 °C	30 sec	x 35
55 °C	30 sec	
72 °C	30 sec	
72 °C	5 min	x 1
4 °C	infinity	x 1

Second PCR cycle		
temperature	length	cycle
95 °C	3 min	x 1
95 °C	30 sec	x 8
55 °C	30 sec	
72 °C	30 sec	
72 °C	5 min	x 1
4 °C	infinity	x 1

Index Name	Index 1 (i7)	Index sequence
N701-A	CAAGCAGAAGACGGCATAACGAGATT <b>TCGCCT</b> TAGTGACTGGAGTTCAGACGTGTG	TAAGGCCGA
N702-A	CAAGCAGAAGACGGCATAACGAGAT <b>CTAGTACG</b> GTGACTGGAGTTCAGACGTGTG	CGTACTAG
N703-A	CAAGCAGAAGACGGCATAACGAGATT <b>TTCTG</b> CCTGTGACTGGAGTTCAGACGTGTG	AGGCAGAA
N704-A	CAAGCAGAAGACGGCATAACGAGAT <b>GCTCAGG</b> AGTGACTGGAGTTCAGACGTGTG	TCCTGAGC
N705-A	CAAGCAGAAGACGGCATAACGAGAT <b>AGGAGTCC</b> GTGACTGGAGTTCAGACGTGTG	GGACTCCT
N706-A	CAAGCAGAAGACGGCATAACGAGAT <b>CATGCCT</b> AGTGACTGGAGTTCAGACGTGTG	TAGGCATG
N707-A	CAAGCAGAAGACGGCATAACGAGAT <b>GTAGAGAG</b> GTGACTGGAGTTCAGACGTGTG	CTCTCTAC
N710-A	CAAGCAGAAGACGGCATAACGAGAT <b>CAGCCTCG</b> GTGACTGGAGTTCAGACGTGTG	CGAGGCTG
N711-A	CAAGCAGAAGACGGCATAACGAGATT <b>GCCTCT</b> TGTGACTGGAGTTCAGACGTGTG	AAGAGGCA
N712-A	CAAGCAGAAGACGGCATAACGAGATT <b>CTCTAC</b> GTGACTGGAGTTCAGACGTGTG	GTAGAGGA
N714-A	CAAGCAGAAGACGGCATAACGAGATT <b>CATGAGC</b> GTGACTGGAGTTCAGACGTGTG	GCTCATGA
N715-A	CAAGCAGAAGACGGCATAACGAGAT <b>CCTGAGAT</b> GTGACTGGAGTTCAGACGTGTG	ATCTCAGG
N716-B	CAAGCAGAAGACGGCATAACGAGATT <b>AGCGAGT</b> GTGACTGGAGTTCAGACGTGTG	ACTCGCTA
N718-B	CAAGCAGAAGACGGCATAACGAGAT <b>GTAGCTCC</b> GTGACTGGAGTTCAGACGTGTG	GGAGCTAC
N719-B	CAAGCAGAAGACGGCATAACGAGATT <b>ACTACGCG</b> TGACTGGAGTTCAGACGTGTG	GCGTAGTA
N720-B	CAAGCAGAAGACGGCATAACGAGAT <b>AGGCTCCG</b> GTGACTGGAGTTCAGACGTGTG	CGGAGCCT
N721-B	CAAGCAGAAGACGGCATAACGAGAT <b>GCAGCGT</b> AGTGACTGGAGTTCAGACGTGTG	TACGCTGC
N722-B	CAAGCAGAAGACGGCATAACGAGAT <b>CTGCGCAT</b> GTGACTGGAGTTCAGACGTGTG	ATGCGCAG
N723-B	CAAGCAGAAGACGGCATAACGAGAT <b>GAGCGCT</b> AGTGACTGGAGTTCAGACGTGTG	TAGCGCTC
N724-B	CAAGCAGAAGACGGCATAACGAGAT <b>CGCTCAGT</b> GTGACTGGAGTTCAGACGTGTG	ACTGAGCG
N726-B	CAAGCAGAAGACGGCATAACGAGAT <b>GTCTTAGG</b> GTGACTGGAGTTCAGACGTGTG	CCTAAGAC
N727-B	CAAGCAGAAGACGGCATAACGAGAT <b>ACTGATCG</b> GTGACTGGAGTTCAGACGTGTG	CGATCAGT
N728-B	CAAGCAGAAGACGGCATAACGAGATT <b>AGCTGCA</b> GTGACTGGAGTTCAGACGTGTG	TGCAGCTA
N729-B	CAAGCAGAAGACGGCATAACGAGAT <b>GACGTCG</b> AGTGACTGGAGTTCAGACGTGTG	TCGACGTC

Index Name	Index 2 (i5)	Index sequence
S502-A	AATGATACGGCGACCACCGAGATCTACAC <b>CTCTCT</b> ATACACTCTTTCCCTACACGACGC	CTCTCTAT
S503-A	AATGATACGGCGACCACCGAGATCTACACT <b>TATCCTCT</b> ACACTCTTTCCCTACACGACGC	TATCCTCT
S505-A	AATGATACGGCGACCACCGAGATCTACAC <b>GTAAGG</b> AGACACTCTTTCCCTACACGACGC	GTAAGGAG
S506-A	AATGATACGGCGACCACCGAGATCTACAC <b>ACTGCATA</b> AACTCTTTCCCTACACGACGC	ACTGCATA
S507-A	AATGATACGGCGACCACCGAGATCTACAC <b>AAGGAGTA</b> AACTCTTTCCCTACACGACGC	AAGGAGTA
S508-A	AATGATACGGCGACCACCGAGATCTACAC <b>CTAAGCCT</b> AACTCTTTCCCTACACGACGC	CTAAGCCT
S510-A	AATGATACGGCGACCACCGAGATCTACAC <b>CGTCTAAT</b> AACTCTTTCCCTACACGACGC	CGTCTAAT
S511-A	AATGATACGGCGACCACCGAGATCTACACT <b>CTCTCCG</b> AACTCTTTCCCTACACGACGC	TCTCTCCG
S513-C	AATGATACGGCGACCACCGAGATCTACACT <b>CGACTAG</b> AACTCTTTCCCTACACGACGC	TCGACTAG
S515-C	AATGATACGGCGACCACCGAGATCTACACT <b>TTCTAGCT</b> AACTCTTTCCCTACACGACGC	TTCTAGCT
S516-C	AATGATACGGCGACCACCGAGATCTACAC <b>CCTAGAGT</b> AACTCTTTCCCTACACGACGC	CCTAGAGT
S517-C	AATGATACGGCGACCACCGAGATCTACAC <b>GCGTAAGA</b> AACTCTTTCCCTACACGACGC	GCGTAAGA
S518-C	AATGATACGGCGACCACCGAGATCTACAC <b>CTATTAAG</b> AACTCTTTCCCTACACGACGC	CTATTAAG
S520-C	AATGATACGGCGACCACCGAGATCTACAC <b>AAGGCTAT</b> AACTCTTTCCCTACACGACGC	AAGGCTAT
S521-C	AATGATACGGCGACCACCGAGATCTACAC <b>GAGCCTTA</b> AACTCTTTCCCTACACGACGC	GAGCCTTA
S522-C	AATGATACGGCGACCACCGAGATCTACACT <b>TATGCGA</b> AACTCTTTCCCTACACGACGC	TTATGCGA

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Sample_ID	Sample_Name	Sample_Plate	Sample_Well	I7_Index_ID	index	I5_Index_ID	index2
1M	1M			N701-A	TAAGGCGA	S502-A	CTCTCTAT
2M	2M			N702-A	CGTACTAG	S503-A	TATCCTCT
3M	3M			N703-A	AGGCAGAA	S505-A	GTAAGGAG
4M	4M			N704-A	TCCTGAGC	S506-A	ACTGCATA
5M	5M			N705-A	GGA CTCCT	S507-A	AAGGAGTA
6M	6M			N706-A	TAGGCATG	S508-A	CTAAGCCT
7M	7M			N707-A	CTCTCTAC	S510-A	CGTCTAAT
8M	8M			N710-A	CGAGGCTG	S513-C	TCGACTAG
9M	9M			N711-A	AAGAGGCA	S502-A	CTCTCTAT
10M	10M			N712-A	G TAGAGGA	S503-A	TATCCTCT
11M	11M			N714-A	GCTCATGA	S505-A	GTAAGGAG
12M	12M			N701-A	TAAGGCGA	S506-A	ACTGCATA
13M	13M			N702-A	CGTACTAG	S507-A	AAGGAGTA
14M	14M			N703-A	AGGCAGAA	S508-A	CTAAGCCT
15M	15M			N704-A	TCCTGAGC	S510-A	CGTCTAAT
16M	16M			N705-A	GGA CTCCT	S513-C	TCGACTAG
17M	17M			N706-A	TAGGCATG	S502-A	CTCTCTAT
18M	18M			N707-A	CTCTCTAC	S503-A	TATCCTCT
19M	19M			N710-A	CGAGGCTG	S505-A	GTAAGGAG
20M	20M			N711-A	AAGAGGCA	S506-A	ACTGCATA



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**Dear Editor**

**We thank you very much for taking your time to review our manuscript and providing us critical suggestions and concerns. We made correction on our manuscript based on your comments as in below Table. We hope these corrections will meet your expectation for the journal publication.**

	<b>Editorial comments</b>	<b>Author's response</b>
1	Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.	✓ We corrected English mistakes and had a native speaker proofread the revision.
2	Corresponding authors are different in the main manuscript (Fumito Maruyama) and the Editorial software (Kyoko Yarimizu, where the authors provide input while uploading the manuscript). Please clarify.	✓ We clarified on the manuscript top page that Fumito and Kyoko are co-corresponding authors.
3	Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).	✓ We removed personal pronouns. We kept " <u>our</u> conventional Chilean HAB monitoring programs" because it must be distinguished from other research groups' monitoring methods.
4	JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. Please sort the Materials Table alphabetically by the name of the material.	✓ We transferred commercial languages to Table of Materials. ✓ Table of Materials was sorted by product name in alphabetic order.
5	Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes.	✓ We removed bullet points and replaced by numbering.
6	Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Accordingly, please include Step 1.1 in the Introduction or in the Discussion section since this is not an action step	✓ We changed the protocol section with imperative tense. ✓ Alternatively, we used "NOTE". ✓ Protocol Step 1.1. Overview was moved to Introduction as suggested.
7	Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material	✓ The video is provided with the revised manuscript.

	specifying how to perform the protocol action. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol	
8	<p>Please add more details to your protocol steps:</p> <p>Step 1.2.2/1.2.4: Please provide the reagents for the PCR reaction in a tabular form. Also, provide details of the primers used.</p> <p>Line 151: Please mention the step number where the DNA sample is obtained.</p> <p>Line 155: How long can the sample be stored?</p> <p>Line 156: Please specify all gel running conditions throughout V/cm, temperature, etc.</p> <p>Line 189: Please provide more details regarding the concentration measurement if this step needs to be filmed</p>	<p>✓ We created Table 2 for PCR master mix, Table 3 for primer sequence, Table 4 for PCR cycles, Table 5 for Index sequences, Table 6 for index position, and Table 7 for example of sample sheet.</p> <p>✓ Step numbers were reassigned.</p> <p>✓ We clarified that DNA sample storage and sequencing samples must not exceed over 1 month and 1 week, respectively.</p> <p>✓ The gel condition “100 volts 30 min” was added.</p> <p>✓ DNA concentration can be measured by any spectrophotometer. Thus, we simply added the device number we used in the Material Table.</p>
9	Please provide the dilution solvents throughout.	<p>✓ We clarified all the dilution solvents, mostly PCR grade water (only one part with TE buffer).</p>
10	In the software, please ensure that all button clicks and user inputs are provided throughout. Also, please ensure that the button clicks are bolded.	<p>✓ Button clicks, software commands, user inputs were added.</p>
11	Please include the details of all the instruments/ reagents/ softwares/ specifications in the Table of Materials.	<p>✓ We updated the Table of Materials.</p>
12	Please include a one-line space between each protocol step and then highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Also, please ensure that it is in line with the title of the manuscript. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.	<p>✓ We added a line between steps.</p> <p>✓ We highlighted the steps used in the video.</p>
13	Figure 5A/5B: Please submit as one JPEG/PDF file. Else Figure 5B can be made Figure 6.	<p>✓ We separated the figures into Figure 5 and Figure 6.</p>
14	Please spell out the journal titles in the References.	<p>✓ All journal names are fully spelled out.</p>

**Dear Reviewer #1**

**We thank you very much for taking your time to review our manuscript and providing us critical suggestions and concerns. We made correction on our manuscript based on your comments, and our answers are written below in green. We hope these corrections will meet your expectation for the journal publication.**

Reviewer #1:

Manuscript Summary:

Described common molecular markers 18S rDNA and 16S rDNA-based metabarcoding analysis for monitoring Harmful Algal Blooms in Chile. Indeed, this method should be applicable to all ocean regions, not only Chile. This method is complementary to existing methods, including morphology-based microscopic observation, and toxin-based analysis. This method is particularly suited for HAB species that are small and are unstable during analysis. It is also a desirable method for detecting HAB cells with low abundance.

Major Concerns:

1. The authors should be clear about databases used for the annotation of HAB species, highlighting the weakness and limitation of the current status of the reference databases. Reference sequences of many HAB species may be missing from the current databases. What is the consequence of such incompleteness?

Thank you for the comments. We incorporated your comments as follows: In section 4.2., we clarified that taxonomy assignment is performed using the SILVA rRNA database for DADA2 (silva\_nr\_v132\_train\_set.fa.gz is set as a default). In discussion, we added the limitation of the database: Newly discovered nucleotide sequences for bacterial and algal species are updated daily. The well-studied algal and bacterial species have reliable sequences in databank. On the other hands, there are many sequences for unknown species in databank, meaning that the resolution of nucleotide sequences are not always enough for species identification (only good for genus identification). Thus microscopic species identification must be orthogonally performed.

2. The resolution of the molecular markers used in this project. Did the authors used the V4 of 18S rDNA, and the V3-V4 of the 16S rDNA?

We used primers for V3-V4 of 16S rDNA and V7-V9 for 18S rDNA.

Regarding resolution, it varies depending on the primer locations (some areas cannot be analyzed at the species level but up to the genus level). Additionally, the resolution is also influenced by how well the species have been studied and registered in databank. Thus, orthogonal analytical method such as microscopy is required to incorporate with metagenome analysis.

For some species, other variable regions may provide better resolution. However, the higher resolution can falsely provide bacteria and archaea names with eukaryotes analyses. On the other hands, there are universal primers targeted for bacteria that do not identify all bacterial species. Thus, setting the resolution cut-off is on-going debates in many studies. In any cases, it is often considered that universal primers are good to identify genus of target organism.

3. The authors should pay attention to the relative abundances of the top most abundant species in water samples. How does the relative abundances correspond to the HAB level?

Metabarcoding analysis provides qualitative data and relative ratio to the total reads. Thus, the relatively most abundant species does not indicate a bloom or bloom level. We estimate a bloom level with microscopy, and alternatively we measure chlorophyll-a concentration, which provide roughly estimated phytoplankton biomass. Then, we use the metabarcoding data for species/genus content of the bloom time. The most abundant species in a bloom sample should match between microscopy and metabarcoding analyses, based on the assumption that reliable base sequences are registered in the databank.

**Minor Concerns:**

The manuscript is generally acceptable for JoVE.

Thank you very much for the encouraging comment.

**Dear Reviewer #2**

**We thank you very much for taking your time to review our manuscript and providing us critical suggestions and concerns. We made correction on our manuscript based on your comments, and our answers are written below in green. We hope these corrections will meet your expectation for the journal publication.**

Reviewer #2:

Manuscript Summary:

The manuscript provides details on the application of a previously developed metabarcoding HAB-monitoring protocol to Chilean monitoring programs. These methods are relatively well-documented in the literature. It will be useful for those working on monitoring to have a visual protocol to help them set-up an important tool to better understand HAB development and composition. Overall, the manuscript is well-written, easy to follow and descriptive enough.

Major Concerns:

The manuscript is missing quality control data (number of reads, filtered reads, OTUs assigned and at what level, number of singletons excluded, etc.) for the metabarcoding. This should be presented in a Table. This will be important as a QC reference to those implementing the protocol in their own labs.

**Thank you for the comments. We made a table containing the QC parameter and results and added to the supplement.**

Minor Concerns:

Figures are low quality and with serif fonts.

16S and 18S (lines 146-147) should be 16S rRNA and 18S rRNA.

**Thank you for the comments. The higher quality of figures will be provided when the manuscript is accepted for publication.**

**We corrected the word 16S and 18S to 16S rRNA and 18S rRNA.**

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