JPR Advance Access published May 8, 2014

Journal of
Plankton Research

plankt.oxfordjournals.org

J. Plankton Res. (2014) 0(0): 1–5. doi:10.1093/plankt/fbu033

SHORT COMMUNICATION

Sampling the limits of species richness in marine phytoplankton communities

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Received November 15, 2013; accepted April 4, 2014

Corresponding editor: John Dolan

We examined large volumes of seawater under the microscope to explore the limits of phytoplankton diversity in a highly dynamic coastal ecosystem. Our analysis showed that conventional sample volumes severely underestimate the species richness of these phytoplankton communities. The number of species observed doubled after a 10-fold increase in sample volume, implying that estimates of phytoplankton species richness depend critically on sampling effort. The volume of sample needed to detect 90% of the species varied between 0.25 and 1 L depending on the concentration of phytoplankton biomass.

KEYWORDS: phytoplankton; sample size; species-accumulation curve; diversity

Traditional methods for the estimation of marine phytoplankton species richness consist of collecting a small volume of seawater which is analysed under the microscope (Lund *et al.*, 1958; Utermöhl, 1958; Sournia, 1978). Typically, the sample volume varies between 5 and 100 mL depending on the concentration of photosynthetic biomass. This method is founded on the assumption that the abundance of phytoplankton species and thus the probability of sampling them increases with the biomass of the community. However, a large body of evidence shows that

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microbial plankton communities both prokaryotic and eukaryotic contain a large pool of rare species with low population abundances (Pedrós-Alió, 2006; Sogin *et al.*, 2006; Caron and Countway, 2009). This observation suggests that improved sampling effort (e.g. larger sample sizes) could retrieve a higher number of species from local phytoplankton communities. However, a quantitative assessment of this effect has not been conducted yet for phytoplankton, casting doubt on the validity of estimates of species richness and data set inter-comparisons (Cermeño *et al.*, 2013).

Species accumulation curves and rarefaction analyses depict the way species richness increases with increasing sampling effort (Gotelli and Colwell, 2001; Magurran, 2004). The result is a curve that increases steeply at first, and then gradually levels off. For decades, terrestrial ecologists have routinely performed this sort of analysis with the aim of defining the optimal sample size, i.e. the minimum sample size needed to obtain meaningful estimates of community diversity. Yet, these methods have been rarely applied by phytoplankton ecologists. Previous studies reported species accumulation curves for phytoplankton and zooplankton by pooling together samples collected from different sampling sites and/or at different times (Margalef, 1969; Shurin *et al.*, 2007; Raybaud *et al.*, 2009; Korhonen *et al.*, 2011; Olli *et al.*, 2013). However, environmental conditions change across space and through time limiting the interpretation of these curves to a combination of species belonging to different spatially structured communities and/or stages of the seasonal succession. A recent study shows that the number of species observed increases notably by pooling samples collected simultaneously from the same sampling device (Rodríguez-Ramos *et al.*, 2014). The number of species kept increasing even after inspecting a total of 500 mL of seawater, emphasizing the need to examine larger sample volumes to establish sampling protocols more adequate to assess the total number of species. Here, we present species accumulation curves for marine phytoplankton from sample volumes exceeding 10 L of seawater and spanning a full annual cycle. Our objectives are (i) to quantify the extent to which traditional sampling methods underestimate the species richness of marine phytoplankton communities, (ii) to determine whether the extent of undersampling varies with the amount of photosynthetic biomass present in the system, and (iii) to establish the optimal sample volume needed to detect a given fraction of the species present in the community.

Nine oceanographic cruises were carried out on board the R/V Mytilus to a central station at Ría de Vigo, NW-Iberian Peninsula (42 14.09°N, 8 47.18°W), where the depth is 45 m at low tide. Sampling was scheduled from February to November 2012 on a monthly basis in order to obtain data from different phases of the annual phytoplankton cycle. On each visit, we recorded vertical profiles of temperature with a Sea Bird Electronics SBE 9/11 conductivity, temperature, depth (CTD) probe attached to a rosette. The vertical distribution of photosynthetically active irradiance (PAR, 400–700 nm) was measured with a spherical quantum sensor connected to a data logger. Seawater samples were collected from 3, 10, 20, 30 and 40 m depth in 12 L Niskin bottles. For the determination of chlorophyll-*a* (Chl-*a*) concentration, two 250 mL replicates were filtered through 0.2-μm polycarbonate filters using low vacuum pressure (~100 mm Hg). Pigments were extracted by placing the filters in 90% acetone overnight. Chl-*a* concentration was determined fluorometrically using a TD-700 fluorometer that had been calibrated with pure Chl-*a* (Sigma).

Microplankton species were identified in two types of samples: (i) bottle samples and (ii) net samples. For bottle samples, seawater was collected from three selected depths (3, 10 and 20 m) using 12 L Niskin bottles as specified above. These three samples were dispensed into a 20 L container and gently mixed to obtain a combined sample of the water column photic layer. From this combined sample, four subsamples of 500 mL were preserved in Lugol's iodine solution (2% final concentration). Aliquots (5–50 mL) of these subsamples were settled in composite sedimentation chambers and examined with an inverted microscope until a total volume of 500–1000 mL was reached, depending on the concentration of organisms (Utermöhl, 1958). The samples were examined under the microscope for species identification and cell counting. Unknown species were classified according to the morphological descriptions, for example "Medium-sized, dark, thecate dinoflagellate". This nomenclature was consistent throughout the study.

Net samples were collected from vertical hauls (from 20 m to surface) with a plankton net (20-μm mesh size) and preserved in Lugol's iodine solution (2% final concentration). Taking into account the volume of seawater filtered through the net and the volume collected, aliquots corresponding to water column volumes of between ~500 and 9000 mL were examined using an inverted microscope. This procedure was repeated until a total visualized volume of ~25 L was reached for each sampling day. Net samples were examined with the objective of detecting species not seen in bottle samples and thus cell counting was not carried out. A total of 290 subsamples including bottle and net samples were inspected under the microscope.

The oceanographic conditions in the Ría de Vigo during the sampling days included winter mixing, spring bloom, summer upwelling and autumn downwelling. The depth of 1% optical light varied between 15 and 25 m. The Chl-*a* concentration at the surface was in the range 0.5 to >10 mg m^{-3} with lower values during

winter and higher in spring and summer matching the spring bloom and summer upwelling, respectively.

Figure 1 shows the species accumulation curves for each sampling day representing sampling effort as total volume of seawater inspected. These curves were constructed by combining data on species occurrences obtained from bottle and net samples. We fitted three mathematical models (negative exponential, Hill and Weibull) and the best fitting model was selected according to Akaike Information Criterion (Supplementary data). In all cases, the increase in number of species $y(x)$ with increasing sampling effort x was best described by the Weibull function,

$$y(x) = a[1 - \exp(-bx^c)]$$

where a, b and c are fitted coefficients, a being the maximum number of species predicted by the model (the asymptotic value). These results provide evidence in support of an appropriate model of species accumulation. However, there is no guarantee that the Weibull model would consistently characterize species accumulation curves for phytoplankton communities in different environments.

Conventional sample sizes, typically within the range of 5–100 mL, produced severe underestimates of species richness. Figure 2 shows the number of undetected species in conventional sample sizes relative to a, the total number of species predicted by the mathematical function described above (i.e. the asymptote of the curve). For instance, during summer upwelling conditions roughly

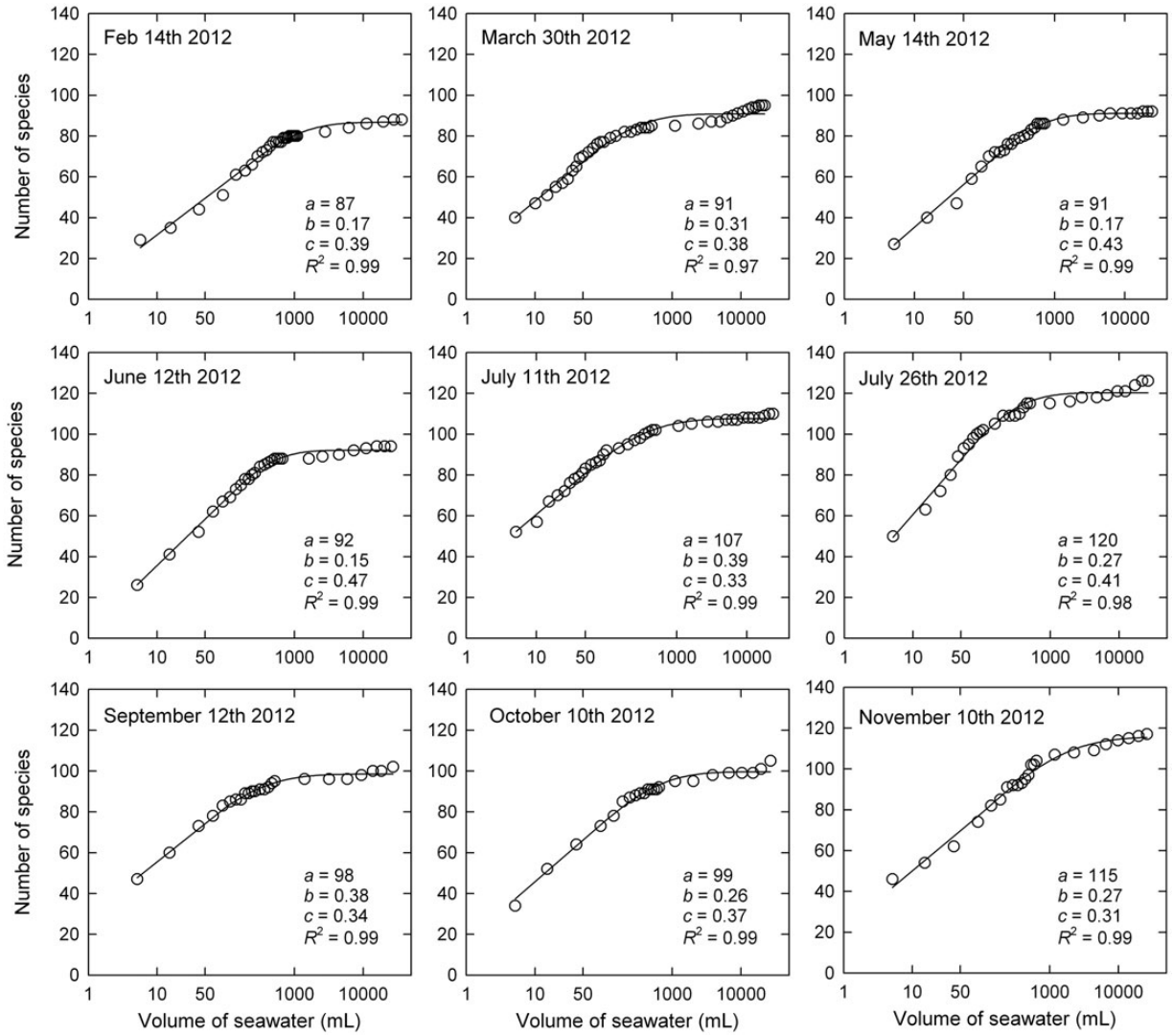

Fig. 1. Species accumulation curves of phytoplankton communities sampled in the Ría de Vigo from February to November 2012. Sampling volume is represented on a log-scale. Statistical values on each panel refer to the parameters of the Weibull function described in the text (see also Supplementary data for details).

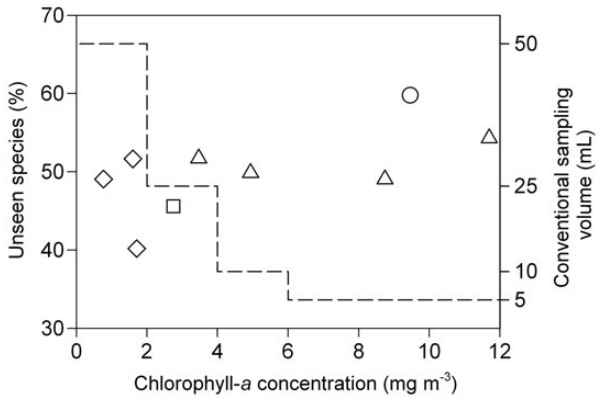

Fig. 2. Undetected species (%) in conventional sample volumes plotted against the concentration of Chl-*a* in seawater (symbols). Each data point represents a sampling day including winter mixing (diamonds), spring bloom (circle), summer upwelling (triangles) and autumn downwelling (square). Conventional sample volumes are depicted as a function of the Chl-*a* concentration (dashed line). It must be noted that the volume of seawater examined decreases with increasing productivity. This partially reduces the differences in the number of undetected species at different concentrations of Chl-*a*.

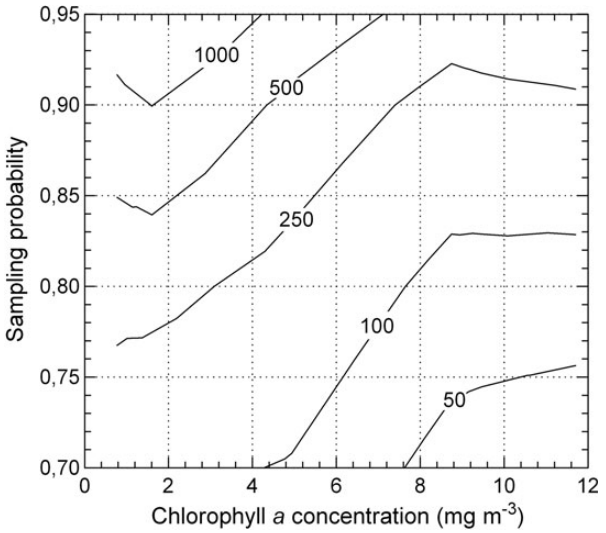

Fig. 3. Sampling probability at different sample volumes (contour labels in millilitres) plotted against the concentration of Chl-*a*. Using conventional volumes (<100 mL), sampling probabilities never exceeded 0.83. To increase the sampling probability and attain a higher percentage of the total number of species present in the community larger volumes must be examined.

50% of the species were not detected by conventional sampling volumes (5–25 mL of seawater). Similar results were obtained during winter mixing conditions and autumn downwelling despite the fact that larger volumes of seawater (25–50 mL) were examined.

We defined sampling probability as the number of species observed in a sample relative to *a*, the total number of species in the community. To increase the sampling probability and hence detect a higher percentage of the total number of species, larger volumes must be examined. We analysed the effect of increasing sample size on sampling probabilities (Fig. 3). For instance, to attain a sampling probability of 0.9 (i.e. 90% of the species detected), we estimated a minimum sample size of ca. 0.25 and 1 L for waters containing high and low Chl-*a* concentrations, respectively (Fig. 3). For a given sample size, the sampling probability increased with the concentration of Chl-*a*. Therefore, for this particular ecosystem, Fig. 3 allows the optimal sample size to be defined by knowing the Chl-*a* concentration.

The species accumulation curves presented here demonstrate that conventional sampling methods underestimate by >40% the species richness of marine phytoplankton communities in a highly dynamic coastal ecosystem (Fig. 2). Rodríguez-Ramos *et al.* (Rodríguez-Ramos *et al.*, 2014) found similar results by performing individual-based rarefaction analyses over two data sets collected during winter mixing and spring bloom conditions in the Ría de Vigo. They show that the main cause of underestimation is related to a systematic undersampling of rare species in small (conventional) sample sizes. Their analysis reveals that even among subsamples collected from the same sampling device, the composition of species differed by >50% (Rodríguez-Ramos *et al.*, 2014). Thus, to obtain better estimates of species richness, it is necessary to examine larger volumes. However, settling large volumes for microscopic analysis is problematic as large amounts of particles can pile up on the plate, hindering the detection of species and making counting protocols difficult. We recommend maintaining traditional sampling/counting methods and repeating the analyses (subsamples) until optimal sample sizes are reached, which must be defined previously by constructing species accumulation curves. Ultimately, quantifying the numerical abundance of rare species (not only their presence) in marine microbial communities is essential to better understand their dynamics and functional role. This will require much larger volumes of seawater than those used in the present study.

Our results illustrate the effect of increasing sample size on estimates of species richness for communities in a highly dynamic coastal ecosystem. The extent to which these results are applicable to other aquatic ecosystems depends largely on the amount of rare species present in the community (Rodríguez-Ramos *et al.*, 2014). Rare species are a common component of marine phytoplankton communities, yet their frequency across ecosystems remains underexplored. We speculate that the effects of undersampling might be more dramatic in open ocean ecosystems wherein (micro)phytoplankton communities are dominated by species with very low population abundances (Cermeño *et al.*, 2006) and thus harder to detect in small sample sizes. A number of non-parametric

estimators of species richness have been proposed to minimize the effect of sample size and obtain estimates of species richness from incomplete censuses (Magurran, 2004). These estimators are based on the assumption that the number of rare species found in a sample can be used to calculate how likely it is there are more undiscovered taxa. However, these estimators that *a priori* are insensitive to sample size produced estimates of species richness distinctly lower than those obtained from the species-accumulation curves (Supplementary data).

Our methodological concerns are important primarily for studies of phytoplankton species richness. Nevertheless, traditional sampling methods remain valid for the study of other properties of marine phytoplankton communities such as the determination of community biomass, the description of dominant patterns of community composition or studies of diversity dynamics based on metrics that give more weight to dominant taxa. These properties are largely unaffected by the addition of rare species and thus their undersampling is not expected to bias the results.

A major issue with estimates of species richness is that sample sizes are smaller than the size of the communities. Our results suggest the need to perform species accumulation curves and rarefaction analyses to define optimal sample sizes. Improved sampling methods will provide more accurate (and higher) estimates of species richness and sampling standardized data sets. Other issues such as wide ranging capabilities to recognize microalgae, different sampling strategies and scales or the commonness of cryptic species in these microbial plankton communities limit our ability to produce reliable estimates of phytoplankton diversity from field data. Addressing all these issues is necessary to gain a better understanding of the mechanisms underlying the assembly of marine phytoplankton communities and the dynamics of diversity across space and through time.

SUPPLEMENTARY DATA

Supplementary data can be found online at http://plankt.oxfordjournals.org.

ACKNOWLEDGEMENTS

The authors thank M.P. Lorenzo and the crew of the R/V Mytilus for their help during the work at sea and sample processing. T. Rodríguez-Ramos helped with statistical analyses. Four anonymous reviewers provided valuable comments on the article.

FUNDING

The research was funded by the Spanish Ministry of Economy and Competitiveness through project DISTRAL (CTM2011-25035).

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