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Assessment of microbial plankton diversity as an ecological indicator in the NW Mediterranean coast

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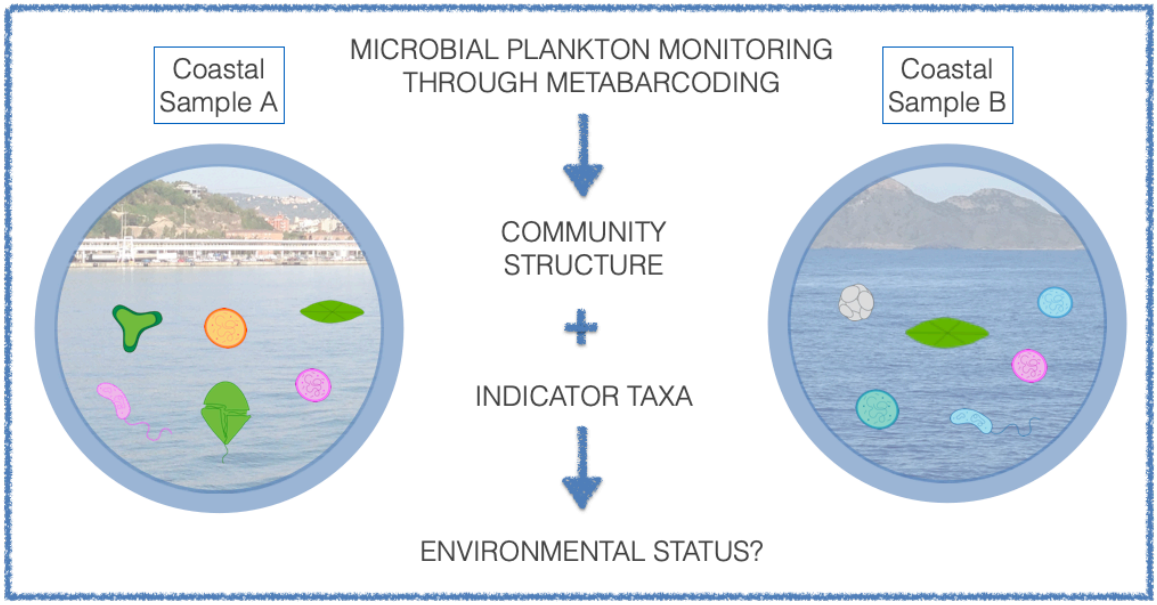
Research highlights

- We evaluated microbial plankton diversity as an ecological indicator in NW Mediterranean coastal waters using metabarcoding of rRNA genes
- Studied samples were subjected to varying degrees of continental pressures
- Diversity metrics from microbial eukaryotic communities displayed more suitability to be used as indicators than those of prokaryotes
- Few microbial planktonic taxa (both from prokaryotes and eukaryotes) showed potential as indicators
- Implementing fast and simple ecological indicators from pico- and nanoplankton diversity is challenging due to the complexity and dynamics of the pelagic communities

Graphical abstract



Abstract

High-throughput sequencing of microbial assemblages has been proposed as an alternative methodology to the traditional ones used in marine monitoring and environmental assessment. Here, we evaluated pico- and nanoplankton diversity as ecological indicators in NW Mediterranean coastal waters by comparing their diversity in samples subjected to varying degrees of continental pressures. Using metabarcoding of the 16S and 18S rRNA genes, we explored whether alphadiversity indices, abundance of Operational Taxonomic Units and taxonomic groups (and their ratios) provide information on the ecological quality of coastal waters. Our results revealed that only eukaryotic diversity metrics and a limited number of prokaryotic and eukaryotic taxa displayed potential in assessing continental influences in our surveyed area, resulting thus in a restrained potential of microbial plankton diversity as an ecological indicator. Therefore, incorporating microbial planktonic biodiversity in environmental assessment could not always result in a significant improvement of current marine monitoring strategies.

Keywords: Plankton diversity, high-throughput sequencing, coastal marine monitoring, ecological indicators, anthropogenic pressures, eutrophication

Introduction

Oceans provide ecosystem services to society in a myriad of ways, from the regulation of the planet's climate to providing resources for human survival and well-being (Liquete et al., 2013). Human-modified coastal areas are experiencing increasing threats due to a continuously growing human population that accelerates resource use, waste production and environmental degradation. For instance, run-off of pollutants and nutrients arriving to coastal waters may alter natural ecosystems by changing productivity and food web dynamics or shifting species distributions among other impacts of unknown consequences (Halpern et al., 2007; Halpern et al., 2008; Hoegh-Guldberg and Bruno, 2010). All biological components of marine ecosystems may be affected by the consequences of human activities, from microbes to large animals (Davidson et al., 2012; Gall and Thompson, 2015; Cavicchioli et al., 2019). Given the importance of the marine ecosystem for the functioning of our planet and for our own welfare and its vulnerability to human impacts, there is a need to report on its condition and on the responses to the exerted pressures. In fact, numerous initiatives regarding the management of the marine environment have been or are being implemented worldwide in order to protect our seas and oceans (e.g., United Nations Convention on the Law of the Sea, the Marine Strategy Framework Directive in Europe or the Oceans Act in the USA, besides several local initiatives) (Birk et al., 2012).

The European Marine Strategy Framework Directive (MSFD, 2008/56/EC) requires European states to maintain their marine waters in 'Good Environmental Status' (GES). The MSFD includes 11 descriptors of GES: biological diversity, marine food webs, seafloor integrity, non-indigenous species introduction, fisheries, human-induced eutrophication, alteration of hydrographical conditions, concentrations of contaminants, contaminants in fish and other seafood, marine litter and introduction of energy and noise. For each descriptor, the status of the marine environment must be assessed using ecosystem criteria and indicators. There are currently multiple indicators being

applied to the MSFD, some of them previously used under the European Water Framework Directive (WFD; 2000/60/EC), such as phytoplankton abundance and zoobenthos species composition, for eutrophication and biodiversity respectively (Borja et al., 2010; Camp et al., 2018). However, in the first case for example, the complexity of interactions between phytoplankton structure and physical, chemical and biological factors hinders the establishment of well-defined relationships between pressures and impacts, and therefore, effective management strategies. In fact, initial assessments during the first implementation phase of the MSFD revealed a general lack of operational indicators (Hummel et al., 2015) and thus, the need to develop alternative and innovative ones that can be implemented in a simple, fast and cheap manner (Caruso et al., 2015). In this regard, adding genetic diversity in marine monitoring is gaining attention and showing promising results, particularly in sediments. For example, the use of genomic-based indices has been proposed as an alternative to the macrobenthos biotic indices commonly applied to coastal waters (Aylagas et al., 2016; Pawlowski et al., 2018). Moreover, using microbial community composition has recently been considered in biomonitoring beyond the traditional use of fecal microorganisms as indicators of contamination (Caruso et al., 2015; Danovaro et al., 2016).

Marine microbes are essential in marine biogeochemical cycles and vital for the functioning of food webs, besides being substantial contributors to global marine biodiversity (Gasol and Kirchman, 2018). These organisms are known to respond rapidly to perturbations, such as increase in nutrient loads or events of acute contamination (Nogales et al., 2011). Placing microbial communities at the base of management decisions has gained attention in recent years, particularly after the advent of molecular approaches and high-throughput sequencing (HTS) that allow to overcome the limitation of identifying environmental microbes. A new and promising genomic-based microbial index was proven to correlate well with sediment quality and could be used to assess the ecological status of estuarine and coastal sediments (Aylagas et al., 2017). Likewise,

diversity surveys of benthic bacterial and protist communities based on DNA sequencing seem to be useful in environmental assessments of fish farming, an industry having serious environmental impacts in marine habitats (Pawlowski et al., 2014; Stoeck et al., 2018).

Contrary to sediments, in which pollution is deposited and accumulated over time, pelagic ecosystems are much more dynamic which, comparatively, makes the determination of environmental status potentially more challenging. In fact, despite the increasing knowledge on the composition of plankton communities in recent times, their use for assessment of environmental status in marine waters is only beginning to be explored. Recently, Pearman et al. (2018) evaluated plankton communities in anthropogenically impacted oligotrophic coastal regions of the Red Sea and concluded that studying changes in the composition of microbial communities could be used to complement the existing approaches used to examine the multiple stresses affecting coastal areas. Nonetheless, given the limited information existing for pelagic ecosystems, more studies are required to better evaluate the usefulness of including small planktonic communities in the assessment of anthropogenic impacts in marine ecosystems.

In this study we explore pico- and nanoplankton diversity as an ecological indicator in the North-western Mediterranean coast. Beforehand, we had compared the performance of two distinct HTS methodologies to study marine picoplanktonic biodiversity and explored their use in ecosystem health assessment (Ferrera et al., 2016). This initial study revealed that certain taxa, as well as the ratio between the abundances of some bacterial groups, had potential for being useful indicators. Yet, the study was limited to a single location – the coast of Barcelona – at a single time point and more extensive surveys were needed to further evaluate the robustness of these findings. Here, we have tested the applicability of microorganisms as operational GES indicators in a survey of 6 locations across the Catalan and Balearic coasts subjected to varying degrees of

continental pressures. In particular, we have explored whether diversity and richness indices, the relative abundance of OTUs (Operational Taxonomic Units) and taxonomic groups, as well as the ratios between the abundances of different planktonic groups respond to coastal impacts thus providing information on the ecological quality of NW Mediterranean coastal waters.

2. Methods

2.1. Study sites.

Surface water samples were collected from six locations located along the Catalan and Balearic coastal areas (Figure 1) that are representative of the NW Mediterranean coast in terms of geography, demography and socioeconomic activities. The choice of these coastal sites was based on previous characterization of the areas in the context of the Water Framework Directive (Table S1; Flo et al. 2011, 2017, 2019) and on Basterretxea et al. (2018). The six areas covered a variety of continental pressures and putatively receive variable nutrient loads and other pollutants from urban, industrial and agricultural activities (domestic waste, organic and inorganic nutrient enrichment among others). First, four cross-shore transects were undertaken in Palma de Mallorca, L'Estartit, L'Hospitalet de l'Infant and Barcelona. Sampling was conducted in summer (June-July 2014 for the Catalan Coast and July 2015 for the Balearic Coast) when temperatures are warm and there is a lack of tidal mixing (Basterretxea et al., 2018). Palma (39°32'N 2°43'E) is an intensive agricultural area in the island of Mallorca with reported nutrient rich groundwater seeps along the shoreline (Rodellas et al., 2014; Tovar-Sánchez et al., 2014). The L'Estartit (42°01'N 3°12'E) coastal area drains from a wetland with some agricultural activity and is also influenced by the Ter river, a low flow nitrate-rich Mediterranean river. L'Hospitalet de l'Infant (40°58'N 0°54'E) is a sparsely populated region with dry land agriculture. While groundwater seeps from nearby coastal aquifers (Fernández Ruiz, 2012), nutrient concentrations along the coast are lower than at the

previously mentioned agricultural areas. Barcelona is a hypothetically more impacted site since it is a highly developed urban area with a population of ~3.2 million inhabitants in the metropolitan area. From each of these four sites, ~10 surface samples were collected from the coastline to about 4-6 miles offshore. In the area of Barcelona, two additional cross-shore transects of 5 samples conducted in June and August 2013 around the PUDEM Coastal Ocean Observatory monitoring station (Arin et al., 2013) have been included in this study, one of them corresponding to the samples analyzed in Ferrera et al. (2016). Sampling cross-shore transects could reveal a continental pressure gradient even within samples collected in one area, since those taken near the coast are presumably more prone to be affected than the corresponding offshore samples. Besides these coast-to-offshore samplings, a transect of 4 stations was conducted in July 2014 in the estuarine Alfacs Bay, located in the Ebro Delta (40°38'N 0°43'E). This represents one of the most riverine-influenced areas of the Catalan coast and was selected to include samples subjected to a large agricultural influence.

In addition to these spatial gradients, samples from two time-series monitoring stations covering contrasting urban scenarios were included in the survey. The Blanes Bay Microbial Observatory (41°40'N 2°48'E) is a coastal oligotrophic site subjected to low anthropogenic pressures (Gasol et al., 2016). The sampling station is located near the town of Blanes of ~40.000 inhabitants; natural disturbances are not frequent in this site since the closest river flows south of the monitoring station and its discharges are taken away by a predominantly south-west surface current. Samples collected from 2004 to 2013 were available for this study (but we excluded those from 2010-2012 due to construction of a nearby harbor during this period). The second location is the abovementioned PUDEM Station, off the coast of Barcelona. Samples collected at this site in 2014 were available for our study. Although monthly sampling is typically conducted in these two monitoring stations, only samples from May to September were used in the analyses to avoid natural seasonal variability from masking the potential

differences found between areas. A total of 93 samples were included in the analyses. Basic environmental data associated to the samples are shown in Table 1.

2.2. Sample collection

About 2 L of 200-μm pre-filtered surface seawater were collected and immediately transported to the laboratory where they were sequentially filtered through a 20-μm mesh followed by a 3-μm and a 0.2-μm pore-size polycarbonate filter (Poretics) using a peristaltic pump. The aim of the serial filtration was to obtain two different microbial size fractions, picoplankton from 0.2 to 3 μm and nanoplankton from 3 to 20 μm. The size filtering separates eukaryotic organisms of different sizes, while in the case of prokaryotes it mostly separates free-living (0.2–3 μm) from particle-attached (3–20 μm) cells (Acinas et al., 1999). Filters were kept at -80°C until processed. Cells were lysed using lysozyme, proteinase K and sodium dodecyl sulfate, and nucleic acids were extracted with phenol and concentrated in an Amicon 100 (Millipore), as described in Massana et al. (1997). The DNA was quantified spectrophotometrically (Nanodrop, Thermo Scientific), and a subsample was sent for sequencing to the Research and Testing Laboratory (rtlgenomics.com/).

A suite of environmental parameters was measured during sample collection. Temperature and salinity were measured with a CTD probe, the concentrations of inorganic nutrients were determined spectrophotometrically using an Alliance Evolution II autoanalyzer according to standard procedures (Grasshoff et al., 1983). In addition, distance to the coastline and freshwater content were taken into account in the analyses. Freshwater content was obtained from the salinity in the water in relation to the maximum salinity in the dataset as follows:

$$\text{Freshwater content} = 1000 - (1000 * S) / \max(S) \text{ where } S \text{ is salinity}$$

2.3. Sequencing and sequence processing

Both Bacteria and Eukarya were amplified from the two size fractions collected. Primers 341F (5'-CCTACGGGNGGCWGCAG-3'; Herlemann et al., 2011) and 806RB (5'-GGACTACNVGGGTWTCTAAT-3'; Apprill et al., 2015) were used to amplify the V3-V4 region of the bacterial 16S rRNA gene, whereas eukaryotic primers TAReuk454FWD1 (5'-CCAGCASCYGCGGTAATTCC-3') and TAReukREV3 (5'-ACTTTCGTTCTTGATYRA-3') (Stoeck et al., 2010) were used to amplify the V4 region of the 18S rRNA gene. Amplicons were sequenced in an Illumina MiSeq 2 x 250 flow cells following protocols described elsewhere (Cúcio et al., 2016).

Illumina reads of both 16S and 18S rRNA genes underwent quality filtering before being analyzed through a custom made pipeline (Logares, 2017). Spades software (Nikolenko et al., 2013) was used to correct errors that may had arisen in the sequencing process; R1 (forward) and R2 (reverse) reads were merged using Pear (Nurk et al., 2013) and the resulting sequences were filtered by quality (expected errors *per* sequence did not exceed 1) with USEARCH. Then, all reads were put into the same direction using a Hidden Markov Model, concatenated, dereplicated with USEARCH and sorted by abundance. Subsequently, reads were clustered into OTUs (Operational Taxonomic Units) using 97% similarity threshold for prokaryotes and 99% for eukaryotes, and possible chimeras were filtered using the version 119 of the SILVA SSU non-redundant database as reference. Singletons were also discarded as a pre-emptive measure to remove OTUs putatively deriving from sequencing errors. Next, the OTU table was generated and OTUs were taxonomically classified by using BLAST against SILVA v119 for prokaryotes and an in-house database for eukaryotes (EukaryotesV4 database; Obiol et al., 2020). Subsequently, all OTUs classified as chloroplast, mitochondria or Archaea, in the case of prokaryotes, and Metazoan, Streptophyta or Nucleomorphs in the case of eukaryotes, were removed. After filtering, the OTU reads for each sample were rarefied to 5000 reads and the resulting table was used for the diversity and richness indices, whereas the other analyses were carried out using the OTU table with relative abundances. OTUs

were collapsed into the main bacterial and eukaryotic taxonomic groups when needed to explore the relative contribution of each group.

2.4. Data analyses

An arcsine, or angular, transformation was applied to the OTU relative abundances in the non-rarefied table. This transformation equals to the inverse sine of the square root of the proportion transformed again from radians to a proportion value, or:

$$2/\pi * \arcsin(\sqrt{p})$$

where *p* is the relative abundance of an OTU. The arcsine transformation spreads the ends of the scale while compressing the middle, and is recommended by many statisticians for proportion data, often improving normality (Sokal and Rohlf, 1995).

In order to categorize the stations depending on their degree of anthropic pressure, we used the FLU and FAN methods developed and validated by Flo (2017) in the same study area. The approach uses physicochemical variables to assess continental urban and fluvial influences in a given site. The method is based on the following assumptions: i) the main pressures on coastal waters are continental influences, which are linked to freshwater inflows and to the nutrients they release into coastal waters, ii) continental influences, through their nutrient contributions, trigger the production of chlorophyll *a* in coastal waters, which may enhance eutrophication, and iii) continental influences on coastal waters can be of urban or fluvial origin. The FLU index, computed mainly based on silicate and nitrate levels as well as on freshwater content describes a gradient related to fluvial continental influences. The FAN index mainly reflects phosphate, ammonium, and nitrite levels and describes a gradient related to urban continental influences of anthropogenic origin. The method was validated along the Catalan coast using a large time series dataset (1994–2014, N=18,102) and can be applied at different spatial and temporal scales and is reproducible, allowing comparisons across

geographical areas and study periods. The indices were calculated as:

$$\text{FLU index} = 0.86*NO_3 - 0.37*NO_2 - 0.52*NH_4 - 0.89*PO_4 + 1.15*SiO_4 + 0.87*FWC - 2.00$$

$$\text{FAN index} = -0.19*NO_3 + 2.86*NO_2 + 1.42*NH_4 + 2.91*PO_4 - 0.27*SiO_4 - 0.35*FWC - 0.60$$

Based on the values of both indices, all samples were classified into three categories (Low, Medium and High) according to the quartile to which they belong. The values belonging to the first quartile were classified as Low, the ones belonging to the two central quartiles as Medium and the ones belonging to the highest quartile were classified as High (Figure S1).

Statistical analyses were performed using the R statistical software (R Development Core Team, 2015) and the packages *ggplot2*, *reshape2*, *phyloseq*, *magrittr*, *labdsv*, *tidyverse*, *dendextend*, *ggfortify*, *FactoMineR*, *lubridate*, *vegan* and *dplyr*. The Shannon and Chao1 indices, for diversity and for richness estimation respectively (Magurran 1988; Chao and Lee, 1992), were calculated for both plankton size fractions of prokaryotes and eukaryotes. These indices are of common use and were obtained through the *phyloseq* package in R. The values were grouped according to the FLU and FAN index category that each sample falls into. Potential indicator OTUs or taxonomic groups were also explored by calculating the Pearson correlation coefficient between the relative abundance of each OTU or taxonomic group and the FLU and FAN values, as well as the concentration of nutrients in the water samples. Additionally, Indicator Value (IndVal; Dufrêne and Legendre, 1997), which use species (or OTU) fidelity and relative abundance to identify indicator species, were calculated in order to identify potential indicators for the three categories (Low, Medium or High) of the impact indices. The tests were carried out separately for each size fraction, since organisms belonging to the same taxonomic group but with substantially different sizes or lifestyles could respond differently to environmental changes. The *p*-values were corrected through the Holm-Bonferroni method (Holm, 1979) for the number of taxonomic units being tested for potential correlations with nutrient

concentrations or impact index values, in order to avoid having spurious significant *p*-values as a consequence of the high number of tests performed. The IndVal results were capped at *p*-value < 0.05 and IndVal value > 0.3, since this is the value that has been proposed as a threshold for indicating habitat specialization (Dufrêne and Legendre 1997). The results were also filtered by the relative abundance of the analyzed OTUs or taxonomic groups, with a threshold of 0.4% as in Ferrera et al. (2016) since the potential as indicator species of rare OTUs is questionable considering the differences found between sequencing methods (Ferrera et al. 2016) and the known biases of the PCR-based methodologies (Polz and Cavanaugh, 1998). Analysis of variance was used to test for differences in the relative abundance of different taxa depending on the impact index category. *P* values were adjusted by the number of ANOVAs performed. Sequence data has been submitted to the European Nucleotide Archive (http://www.ebi.ac.uk/ena) under accession numbers PRJEB23788, PRJEB38773, PRJEB38800 and PRJEB38808.

Results

Impact indices. For the purpose of categorizing the samples in relation to continental pressures, two impact indices were calculated: the FLU and the FAN indices (Table 1). The values were plotted by location (Figure 2); FLU and FAN values were significantly different among sampling locations (ANOVA, *p* values = $1.76e^{-11}$ and $2.88e^{-13}$ respectively). In particular, samples from Alfacs, L'Estartit and L'Hospitalet de l'Infant displayed higher FLU values than other locations. These sites are located in areas with either riverine (Alfacs, L'Estartit) or groundwater (Alfacs, L'Hospitalet de l'Infant) influence. At the same time, these three locations displayed lower FAN values whilst the values in Barcelona and Blanes were significantly higher than in the other locations (Tukey HSD test at $p<0.05$). FAN values for Barcelona and Blanes were however within the same range despite the diverging continental pressures expected. Palma presented intermediate FLU and FAN values (indicating a mixed influence of urban and freshwater

pressures). The values of the indices were pooled and classified into Low, Medium and High categories (see *Materials and Methods,* Figure S1) in order to explore the response of the biological variables (i.e. diversity data) in relation to these indices. Most samples from Alfacs, L'Estartit and L'Hospitalet de l'Infant fell within the High category of the FLU index. Barcelona and Palma samples belonged mainly to the Medium impact category. For Blanes, FLU values were variable; while many samples fell into the Low category, some of them also belonged to the Medium or High categories. The opposite trend was observed for the FAN index from Alfacs, L'Estartit and L'Hospitalet de l'Infant that fell mostly in the Low FAN category. As for the FLU index, Palma samples were categorized as Medium FAN impact, while Barcelona and Blanes samples were distributed between the Medium and High FAN impact categories.

Diversity indices. Biological diversity is one of the descriptors included in the European MSFD for the assessment of 'Good Environmental Status'. We thus explored whether common alphadiversity metrics (i.e. Chao1 index for richness and Shannon index for diversity) responded to the computed FLU and FAN indices (Figures 3 and 4). For prokaryotes, Chao1 and Shannon indices displayed higher values for the particle-attached bacteria (nanoplankton fraction) than for the free-living one (picoplankton), regardless of the category of the FLU or FAN indices. The response of the alphadiversity indices to the degree of impact estimated by the FLU and FAN indices was however little. No significant differences were found for alphadiversity indices of bacterioplankton (neither for the free-living nor for the particle-attached bacteria) as a function of the FLU or FAN categories (ANOVA, $p>0.05$, Figure 3a). Compared to prokaryotes, eukaryotes displayed overall higher values of alphadiversity. Eukaryotic nanoplankton presented higher Chao1 values than picoplankton but this trend was not observed for the Shannon diversity values (Figure 4). As for the differences in relation to the impact indices, greater differences were observed for eukaryotes than for prokaryotes. In particular, significant differences were found for picoplankton in Chao1 and Shannon indices for the different

categories of the FLU (ANOVA, p=5.82e^{-03} and p=9.36e^{-04} respectively) and FAN (ANOVA, p=4.59e^{-05} and p=3.50e^{-05} respectively) indices whereas these differences were only significant for Shannon diversity in the nanoplankton fraction (ANOVA, p=2.85e^{-03} and p=4.22e^{-04} for FLU and FAN, respectively). Interestingly, contrary to prokaryotes, lower values of alphadiversity corresponded to higher values of the FLU index while the FAN categories followed the opposite trend.

Potential indicator taxa. Given that microbes respond rapidly to variations in environmental conditions, including nutrient inputs, we explored whether the relative abundances of the occurring taxa were related to the impact indices, both at the broad taxonomic group and at the OTU level. Figure 5 shows the relative contribution of major prokaryotic and eukaryotic taxa to planktonic community structure grouped by the category of the impact indices that the samples belong to (Low, Medium or High categories of FLU and FAN). Although no major changes in the taxonomic composition of the samples were observed regardless of their category, analysis of variance revealed that significant differences existed for the Actinobacteria, Rickettsiales (Alphaproteobacteria) and Sphingobacteriia (Bateroidetes) in relation to the FLU and FAN categories (Figure S2, Table S2). Within the eukaryotic taxa, analyses of variance only revealed significant differences in the abundance of the Basal Fungi (Opisthokonta) in the nanoplankton size in relation to the impact indices. Besides, we tested for differences at the OTU level and only found positive correlations between a nanoeukaryotic OTU affiliated to *Gymnodinium litoralis* (Dinoflagellata) and the concentrations of phosphate, nitrate and silicate (N=36, R>0.5, $p < 1e^{-13}$, Table S3).

We further explored potential 'indicator taxa' through the Indicator Value (IndVal) from Dufrêne and Legendre (1997). This value identifies indicator taxa fidelity and relative abundance and is a popular measure to express taxa importance in community ecology. Likewise, its potential to reflect environmental quality has been explored in biodiversity surveys (Ferrera et al., 2016; Lumbreras et al., 2016; Cordier et al., 2020). A total of 9

bacterial and 6 eukaryotic taxonomic groups were found with significant IndVal and relative abundances higher than 0.4% (Table 2). These groups have explanatory power mostly for either the Low and High categories of the impact indices, and most often for the FLU index (Table 2). More significant IndVal were detected among the prokaryotes than the eukaryotes. Moreover, significant IndVal were found in the two analyzed size fractions of the bacterial dataset but only in the picoplankton fraction of the eukaryotes. Most prokaryotic indicator taxa of the High FLU impact were at the same time indicators of the Low FAN index category (i.e., the Actinobacteria, Planctomycetes and Sphingobacteriia). Additionally, the Flavobacteriia were indicator only for the FLU index (Medium impact). For eukaryotes, the Rhizaria (Cercozoa) and Stramenopiles (MAST-3 and MAST-4) appeared as potential indicators for the Low category of the FLU index while the Telonema (Hacrobia) were indicator for the High category of this index and, at the same time, for the Low FAN impact category.

Besides the concept of indicator species or taxa, the potential of quality indicators based on the ratio of different taxa was investigated. In particular, we explored the ratios of the bacterial groups Alphaproteobacteria / Gammaproteobacteria, *Alteromonas* / SAR11, and *Alteromonas* + Oceanospirillales / SAR11 that had been proposed in our previous work (Ferrera et al., 2016) together with various alternative potential indices based on the abundance of those groups that appeared as indicator taxa. We found that, from all those tested, only the ratio Actinobacteria / Rickettsiales, calculated by dividing the relative abundances of Actinobacteria by that of Rickettsiales in the picoplankton fraction, was higher at high FLU values (Figure 6). No ratios with indicator potential were found within the eukaryotes.

Discussion

We explored the informative potential of pico- and nanoplankton communities for environmental status assessment using pelagic samples collected from diverse areas of the

NW Mediterranean. As recently reviewed by Cordier et al. (2020), various strategies to explore indicators based on environmental genetic data exist. Here, we explored the so-called 'structural community metrics strategy' by examining the potential of diversity and richness indices, and the '*de novo* strategy' aimed at discovering new indicators of environmental status in the water column by analyzing the abundances of OTUs and taxonomic groups (and their ratios). To do so, we classified the sampled stations based on the FLU and FAN impact indices, which indicate the origin of the land influences to the coast, derived from physicochemical variables as previously described (Flo 2017) and explored whether the biological variables responded to them. These indices were developed to distinguish between natural and cultural eutrophication, which is key to management planning. The FLU index clearly distinguished samples from Alfacs Bay and L'Estartit, both influenced by rivers, as well as from L'Hospitalet de l'Infant which could be explained by the presence of nearby groundwater seeps (Fernández-Ruiz 2012, Basterretxea et al., 2018). Likewise, the FAN values were overall different among sites but varied slightly between Blanes and Barcelona despite these are a small and a large city, respectively. In any case, the highest values were found for samples off the coast of Barcelona, particularly those closest to shore (Figure S3). Contrarily, samples collected at ~200 m from the coast line of Barcelona showed values within the range of low populated areas (Figure S3). The lack of differences between these sites may be related to the implementation of policies to reduce the impact of urban areas on coastal systems (i.e., wastewater treatment plants, sewage management, etc.) that combined with natural processes challenge the reliable discrimination between natural variability and human effects in the water column. For instance, one station statistically considered to be in good environmental status can episodically present low values of water quality (in our case would be reflected by high FAN values) that fall within the range of the best values from another location considered to be in bad environmental status, and vice versa. The large variability of FAN values from for Blanes and Barcelona could be examples of this scenario.

The measurement of species diversity of an ecosystem has been proposed as a useful tool for assessing the impacts of human activities on marine ecosystems. The strategy based on community metrics aims at discovering and understanding the ecological processes shaping communities and their response to disturbances (see Cordier et al., 2020). Actually, the results of our previous work (Ferrera et al., 2017) indicated that it could be worth exploring the links between microbial diversity and environmental status of coastal waters. Here, we found that both Chao1 and Shannon indices from eukaryotic communities showed power as indicators for assessing continental influences. These findings are contrary to those reported by Pearman et al. (2018) that found no differences in alphadiversity in a study assessing plankton community in anthropogenic-impacted coastal regions of the Red Sea. Likewise, opposed results have been reported in marine sediments; alphadiversity has been found to decrease in bacterial communities impacted by aquaculture (Stoeck et al., 2018) but disturbances can also trigger increases in bacterial diversity (Galand et al., 2016). These evidences thus challenge the implementation of using diversity metrics in environmental monitoring.

Regarding the '*de novo* strategy', differences in the abundance of certain taxa were observed in relation to nutrient values and impact indices. Both prokaryotes and eukaryotes showed potential as indicators. Within the prokaryotes, the relative abundance of Actinobacteria in the picoplankton displayed the highest Indicator Value, particularly as indicator of the High category of the FLU and the Low FAN impact indices. Actinobacteria, are widely distributed in both terrestrial and aquatic (marine and freshwater) ecosystems. Genomic analyses reveal a remarkable potential capacity to transform recalcitrant detrital material, particularly lignin-derived compounds, suggesting close linkages between the terrestrial and aquatic realms (Ghai et al., 2014). Their correlation with high FLU and low FAN values may indicate that these organisms are transported from freshwater to coastal ecosystems. Likewise, the Sphingobacteria that presented a significant IndVal in samples of High FLU and Low FAN categories are

capable of degrading polymeric matter (Bergauer et al., 2018). Within the eukaryotes, our results indicate that abundances of an OTU attributed to the dinoflagellate *Gymnodinium litoralis* were positively correlated with concentrations of nitrate, silicate and phosphate. This species is known to produce recurrent near-shore high-biomass blooms in L'Estartit (Reñé et al., 2011), an area shown to have riverine influence and high availability of these inorganic nutrients (Tables 1 and S1). At broad taxonomic levels, Basal Fungi (Ophisthokonta) from nanoplankton were correlated with nitrate, silicate and phosphate. This lineage comprises a diverse group of heterotrophic, saprophytic and parasitic organisms, including the Chytridiomycota that contains many parasites of phytoplankton (Frenken et al., 2017, Grossart et al., 2019). The fact that they show positive correlations with inorganic nutrients could reflect their coupling with the higher abundances of potential hosts, like dinoflagellates. Likewise, other eukaryotic taxa exhibit significant IndVal scores confirming the potential to unveil indicators through the '*de novo* approach'. Among these, the Chlorodendrophyceae (Archaeplastida) were indicator for samples subjected to low FAN and high FLU impacts. This group of prasinophytes (green algae) can be abundant in certain Mediterranean coastal stations (Tragin and Vaulot, 2018). Given their IndVal score in stations linked to a gradient of freshwater content as well as nitrate and silicate concentrations, their presence could be related to natural continental influences of fluvial origin. The uncultured marine stramenopiles MAST-3 and MAST-4 also displayed significant IndVal scores. These clades represent heterotrophic small protists that appear as common members in molecular surveys of marine picoplankton (Massana et al., 2004). Noteworthy, these taxa were indicative of water under low FLU impact (that is low freshwater, nitrate and silicate content). While certain clades of MAST have shown preference for brackish or freshwater environments, MAST-3 and MAST-4 have a clear preference for marine waters (Massana et al., 2014).

Besides indicator species or taxa, the potential of using the ratio between different

groups of microorganisms as an alternative indicator of environmental status has been proposed (Garrido et al., 2014). In fact, in our previous survey we concluded that some bacterial indices, i.e. the ratio of Alphaproteobacteria / Gammaproteobacteria, *Alteromonas* / SAR11 and *Alteromonas* + Oceanospirillales / SAR11 could potentially become new tools in marine monitoring (Ferrera et al., 2016). Despite the promising results found in that proof-of-concept study, here we found that when comparing a range of conditions and accounting for certain temporal variation, these indices lost significance. Contrarily, the Actinobacteria / Rickettsiales ratio appeared to be correlated with the FLU index, and could potentially reflect continental pressures, particularly associated to areas of riverine influence (i.e., Alfacs and L'Estartit). On the other hand, no ratios with indicator potential were found within the small eukaryotes (up to 20 μm) although previous studies have claimed the potential of protists as indicators (see Pawlowski et al., 2018). This lack of consistency highlights the difficulty of finding operational indicators that can be widely used.

Metabarcoding of environmental DNA provides a cost efficient approach for biodiversity monitoring and overcome many of the problems associated with traditional monitoring, offering the possibility to explore the use of microorganisms as bioindicators. In fact, its application has resulted in promising results in areas subjected to acute contamination but also along eutrophication gradients, particularly in sediments (Pawlowski et al., 2014; Aylagas et al., 2017; Stoeck et al., 2018). Although potential bioindicators were also unveiled in our study, the results are not as striking as those recently published by others. The structure and composition of the studied planktonic communities changed only slightly in areas of riverine influence and the shifts were even more negligible among sites under contrasting degrees of urban influence, represented by elevated values of nitrite and ammonia. A possible explanation for the differences in our results and those by other authors is the range of environmental pressures evaluated. Even though our study covers contrasting locations in terms of continental pressures, from

hardly populated sites to the large city of Barcelona, the evaluated pressures here may have been more restrained. The study of Pearman et al. (2018) compared nearly pristine sites to areas impacted by a wastewater treatment plant effluent or the pressure from container ships calling the port of Jeddah, Saudi Arabia, and were able to detect taxa associated to sewage or fecal matter. Likewise, the microgAMBI (Aylagas et al., 2017) index was developed for the evaluation of anthropogenic impacts occurring in sediments subjected to a wide range of human pressures derived from industrial activities such as the presence of metals and chemical pollutants (PCB among others). Our study, moreover, covers from nearshore coastal sites to offshore stations and, even though we limited our study to end of spring and summer, we observed certain spatial and temporal variability in the FLU and FAN index values in each location (see Figures 2 and S3), supporting the known difficulties of setting ecological status boundaries in areas subjected to moderate degrees of impacts. It is possible that the natural spatial and seasonal variability (succession of continually changing communities) of the studied area may be constraining the potential of pico- and nanoplankton as indicators. A good biodiversity indicator should be able to distinguish the anthropogenic impact from natural variability (Borja et al., 2012). Microbial communities are known to display natural seasonality (Furhman et al., 2015; Auladell et al., 2019; Giner et al., 2019) which may challenge using these assemblages in environmental assessments unless baseline conditions are well known. In fact, community composition cannot be used as a quality indicator in an absolute sense but only in relation with known environmental conditions, and thus, previous information on the natural spatial and temporal variability of an area is necessary to establish a baseline of knowledge that allows to discriminate the natural from the human-derived variability. Yet, an operational indicator by definition must be implemented in a simple, fast and cheap manner. Requiring large efforts to establish a knowledge baseline for an indicator compromises its usefulness, which could be the case for pico- and nanoplankton, at least based in our results. Further yet, a recent study conducted in the Bay of Pozzuoli (Gulf of Naples, Mediterranean Sea) revealed that,

even when taking into consideration natural seasonality, plankton biomass and diversity (bacteria, phytoplankton and mesozooplankton) did not reflect the environmental status even in areas showing signals of current anthropogenic pressure (Margiotta et al., 2020).

Traditionally, with the exception of fecal indicators, microbes have not been used as indicators due to the difficulties in the taxonomic identification of environmental microorganisms. Nowadays, the use of sequencing technologies overcomes these limitations and allow to assess microbial community patterns in coastal regions in a faster and cheaper manner. In that sense, microbes have been proposed as indicators of marine environmental quality because they are known to react quickly to environmental changes, which makes them sensitive to disturbances. At the same time, however, communities have a large resilience and they are able to recover fast if the pressure is not permanent. As a result, in highly dynamic environments such as the pelagic realm, the small organisms of the plankton compartment may bear short-term memory of impact events and be poor indicators of environmental status, at least in areas of moderate impact. We thus conclude that in spite of the usefulness of environmental genomic-based approaches for biodiversity monitoring, translating pico- and nanoplankton diversity into fast and simple ecological indicators is challenging, in part due to the complexity and dynamics of these pelagic communities. Increasing our knowledge on plankton species responses to the natural environmental could however strengthen their potential as ecological indicators.

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Author statement

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Table 1. Values (average ± standard deviation, minimum to maximum) of the FLU and FAN impact indices as well as of the variables taken into consideration for calculating them in each sampling area.

Site	Number of samples	Salinity (psu)	PO_4^{3+} (μM)	NH_4^+ (μM)	NO_2^- (μM)	NO_3^- (μM)	Si (μM)	FLU	FAN
Alfacs	4	35.85 ± 0.42 (35.30 to 36.3)	0.16 ± 0.02 (0.13 to 0.18)	0.84 ± 0.72 (0.04 to 1.67)	0.12 ± 0.04 (0.09 to 0.18)	0.60 ± 0.40 (0.33 to 1.19)	8.08 ± 4.37 (2.65 to 11.88)	60.1 ± 14.6 (42.9 to 77.2)	-22.2 ± 6.0 (-29.5 to -15.4)
Barcelona	23	37.79 ± 0.19 (37.04 to 38.01)	0.12 ± 0.08 (0.02 to 0.35)	1.15 ± 1.12 (0.12 to 4.01)	0.21 ± 0.21 (0.00 to 0.98)	0.73 ± 0.84 (0.04 to 3.91)	1.16 ± 2.01 (0.10 to 9.17)	7.7 ± 4.4 (2.4 to 24.2)	-1.9 ± 2.8 (-9.8 to 4.1)
Blanes	35	37.59 ± 0.71 (35.08 to 38.17)	0.10 ± 0.06 (0.02 to 0.23)	1.06 ± 0.83 (0.1 to 3.48)	0.13 ± 0.16 (0.01 to 0.87)	0.34 ± 0.28 (0.03 to 1.38)	0.87 ± 0.54 (0.04 to 2.22)	11.7 ± 16.1 (-0.85 to 69.4)	-4.0 ± 6.5 (-27.1 to 3.1)
L'Estartit	10	36.62 ± 0.79 (35.14 to 37.47)	0.41 ± 0.40 (0.11 to 1.23)	0.23 ± 0.20 (0.03 to 0.62)	0.25 ± 0.23 (0.07 to 0.79)	7.90 ± 10.30 (0.18 to 31.27)	15.15 ± 17.09 (1.36 to 52.96)	56.9 ± 44.7 (15.5 to 144.4)	-18.1 ± 11.4 (-38.6 to -6.8)
L'Hospitalet de l'Infant	10	36.65 ± 0.42 (35.53 to 37.14)	0.13 ± 0.02 (0.11 to 0.17)	0.24 ± 0.18 (0.02 to 0.57)	0.12 ± 0.02 (0.08 to 0.15)	0.88 ± 0.78 (0.32 to 2.82)	0.81 ± 0.70 (0.32 to 2.14)	34.0 ± 10.8 (22.0 to 62.4)	-13.8 ± 4.2 (-25.0 to -9.5)
Palma	11	37.6 ± 0.05 (37.46 to 37.64)	0.17 ± 0.05 (0.11 to 0.26)	0.29 ± 0.28 (0.06 to 1.02)	0.10 ± 0.14 (0.02 to 0.51)	0.96 ± 1.16 (0.06 to 4.04)	1.02 ± 0.40 (0.72 to 2.06)	12.7 ± 1.58 (10.8 to 15.3)	-5.1 ± 0.4 (-5.5 to -4.1)

Table 2. Potential indicator taxonomic groups identified by significant IndVal for the various categories and the FLU and FAN impact indices that each sample falls into. Pico (picoplankton) corresponds to the 0.2 – 3 μm fraction; nano (nanoplankton) corresponds to the 3 – 20 μm fraction. Abundance (%) indicates the mean relative abundance of that taxon in the corresponding fraction.

Domain	Size Fraction	Taxonomic group	Rel. Abund. (%)	Variable	Category	IndVal	P-value
Prokaryotes	pico	Actinobacteria	4.37	FAN	Low	0.59	$3.0e^{-04}$
	pico	Sphingobacteriia	2.05	FAN	Low	0.48	$5.0e^{-04}$
	pico	Actinobacteria	4.37	FLU	High	0.60	$3.0e^{-04}$
	pico	Sphingobacteriia	2.05	FLU	High	0.49	$3.0e^{-04}$
	nano	Sphingobacteriia	5.68	FAN	Low	0.53	$3.0e^{-04}$
	nano	Planctomycetes	2.42	FAN	Low	0.46	$1.5e^{-03}$
	nano	Flavobacteriia	15.05	FLU	Medium	0.41	$2.4e^{-02}$
	nano	Sphingobacteriia	5.68	FLU	High	0.53	$3.0e^{-04}$
	nano	Planctomycetes	2.42	FLU	High	0.44	$1.4e^{-02}$
Eukaryotes	pico	Chlorodendrophyceae (Archaeplastida)	4.76	FAN	Low	0.73	$5.8e^{-03}$
	pico	Chlorodendrophyceae (Archaeplastida)	4.76	FLU	High	0.68	$3.4e^{-02}$
	pico	Telonema (Hacrobia)	0.67	FLU	Low	0.52	$3.4e^{-02}$
	pico	Cercozoa (Rhizaria)	1.7	FLU	Low	0.55	$1.9e^{-03}$
	pico	MAST_3 (Stramenopiles)	1.94	FLU	Low	0.51	$1.2e^{-02}$
	pico	MAST_4 (Stramenopiles)	0.77	FLU	Low	0.54	$2.9e^{-02}$

Supplementary Table 1. Table showing the results of an integrated assessment of coastal waters using a dataset from the Catalan Water Agency (ACA) from 2011 to 2016 and thus corresponding to the 6-year evaluation period mandated by the Water Framework Directive to assess water status*. The data from Palma de Mallorca correspond to a report from the Balearic Islands Government**. The information was used to choose the study area included in this work. FAN and FLU indexes, LUSI and Chlorophyll *a* (Chl-*a*) concentration were applied to a dataset from 2011 to 2016 of the Catalan water bodies (WBs) and to the 2009-2015 period for the Balearic coast. The LUSI method assesses the continental pressures on coastal waters, which are linked to continental influences (Flo et al., 2019). The FAN and FLU indexes method assesses the water quality, the anthropogenic component of the trophic state, the fluviality and the continental influences on coastal waters. WBs code and name are indicated, together with the salinity and Chl-*a* concentration (μg L^{-1}).

WB code	WB name	Correspondence to this work	LUSI	FAN INDEX	FLU INDEX	SALINITY	Chl-*a*	FLUVIALITY	CONTINENTAL INFLUENCES
C11	Torroella de Montgri	L'Estartit	6.25	0.75	0.88	34.09	0.9	High	Fluvial influence
C15	Blanes-Pineda de Mar	Blanes	4	1	0.75	36.99	0.71	Low	None
C19	Sant Adrià de Besòs-Barceloneta	Barcelona	4	0.13	0.63	37.43	1.16	Medium	Mixed
C31	Vandellós i L'Hospitalet de l'Infant	L'Hospitalet de l'Infant	2	1	1	37.71	0.65	Medium	None
T03	Badia Alfacs	Alfacs	6.25	0.75	0.13	33.39	6.45	Very high	Fluvial influence
MAMC15M3	Palma de Mallorca	Palma	6			36	0.9	Medium	Mixed

*ACA 2005 CARACTERITZACIÓ DE MASSES D'AIGUA I ANÀLISI DEL RISC D'INCOMPLIMENT DELS OBJECTIUS DE LA DIRECTIVA MARC DE L'AIGUA (2000/60/CE) A CATALUNYA (conques intra i intercomunitàries) En compliment als articles 5, 6 i 7 de la Directiva. Generalitat de Catalunya. http://aca.gencat.cat/web/.content/30_Plans_i_programes/10_Pla_de_gestio/document_IMPRESS/IMPRESS_2005.pdf

** Plan Hidrológico de las Illes Balears2015-2021 Memoria, Govern de les Illes Balears. http://observatoriaigua.uib.es/repositori/phib_2015_memoria.pdf

Supplementary Table 2. Taxa displaying significant correlation values between their relative abundance and the impact indices or nutrient concentrations measured in sampled waters. Size fraction indicates picoplankton (pico) or nanoplankton (nano). Abundance (%) represents the mean relative abundance of that taxa in that size fraction. Pearson correlations and *p*-values are shown.

Taxonomic Group		Size fraction	Abundance (%)	Variable	Correlation	p-value
Prokaryotes	Actinobacteria	pico	4.37	FAN Index	0.60	2.1E-19
	Actinobacteria	pico	4.37	FLU Index	0.59	7.7E-19
Eukaryotes	BasalFungi (Opisthokonta)	nano	0.38	PO_4^{3-}	0.50	2.5E-13
	BasalFungi (Opisthokonta)	nano	0.38	NO_3^-	0.74	1.6E-26
	BasalFungi (Opisthokonta)	nano	0.38	Si	0.62	8.1E-19

Supplementary Table 3. OTUs showing significant Pearson correlation values between their relative abundance in the nanoplankton fraction and the nutrient loads measured in sampled waters.

OTU	Rel. Ab. (%)	Variable	Correlation	p-value	Taxonomy
OTU9	0.5	PO_4^{3-}	0.55	2.33E-15	Alveolata;Dinoflagellata;Dinophyceae;Gymnodiniphycidae; *Gymnodinium litoralis*
OTU9	0.5	NO_3^-	0.74	6.44E-27	Alveolata;Dinoflagellata;Dinophyceae;Gymnodiniphycidae; *Gymnodinium litoralis*
OTU9	0.5	Si	0.52	3.31E-14	Alveolata;Dinoflagellata;Dinophyceae;Gymnodiniphycidae; *Gymnodinium litoralis*

Figure Legends

Figure 1. Map of the NW Mediterranean area showing the sampled areas (source: QGIS Geographic Information System, http://qgis.osgeo.org).

Figure 2. Box plots of the FLU and FAN impact indices for each sampling location. Letters shown in the boxes represent the results of a Tukey HSD test. Areas not connected by the same letter are significantly different ($p<0.05$).

Figure 3. Box plots of bacterial richness (Chao1) and diversity (Shannon) indices depending on the categories of the FLU and FAN impact indices that each sample falls into (Low, Medium, High). Two bacterioplankton size fractions were analyzed separately (nano: nanoplankton; pico: picoplankton). No significant differences were found (ANOVA, $p<0.05$).

Figure 4. Box plots of bacterial richness (Chao1) and diversity (Shannon) indices depending on the categories of the FLU and FAN impact indices that each sample falls into (Low, Medium, High). Two eukaryotic plankton size fractions were analyzed separately (nano: nanoplankton; pico: picoplankton). *Asterisks indicate that differences for that category and size fraction were significant (Tukey HSD test at $p<0.05$).

Figure 5. Bar plots showing the relative abundances of bacterial (left) and eukaryotic (right) taxa depending on the FLU and FAN impact index categories (Low, Medium or High). Two plankton size fractions were analyzed separately (nano: nanoplankton; pico: picoplankton).

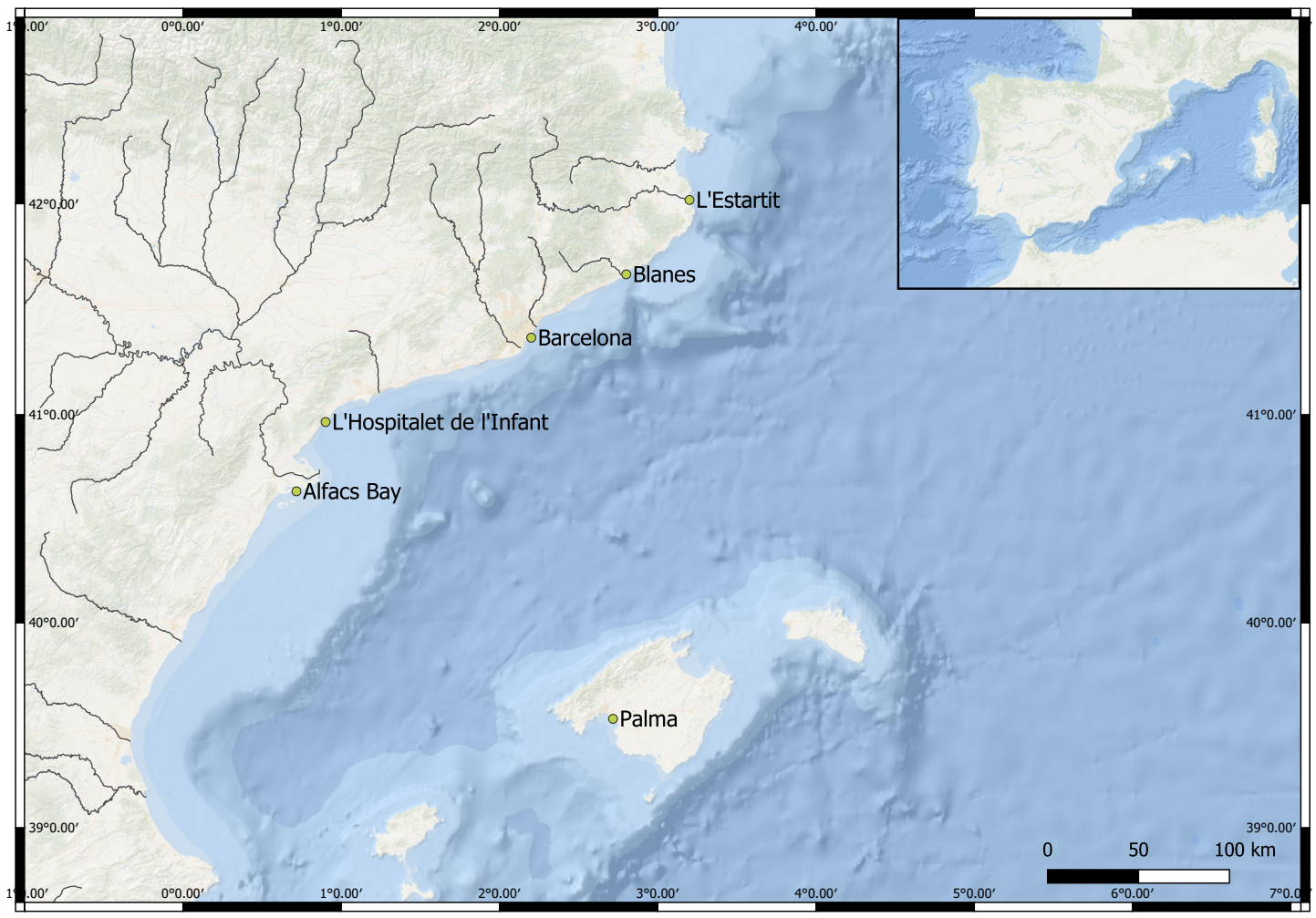
Figure 6. Box plot showing the differences in the Actinobacteria / Rickettsiales ratio in the picoplankton fraction along the FLU and FAN index categories.

Supplementary Figure Legends

Figure S1. FLU and FAN impact index values for each sample grouped by the index category. Each sample is colored according to the sampling area.

Figure S2. Box plots of bacterial and eukaryotic taxa showing significant differences (ANOVA, $p<0.05$) in the normalized relative abundances depending on the categories of the devised FLU and FAN indices (Low, Medium or High). The plankton size fraction is also indicated (nano: nanoplankton; pico: picoplankton).

Figure S3. FLU and FAN values for each sample depending on the distance to the coast at which the samples were collected. Samples are colored by sampling area. Alfacs Bay samples are not shown since those samples belong to an estuary.



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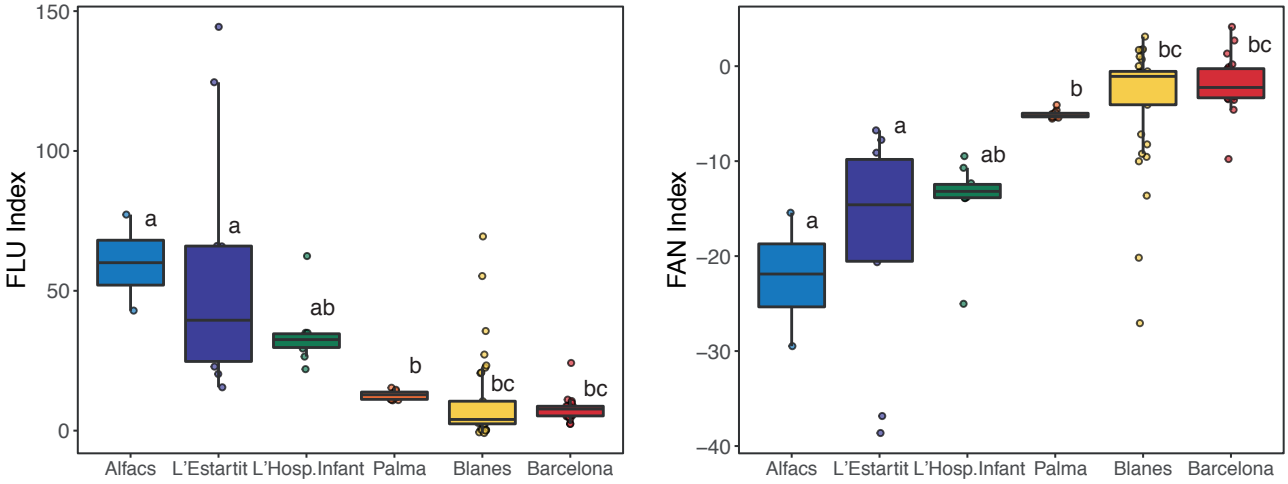

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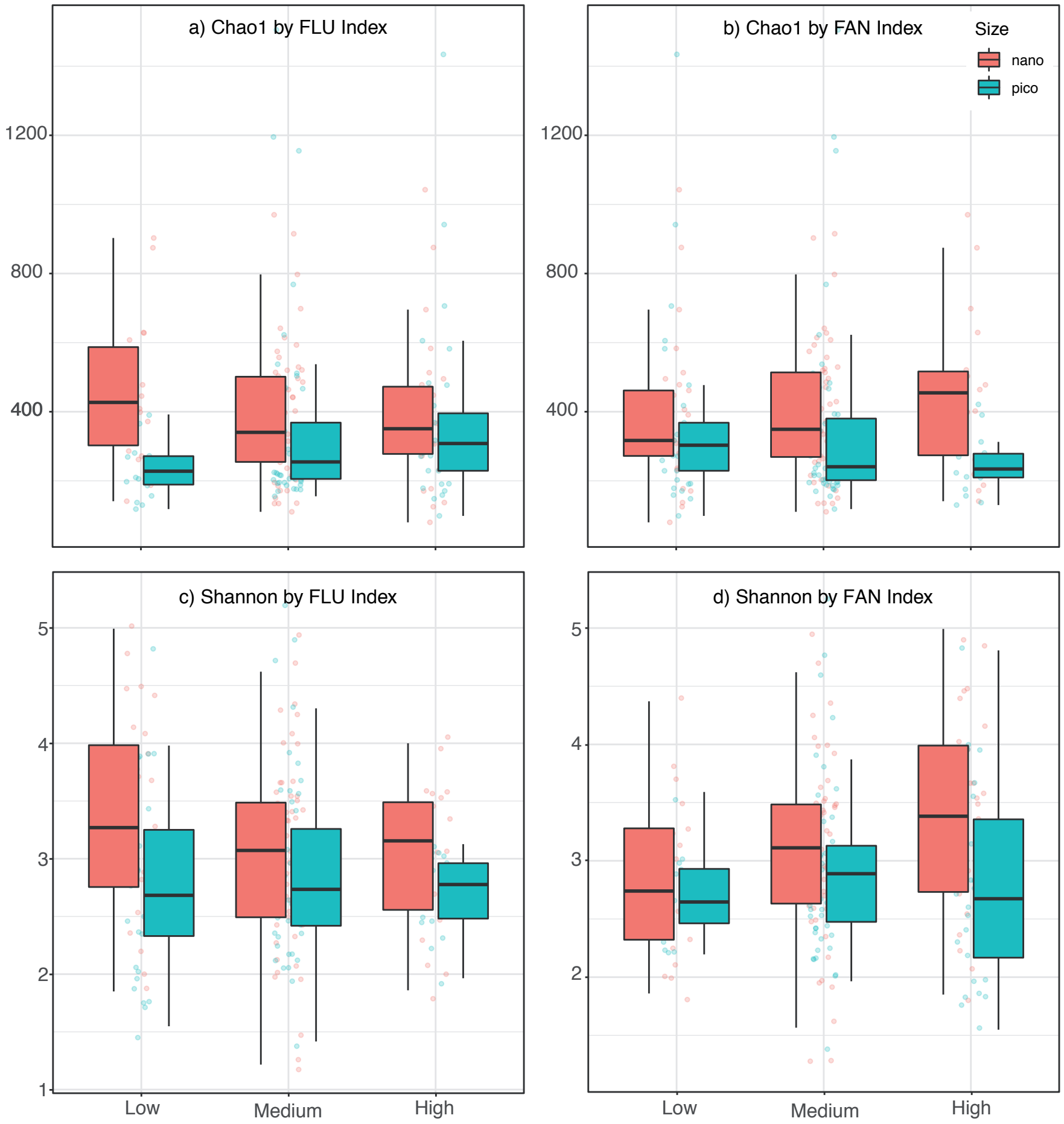

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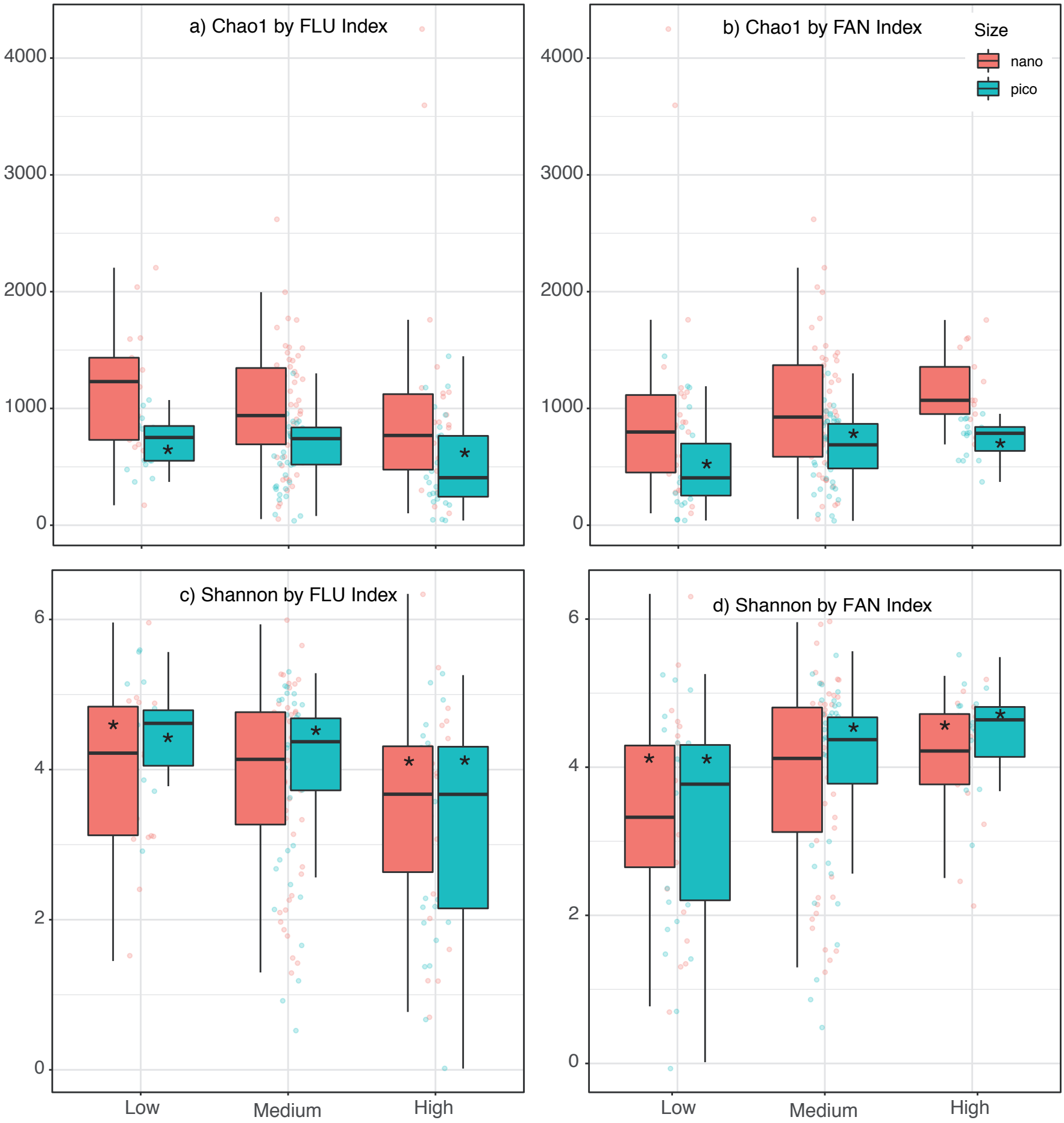

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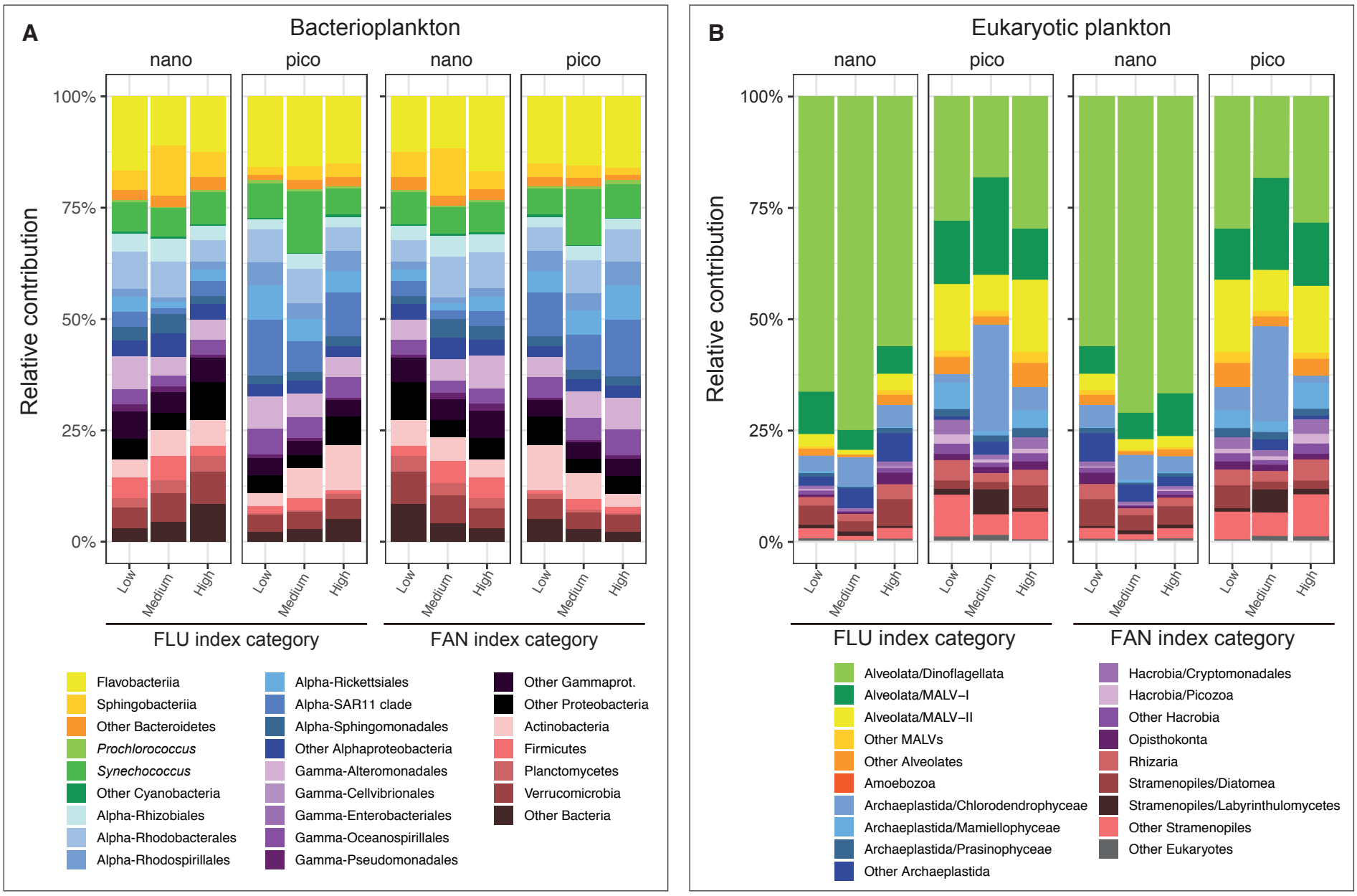

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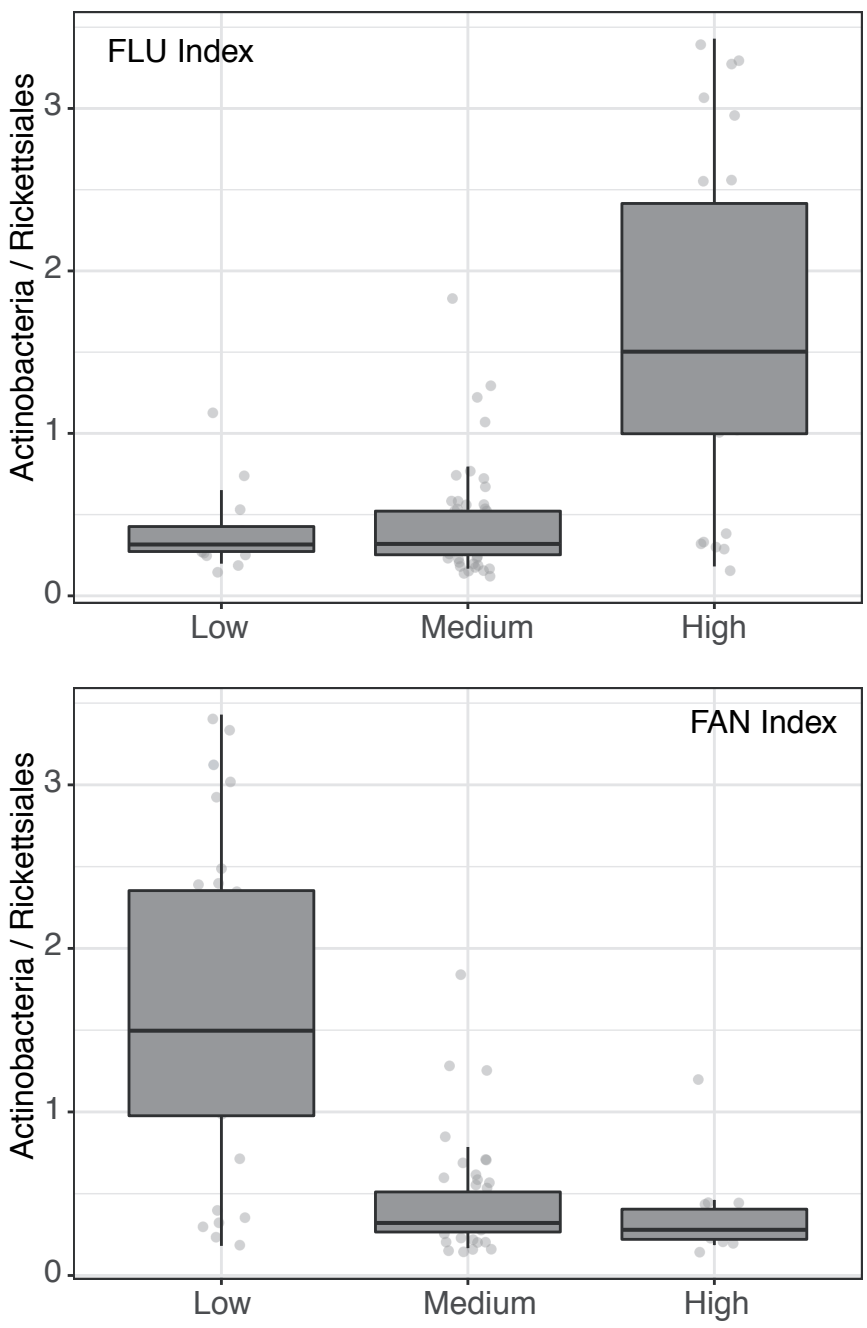

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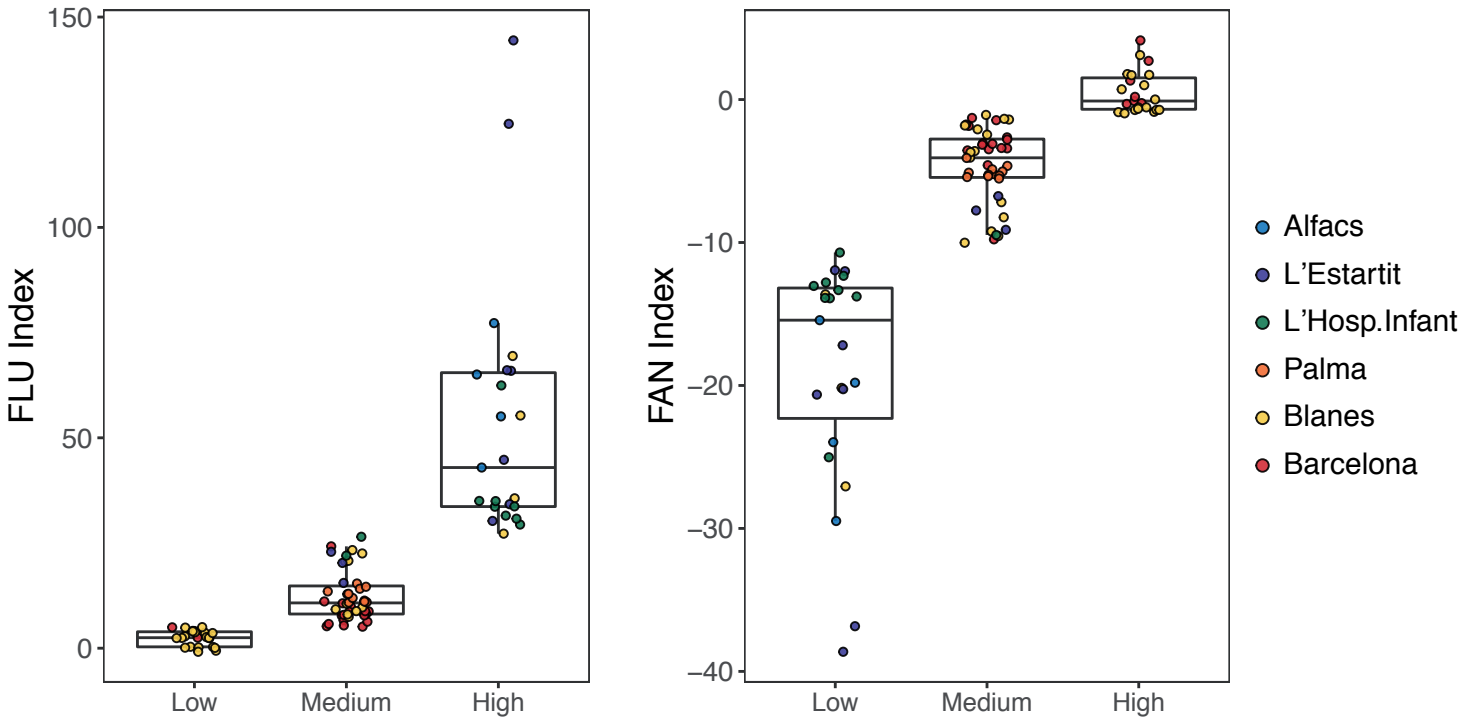

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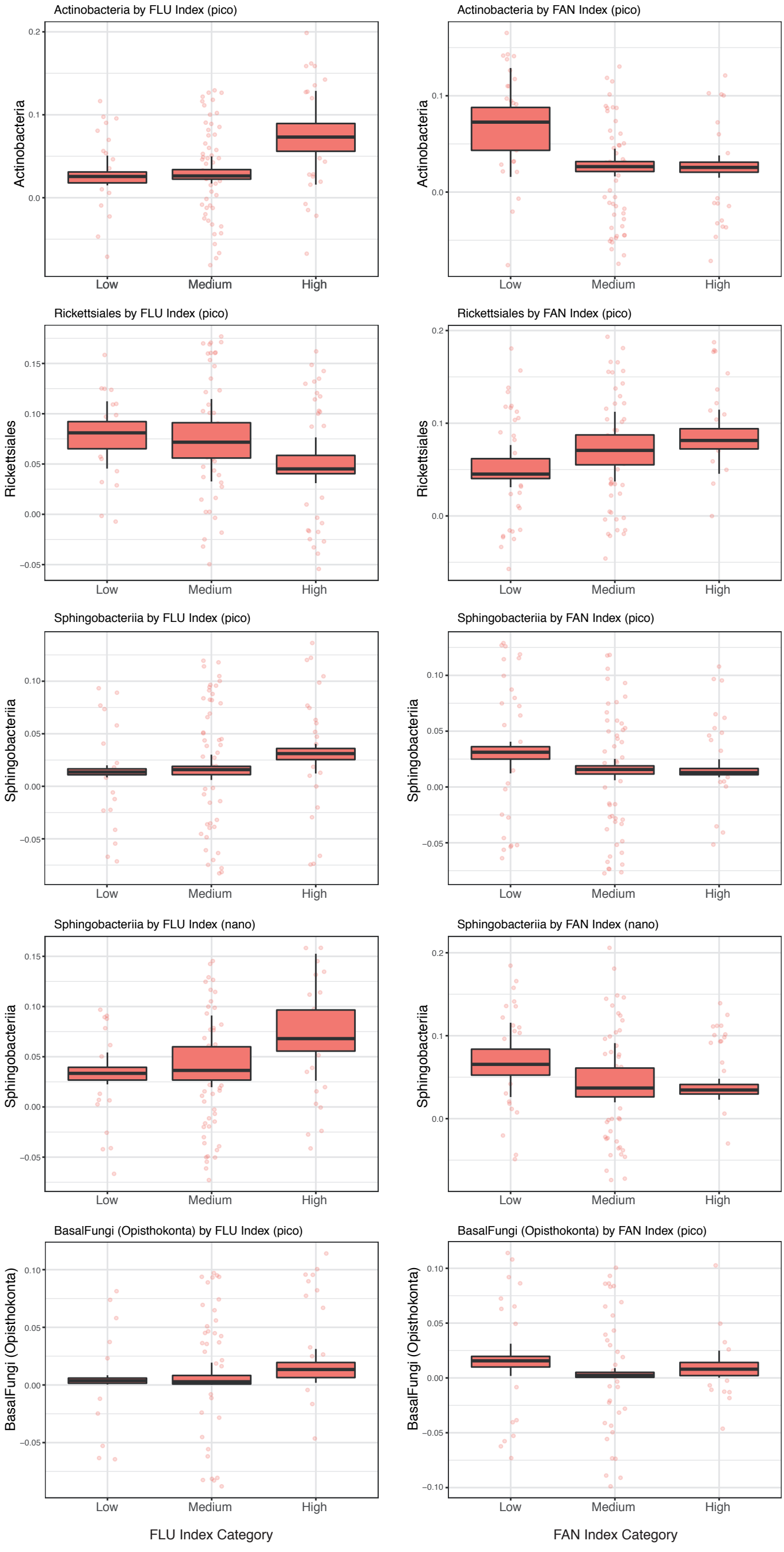

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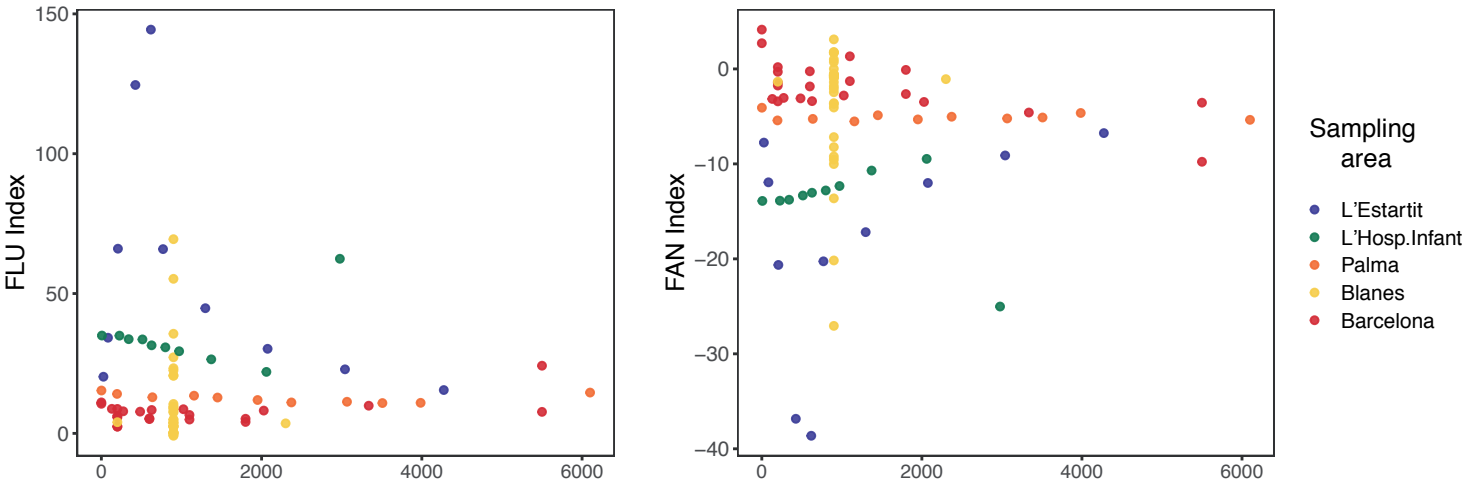

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