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► **To cite this version:**

Ina Maria Deutschmann, Anders Krabberød, Francisco Latorre, Erwan Delage, Cèlia Marrasé, et al..
Disentangling temporal associations in marine microbial networks. 2022. hal-03781901v1

HAL Id: hal-03781901

<https://hal.science/hal-03781901v1>

Preprint submitted on 16 Nov 2022 (v1), last revised 7 Sep 2023 (v2)

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Disentangling temporal associations in marine microbial networks

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The authors declare that they have no competing interests.

36 **ABSTRACT**

37 *Background*

38 Microbial interactions are fundamental for Earth's ecosystem functioning and
39 biogeochemical cycling. Nevertheless, they are challenging to identify and remain barely
40 known. Omics-based censuses are helpful in predicting microbial interactions through the
41 statistical inference of single (static) association networks. Yet, microbial interactions are
42 dynamic and we have limited knowledge of how they change over time. Here we investigate
43 the dynamics of microbial associations in a 10-year marine time series in the Mediterranean
44 Sea using an approach inferring a time-resolved (temporal) network from a single static
45 network.

46

47 *Results*

48 A single static network including microbial eukaryotes and bacteria was built using
49 metabarcoding data derived from 120 monthly samples. For the decade, we aimed to identify
50 persistent, seasonal, and temporary microbial associations by determining a temporal
51 network that captures the interactome of each individual sample. We found that the temporal
52 network appears to follow an annual cycle, collapsing and reassembling when transiting
53 between colder and warmer waters. We observed higher association repeatability in colder
54 than in warmer months. Only 16 associations could be validated using observations reported
55 in literature, underlining our knowledge gap in marine microbial ecological interactions.

56

57 *Conclusions*

58 Our results indicate that marine microbial associations follow recurrent temporal dynamics
59 in temperate zones, which need to be accounted for to better understand the functioning of

60 the ocean microbiome. The constructed marine temporal network may serve as a resource for
61 testing season-specific microbial interaction hypotheses. The applied approach can be
62 transferred to microbiome studies in other ecosystems.

63

64 **Keywords:** association network; temporal network; time series; microbial interactions;
65 microorganisms; ocean; plankton

66

67 INTRODUCTION

68 Microorganisms are the most abundant life forms on Earth, being fundamental for global
69 ecosystem functioning [1–3]. The total number of microorganisms on the planet is estimated
70 to be $\approx 10^{12}$ species [4] and $\approx 10^{30}$ cells [5, 6]. In particular, microorganisms dominate the
71 largest biome, the ocean, which harbors $\approx 10^{29}$ microbial cells [6] accounting for $\sim 70\%$ of
72 the total marine biomass [7, 8].

73 Microbial communities are highly dynamic and their composition is determined
74 through a combination of ecological processes: selection, dispersal, drift, and speciation [9].
75 Selection is a prominent community structuring force exerted via multiple abiotic and biotic
76 factors [10, 11]. Several studies have addressed the role of *abiotic* factors in structuring
77 microbial communities. For example, temperature is one of the main factors exerting
78 selection in the ocean microbiome over spatiotemporal scales [12–15]. *Biotic* factors can also
79 strongly affect microbial communities [16]. However, a mechanistic understanding of how
80 they affect community structure is currently lacking, as the diversity of microbial interactions
81 is barely known [3, 17].

82 The vast microbial diversity and the fact that most microorganisms are still uncultured

83 [18, 19] make it impossible to experimentally test all potential interactions between pairs of
84 microbes. However, omics-technologies allow estimating microbial relative abundances over
85 spatiotemporal scales, which permits determining statistical associations between taxa. These
86 associations can be summarized as a network with nodes representing microorganisms and
87 edges representing potential interactions [20, 21].

88 As microorganisms are highly interconnected [21], association networks provide a
89 general overview of the entire microbial system and have been tremendously valuable for
90 generating novel hypotheses about putative interactions. In particular, time series have
91 allowed identifying potential ecological interactions among marine microorganisms [22–28].
92 For example, previous work characterized ecological links between marine archaea, bacteria,
93 and eukaryotes [22], including links with viruses [24, 26], also investigating within- and
94 between ocean-depth relationships [25, 27]. These studies not only identified time-dependent
95 associations among ecologically important taxa, but also potential synergistic or antagonistic
96 relationships, as well as possible ‘keystone’ species and potential niches [22, 23]. Moreover,
97 several studies have reported more associations among microorganisms than between
98 microorganisms and environmental variables, suggesting the importance of biotic
99 relationships in structuring microbial community assemblages [22, 28].

100 Previous studies have used temporal microbial abundance data to infer static
101 networks summarizing all potential associations in space and time. This static abstraction
102 assumes that the network topology does not change (static) and edges represent persistent
103 associations assumed as interactions [29]; that is, edges are present throughout time and
104 space. This assumption cannot represent the reality of most microbial interactions. Thus, a
105 single static network usually contains persistent, temporary, and recurring (including
106 seasonal) associations that need to be disentangled.

107 Despite the contribution of static networks to our understanding of microbial
108 interactions in the ocean, it is necessary to incorporate the temporal dimension. Using a time-
109 resolved, i.e., temporal network instead of a single static network would allow investigation
110 of the dynamic nature of microbial associations and how they change over time, whether the
111 change is deterministic or stochastic, and how environmental selection influences network
112 architecture. Addressing these questions is fundamental for a better understanding of the
113 dynamic interactions that underpin ecosystem function in the ocean. Here, we investigated
114 marine microbial associations through time by determining a temporal network from a single
115 static network.

116

117 **MATERIALS AND METHODS**

118 *The Blanes Bay Microbial Observatory (BBMO)*

119 The BBMO is a coastal oligotrophic site in the North-Western Mediterranean Sea (41°40'N
120 2°48'E) without major natural disturbances and little anthropogenic pressure, except for the
121 construction of a nearby harbor between 2010 and 2012 [30, 31]. The seasonal cycle is
122 typical for a temperate coastal system [30], and the main environmental factors influencing
123 seasonal microbial succession have been well studied and are known [12]. Shortly, the water
124 column is slightly stratified in summer before it destabilizes and mixes with water from
125 offshore in late fall, increasing the availability of inorganic nutrients with maximum
126 concentrations in winter, between November and March. The high amount of nutrients and
127 increasing light induce phytoplankton blooms, mostly in late winter-early spring. During
128 summer, inorganic nutrients become limiting, the primary production is minimal, and
129 dissolved organic carbon accumulates [30].

130

131 *From sampling to microbial relative abundances*

132 We sampled surface water (\approx 1m depth) monthly from January 2004 to December 2013 to
133 determine microbial community composition and also measured ten environmental variables,
134 which were previously described [13, 30]: water temperature ($^{\circ}$ C) and salinity (obtained *in*
135 *situ* with a SAIV-AS-SD204 Conductivity-Temperature-Depth probe), day-length (hours of
136 light), turbidity (Secchi depth in meters), total chlorophyll-a concentration (μ g/l, fluorometry
137 of acetone extracts after 150 ml filtration on GF/F filters [30]), and five inorganic nutrients:
138 PO_4^{3-} , NH_4^+ , NO_2^- , NO_3^- and SiO_2 (μ M, determined with an Alliance Evolution II
139 autoanalyzer [32]).

140 Sampling of microbial communities, DNA extraction, rRNA-gene amplification,
141 sequencing, and bioinformatic analyses are explained in detail in [28]. In short, 6 L of water
142 were prefiltered through a 200 μ m nylon mesh and subsequently filtered through another 20
143 μ m nylon mesh and separated into nanoplankton (3 – 20 μ m) and picoplankton (0.2 – 3 μ m)
144 using a 3 μ m and 0.2 μ m pore-size polycarbonate and Sterivex filters, respectively. Then, the
145 DNA was extracted from the filters using a phenol-chloroform protocol [33], which has been
146 modified for purification with Amicon units (Millipore). We amplified the 18S rRNA genes
147 (V4 region) with the primers TAREukFWD1 and TAREukREV3 [34], and the 16S rRNA
148 genes (V4 region) with Bakt 341F [35] and 806RB [36]. Amplicons were sequenced in a
149 MiSeq platform (2x250bp) at RTL Genomics (Lubbock, Texas). Read quality control,
150 trimming, and inference of Operational Taxonomic Units (OTUs) delineated as Amplicon
151 Sequence Variants (ASVs) were done with DADA2 [37], v1.10.1, with the maximum number
152 of expected errors set to 2 and 4 for the forward and reverse reads, respectively.

153 Microbial sequence abundance tables were obtained for each size fraction for both
154 microbial eukaryotes and prokaryotes. Before merging the tables, we subsampled each table
155 to the lowest sequencing depth of 4907 reads with the *rrarefy* function from the Vegan R-
156 package [38], v2.4-2, (see details in [28]). We excluded 29 nanoplankton samples (March
157 2004, February 2005, May 2010 - July 2012) due to suboptimal amplicon sequencing. In
158 these samples, abundances were estimated using seasonally aware missing value imputation
159 by the weighted moving average for time series as implemented in the *imputeTS* R-package,
160 v2.8 [39]. These imputed values did not introduce biases in the analyses [28].

161 Sequence taxonomy was inferred using the naïve Bayesian classifier method [40]
162 together with the SILVA database [41], v.132, as implemented in DADA2 [37]. Additionally,
163 eukaryotic microorganisms were BLASTed [42] against the Protist Ribosomal Reference
164 (PR2) database [43], v4.10.0. The PR2 classification was used when the taxonomic
165 assignment from SILVA and PR2 disagreed. We removed ASVs that were identified as
166 Metazoa, Streptophyta, plastids, mitochondria, and Archaea since the 341F-primer is not
167 optimal for recovering this domain [44]. Besides, Haptophyta is known to be missed by the
168 primer TAREukREV3 [45].

169 The resulting table contained 2924 ASVs (Table 1A). Next, we removed rare ASVs
170 keeping ASVs with sequence abundance sums above 100 reads and prevalence above 15%
171 of the samples, i.e., we considered taxa present in at least 19 months. The resulting table
172 contained 1782 ASVs (Table 1B). An ASV can appear twice, in the nanoplankton and
173 picoplankton size fractions. However, an ASV may be detected in both size fractions due to
174 dislodging cells or particles and filter clogging, which can introduce biases in our analysis.
175 To reduce these biases, and as done previously [28], we divided the abundance sum of the

176 larger by the smaller size fraction for each ASV appearing in both size fractions and set the
177 picoplankton abundances to zero if the ratio exceeded 2. Likewise, we set the nanoplankton
178 abundances to zero if the ratio was below 0.5. This operation removed two eukaryotic ASVs
179 and 41 bacterial ASVs from the nanoplankton, and 30 bacterial ASVs from the picoplankton
180 (Table 1C). The resulting abundance table was used for network inference.

181

182 *From sequence abundances to the single static network*

183 First, we constructed a preliminary network using the tool eLSA [46, 47], as done in [28, 48],
184 including default normalization and z-score transformation, using median and median absolute
185 deviation. Although we are aware of time-delayed interactions, we considered our 1-month
186 sampling interval too large for inferring time-delayed associations with a solid ecological
187 basis, and focused on contemporary interactions between co-occurring microorganisms.
188 Using 2000 iterations, we estimated p-values with a mixed approach that performs a random
189 permutation test of a co-occurrence if the comparison's theoretical p -values are below 0.05.
190 The Bonferroni false discovery rate (q) was calculated based on the p -values using the
191 *p.adjust* function from the stats R-package [49]. We used the 0.001 significance threshold for
192 the p and q values, as suggested in other studies [20]. We refrained from using an association
193 strength threshold since it may not be appropriate to differentiate between true interactions
194 and environmentally-driven associations [48]. Furthermore, changing thresholds have been
195 shown to lead to different network properties [50]. The preliminary network contained 754
196 nodes and 29820 edges (24458, 82% positive, and 5362, 18% negative).

197 Second, for environmentally-driven edge detection, we applied EnDED [48],
198 combining the methods Interaction Information (with a 0.05 significance threshold and
199 10000 iterations) and Data Processing Inequality. We inserted artificial edges connecting

200 each node to each environmental parameter. We identified and removed 3315 (11.12%)
201 edges that were environmentally driven; 26505 edges (23405, 88.3% positive, and 3100,
202 11.7% negative) remained (Supplementary Tables 3 and 4).

203 Third, we determined the Jaccard index, J , for each microorganisms pair associated
204 through an edge, in order to remove associations between microorganisms that have a low
205 co-occurrence. Let S_i be the set of samples in which both microorganisms are present
206 (sequence abundance above zero), and S_u be the set of samples in which one or both
207 microorganisms are present. Then, we can calculate the Jaccard index as the fraction of
208 samples in which both appear (intersection) from the number of samples in which at least
209 one appears (union): $J = S_i/S_u$. We chose $J > 0.5$ as in previous work [48], which removed
210 9879 edges and kept 16626 edges (16481, 99.1% positive and 145, 0.9% negative). We
211 removed isolated nodes, i.e., nodes without an associated partner in the network. The number
212 and fraction of retained reads are listed in Table 1. The resulting network is our single static
213 network.

214

215 *From the single static network to the temporal network*

216 We determined the temporal network comprising 120 sample-specific (monthly)
217 subnetworks through the three conditions indicated below and visualized in Figure 1. The
218 subnetworks are derived from the single static network and contain a node subset and an edge
219 subset of the static network. Let e be an association between microorganisms A and B , with
220 association duration $d = (t_1, t_2)$, i.e., the association starts at time point t_1 and ends at t_2 . Then,
221 considering month m , the association e is present in the monthly subnetwork N_m , if

222 1) e is an association in the single static network,

223 2) the microorganisms A and B are present within month m , and

224 3) m is within the duration of association, i.e., $t_1 \leq m \leq t_2$.

225 With the 2nd condition, we assumed that an association was present in a month if both
226 microorganisms were present, i.e., the microbial abundances were non-zero for that month.

227 However, we cannot assume that microbial co-occurrence is a sufficient condition for a
228 microbial interaction because different mechanisms influence species and interactions, and
229 the environmental filtering of species and interactions can differ [51]. Using only the species
230 occurrence assumption would increase association prevalence. To lower this bias, we also
231 required that the association was present in the static network, 1st condition, and within the
232 association duration, 3rd condition, both inferred by eLSA [46, 47]. Lastly, we removed
233 isolated nodes from each monthly subnetwork.

234

235 *Network analysis*

236 We computed global network metrics to characterize the single static network and each
237 monthly subnetwork using the igraph R-package [52]. Some metrics tend to be more
238 correlated than others implying redundancy between them, grouping them into four groups
239 [53]. Thus, we selected one metric from each group: *edge density*, *average path length*,
240 *transitivity*, and *assortativity* based on node degree. In addition, we also computed the
241 *average strength of positive associations* between microorganisms using the mean, and
242 *assortativity* based on the nominal classification of nodes into bacteria and eukaryotes.
243 Assortativity (bacteria vs. eukaryotes) is positive if bacteria tend to connect with bacteria and
244 eukaryotes tend to connect with eukaryotes. It is negative if bacteria tend to connect to
245 eukaryotes and vice versa. We also quantified associations by calculating their prevalence as

246 the fraction of monthly subnetworks in which the association was present for all ten years
247 (recurrence), and monthly. We visualized highly prevalent associations with the *circlize* R-
248 package [54]. We tested our hypotheses of environmental factors influencing network
249 topology by calculating the Spearman correlations between global network metrics and
250 environmental data. We used Holm’s multiple test correction to adjust p-values [55], with the
251 function *corr.test* in the *psych* R-package [56]. We used Gephi [57], v.0.9.2, and the
252 Fruchterman Reingold Layout [58] for network visualizations.

253

254 *Test of network construction tool*

255 We have used eLSA to estimate the duration of an association, which we used as the third
256 condition (m is within the duration of association, i.e., $t_1 \leq m \leq t_2$) to infer the sample-specific
257 subnetworks. Other methods may perform better on compositional data such as ours [59]
258 (although this is not necessarily the case; see [60]). Therefore we tested another network
259 construction approach (FlashWeave [61]) for comparative purposes. FlashWeave performed
260 better than eLSA in some benchmark tests run by other authors, while eLSA performed better
261 than FlashWeave in other tests [61]. FlashWeave can handle sparse datasets taking zeros into
262 account and avoiding spurious correlations between ASVs that share many zeros. However,
263 it neglects the temporal variation. To control data compositionality [59], we applied a
264 centered-log-ratio transformation separately to the bacterial and eukaryotic read abundance
265 tables before merging them. Then, we inferred a network using FlashWeave [61], selecting
266 the options “heterogeneous” and “sensitive”. We have run analyses including the
267 environmental data (10 variables; see above). The resulting network had 932 nodes and 1440
268 edges. Next, we determined a temporal network using conditions 1) and 2) but not 3) since

269 the temporal duration is not estimated by FlashWeave. FlashWeave results are used hereafter
270 to compare against eLSA, although eLSA is kept as the main network construction tool in
271 our work, given that it allows determination of the duration of the associations and there is
272 no evidence suggesting a poor performance of this tool. Thus, unless specified otherwise, we
273 refer to the static and temporal network determined by eLSA.

274

275 *Cyanobacteria*

276 Our dataset contained 19 cyanobacterial ASVs, which all appeared in the nano-, and nine in
277 the picoplankton. We blasted the sequences against the Cyanorak database [62], v.2. against
278 the nucleotide database containing all *Synechococcus* and *Prochlorococcus* RNAs with the
279 option `-evalue 1.0e-5`. We found 2812 sequences comprising 95 different ecotypes
280 (considering name, clade and subclade), with 93.84-100% identity. A total of 11 BBMO
281 ASVs obtained 63 hits with 100% identity, and within these 63 reference sequences there
282 were 34 different ecotypes. Most matching sequences were found for *Synechococcus* ASV_1.
283 While *Synechococcus* ASV_5 had only two 100% hits, they did not 100% match ASV_1
284 (Supplementary Table 5). Finding *Synechococcus* in both size fractions was against
285 expectations, as this genus is part of the pico-plankton. Yet, they have been observed in
286 fractions above 3 μm at BBMO [63]. Recovering *Synechococcus* ASVs from the
287 nanoplankton may be due to cell aggregation, particle attachment, clogging of filters, or being
288 prey to larger microorganisms. *Synechococcus* could be also picked up in the 3 μm filters
289 during cell division.

290

291 *Validated associations*

292 As a general rule, the validation of associations tends to be limited as both true interactions
293 and microorganisms that do not interact with each other are poorly known. As done in [48],
294 we determined true genus-genus interactions as those known in the literature, which are
295 compiled within the Protist Interaction Database, PIDA [17]. On October 15th 2019, PIDA
296 contained 2448 interactions. Although our dataset contains protists and bacteria, we could
297 not evaluate interactions between them through PIDA. The ambiguity in taxonomic
298 classification and the large number of edges challenged the validation. We validated
299 associations between microbial eukaryotes via exact string matching as done previously [48].

300

301 **RESULTS**

302 *Extracting a temporal network from a single static association network*

303 From ten years of monthly samples from the Blanes Bay Microbial Observatory (BBMO) in
304 the Mediterranean Sea [30], we computed sequence abundances for 488 bacteria and 1005
305 microbial eukaryotes from two organismal size-fractions: picoplankton (0.2 – 3 μm) and
306 nanoplankton (3 – 20 μm). We removed Archaea since they are not very abundant in the
307 BBMO surface and primers were not optimal to quantify them. We inferred Amplicon
308 Sequence Variants (ASVs) using the 16S and 18S rRNA-gene. After filtering the initial ASV
309 table for sequence abundance and shared taxa among size fractions, we kept 285 and 417
310 bacterial and 526 and 481 eukaryotic ASVs in the pico- and nanoplankton size fractions,
311 respectively. We found 214 bacterial ASVs that appeared in both size fractions, but only two
312 eukaryotic ASVs: a *Cryothecomonas* (Cercozoa) and a dinoflagellate (Alveolate).

313 We used 1709 ASVs to infer a preliminary association network with the tool eLSA
314 [46, 47]. Next, we removed environmentally-driven edges with EnDED [48]. We only

315 considered edges involving partners that co-occurred more than half of the times together
316 than alone (see Methods and Figure 1A-B). Our filtering strategy removed a higher fraction
317 of negative than positive edges (see Methods and Supplementary Table 1). The resulting
318 network is our single static network connecting 709 nodes via 16626 edges (16481 edges,
319 99.1%, positive and 145, 0.9% negative).

320 Next, we developed an approach to determine a temporal network. Building upon the
321 single static network, we determined 120 sample-specific (monthly) subnetworks (see
322 Methods for details). These monthly subnetworks represent the 120 months of the time series
323 and together comprise the temporal network. Each monthly subnetwork contains a subset of
324 the nodes and a subset of the edges of the single static network. We used the ASV abundances
325 indicating the presence (ASV abundance > 0) or absence (ASV abundance = 0) as well as the
326 estimated start and duration of associations inferred with the network construction tool eLSA
327 [46, 47] for determining which nodes and edges are present each month (Figure 1, see
328 Methods).

329

330 *The single static network metrics differed from most monthly subnetworks*

331 Since each monthly subnetwork was derived from the single static network, they were
332 smaller, containing between 141 (August 2005) and 571 (January 2012) nodes, median ≈ 354
333 (Figure 2A), and between 560 (April 2006) to 15704 (January 2012) edges, median ≈ 6052
334 (Figure 2B). For further characterization, we computed six global network metrics (Figure
335 2C and Methods). The results indicated that the single static network differed from most
336 monthly subnetworks and it also differed from the average. In general, the single static
337 network was less connected (edge density) and more clustered (transitivity) with higher

338 distances between nodes (average path length) and stronger associations (average positive
339 association score) than most monthly subnetworks (Figure 2C). In addition, the single static
340 network was usually more assortative according to the node degree but less assortative
341 according to the domain (bacteria vs. eukaryote) than most monthly subnetworks (Figure
342 2C). High assortativity indicates that nodes tend to connect to nodes of a similar degree and
343 domain.

344

345 *Monthly subnetworks display seasonal behavior with yearly periodicity*

346 Over the analyzed decade, the network became more connected and clustered in colder
347 months, with stronger associations and shorter distances between nodes (Figure 2C,
348 Supplementary Figures 1 and 2). Most global network metrics indicated seasonal behavior
349 with yearly periodicity (Figure 2C). For instance, edge density, average positive association
350 score, and transitivity were highest at the beginning and end of each year, while average
351 path length and assortativity (bacteria vs. eukaryotes) were highest in the middle of each
352 year. Assortativity (degree), in contrast to other metrics, usually had two peaks per year
353 corresponding to April-May, and November (Figure 2C). Some metrics (number of nodes
354 and edges, and average path length) presented similar seasonal behavior with yearly
355 periodicity in the temporal network determined from the single static FlashWeave network
356 (Supplementary Figure 3). However, edge density and transitivity displayed patterns
357 contrary to those observed in the temporal network determined from the single static eLSA
358 network.

359 We found mainly temperature and day length, and to a lesser extent nutrient
360 concentrations (mainly SiO₂, NO₃⁻ and NO₂⁻, less PO₄³⁻), and total chlorophyll-a

361 concentration to affect network topologies as indicated by correlation analyses
362 (Supplementary Figure 2). For example, edge density was highest and temperature lowest in
363 January-March. Then, edge density dropped as temperature increased. April-June displayed
364 edge densities slightly above or similar to those in the warmest months July-September, while
365 October-December had similar or slightly lower edge densities than the coldest months
366 January-March. Edge density vs. hours of light (day length) indicated a yearly recurrent
367 circular pattern for September-April (Supplementary Figure 1). Yet, May-August were not
368 part of the circular pattern. May-August had the highest day length and their corresponding
369 networks low edge density (Supplementary Figure 1).

370 Next, we quantified how many edges were preserved (kept), lost, and gained (new)
371 in consecutive months. We found the highest loss of edges in April, pointing to a network
372 collapse. The overall number of edges (preserved and gained) was lowest during April-
373 September and increased towards the end of each year (Figure 2B). The number of
374 associations changed over time in a yearly recurring pattern with few associations being
375 preserved when transitioning from colder to warmer waters. We observed a steep network
376 change when transiting from colder to warmer months, reflecting a large reorganization. In
377 turn, the network change from warmer to colder months was less abrupt. Thus, network
378 change between cold and warm waters was not symmetrical over the studied decade at
379 BBMO.

380 We defined summer and winter as in [28] and compared both seasons between
381 consecutive years in terms of preserved, gained, and lost associations and ASVs. We
382 observed higher repeatability in edges (Supplementary Figure 4) and ASVs (results not
383 shown) in colder than in warmer months, indicating higher predictability during low-
384 temperature seasons.

385

386 *Potential core associations*

387 A single static network can comprise permanent, seasonal, and temporary associations. By
388 comparing monthly subnetworks, we identified edges that remain (preserved), appear
389 (gained), or disappear (lost) over time (Figure 2B). Intuitively, we would classify permanent
390 associations through 100% recurrence. However, no association fulfilled the 100% criteria.
391 Most associations had a low recurrence, with three-quarters of the associations present in no
392 more than 38% (total 46) of the monthly subnetworks. The average association prevalence
393 was similar across taxonomic ranks (Supplementary Figure 5). Considering the 100 most
394 prevalent associations, which appeared in 71.7-98.3% (total 86-118) of the monthly
395 subnetworks, 87 were associations among bacteria (Supplementary Table 2).

396 Although the temporal recurrence of associations over the ten years was low, we
397 found high recurrence in corresponding months from different years. We quantified the
398 fraction of subnetworks in which each association appeared (Supplementary Figure 6). We
399 observed the highest prevalence from December to March, and the lowest prevalence from
400 June to August (Supplementary Figure 6). For each month, we taxonomically characterized
401 prevalent associations appearing in at least nine out of the ten monthly subnetworks (e.g., 9
402 out of 10 Januarys; Figure 3). We found a larger number of prevalent associations in colder
403 waters compared to warmer waters, with Alphaproteobacteria dominating these associations,
404 especially in April and May (Figure 3). The Alphaproteobacteria ASVs featuring highly
405 prevalent associations belonged to *Pelagibacter ubique* (SAR11 Clades Ia & II),
406 Rhodobacteraceae, *Amylibacter*, Puniceispirillales (SAR116), *Ascidiaceihabitans*,
407 *Planktomarina*, Parvibaculales (OCS116), and *Kiloniella*. Between April and May, we

408 noticed a large increase in the fraction of associations including Cyanobacteria or
409 Bacteroidetes as association partners. While Cyanobacteria associations were a small fraction
410 during November-April, they had a dominant role from May-October along with
411 Bacteroidetes and Alphaproteobacteria associations (Figure 3). Overall, this underlines the dynamic
412 nature of associations over the year, pointing to recurring annual associations that may be essential for
413 ecosystem function.

414

415 *Dynamic associations within main taxonomic groups: the case of Cyanobacteria*

416 Our results indicated that associations are dynamic within specific taxonomic groups.
417 Therefore, we investigated their behavior in Cyanobacteria given the importance of this
418 group as primary producers in the ocean. We found 661 associations for *Synechococcus*,
419 *Prochlorococcus*, and *Cyanobium* ASVs (Figure 4 and Supplementary Figure 7). Most
420 associations between cyanobacterial ASVs were positive (63 of 65), and only a
421 *Synechococcus* (referred to as bn_ASV_5) was negatively associated (association score -0.5)
422 with other *Synechococcus* (bn_ASV_1 and bn_ASV_25), which, in turn, were positively
423 associated (association score 0.8). While bn_ASV_5 appeared mainly in colder months, the
424 other two appeared mainly in warmer months (Supplementary Figure 7). All Cyanobacteria
425 had more associations with other bacteria (in total 433) than with eukaryotes (in total 163).

426 Within the temporal network, the fraction of Cyanobacteria associations was highest
427 in April-October (Figure 4A), which are the months with the largest cyanobacterial
428 abundances (Supplementary Figure 7), and the fewest edges in the entire temporal network
429 (Figure 2B), for example, in the year 2011 (Figure 4B). We found that cyanobacterial ASVs,
430 although being evolutionarily related, behaved differently in terms of the number of
431 associations over time, and association partners (Supplementary Figure 7). For example,

432 *Synechococcus* bn_ASV_5 had fewer partners than bn_ASV_1 according to numbers of
433 associations, but more according to taxonomic variety (Supplementary Figure 7). Only a tiny
434 fraction of *Prochlorococcus* (e.g. bp_ASV_18) association partners were other
435 Cyanobacteria, which contrasted with *Synechococcus* and *Cyanobium* (Supplementary
436 Figure 7). Moreover, we observed that *Cyanobium* (bn_ASV_20) connected to one
437 Deltaproteobacteria (SAR324) ASV during the first eight years, but the association
438 disappeared in the last two years. In particular, the inferred association duration was 101
439 months, starting in March 2004 and ending in July 2012. After summer 2012, the
440 Deltaproteobacteria ASV was not detected except a few reads in November and December
441 2012 and 2013. This Cyanobacteria example may also illustrate the dynamics of associations
442 within other main taxonomic groups.

443

444 *Validating associations using known ecological interactions*

445 We checked how many potential interactions could be validated using a database of observed
446 ecological interactions (PIDA; [17]). In total, 16 associations (out of 16626) in the temporal
447 network were validated by PIDA (Supplementary Table 6). These 16 associations describe
448 six unique interactions between seven taxa (at the genus-level). For instance, the reoccurring
449 association between a diatom from genus *Thalassiosira* and a Flavobacteriia starts mainly
450 around October and often ends around March (Supplementary Figure 8). In contrast, the
451 reoccurring association between a dinoflagellate from genus *Gyrodinium* and one from
452 *Heterocapsa* appears for a shorter time and during the summer months (Supplementary
453 Figure 8).

454

455 **DISCUSSION**

456 Previous work identified yearly recurrence of microbial community composition at the
457 BBMO [13, 28, 64], and similarly at the nearby Bay of Banyuls [14], both in the North-West
458 Mediterranean Sea and in other temperate sites around the world [12, 65]. We focused here
459 in the connectivity of microorganisms and how they organize themselves from a network
460 perspective. In general, the measured global network metrics (edge density, transitivity, and
461 average path length) are within the range reported in previous studies [22–25, 66–68] (Table
462 2). Contrary to early studies reporting biological networks generally being disassortative
463 (negative assortativity based on degree) [69], our single static network and the monthly
464 subnetworks were assortative. Microorganisms had more and stronger connections and a
465 tighter clustering in colder than in warmer waters. To some extent, this might reflect species
466 richness, which has been shown for the resident microorganisms to increase during the colder
467 months at BBMO using the same dataset [28]. However, the exact effect of richness on
468 ecological interactions among microorganisms needs further analysis. Seasonal bacterial
469 freshwater networks [67] also showed higher clustering in fall and winter than in spring and
470 summer, but, in contrast to our results, networks were most extensive in summer and smallest
471 in winter. In agreement with our results, Chaffron *et al.* [68] reported higher association
472 strength, edge density, and transitivity in cold polar regions compared to other warmer
473 regions of the global ocean. Colder waters in the Mediterranean Sea are milder than polar
474 waters. However, together, these results suggest that either microorganisms interact more in
475 colder environments, or that their recurrence is higher due to higher environmental selection
476 exerted by low temperatures. Additionally, limited resources (mainly nutrients) in summer
477 or in the tropical and subtropical open ocean may prevent the establishment of several
478 microbial interactions. In any case, temperature is likely not the only driver of network

479 architecture [68].

480 The effects of environmental variables on network metrics are unclear [70], yet, our
481 approach allowed us to identify potential environmental drivers of network architecture.
482 Correlation analyses pointed to variables that have been found to influence microbial
483 abundances in the ocean. For instance, our results indicated that temperature and day length,
484 key variables driving microbial assemblages in seasonal time series [12–14], and to a lesser
485 extent inorganic nutrients, were the main factors influencing global network metrics. It also
486 agrees with earlier works indicating that phosphorus and nitrogen are the primary limiting
487 nutrients in the Western Mediterranean Sea [71, 72]. Altogether, our correlation analysis is a
488 step forward towards elucidating the effects of environmental variables on network metrics.
489 However, we did not consider several other variables that could affect network architecture
490 (e.g. organic matter).

491 Our preliminary network (significant associations derived with eLSA) contained 18%
492 negative edges compared to 0.9% in the single static network (after filtering). Thus, our
493 filtering strategy removed proportionally more negative edges. Associations may represent
494 positive or negative interactions, but they can also indicate high niche overlap (positive
495 association) or divergent niches (negative association) between microorganisms [73]. We
496 hypothesize that most of the removed negative edges represented associations between
497 microorganisms from divergent niches, most likely corresponding to colder or warmer
498 months.

499 We found more highly prevalent associations within specific months than when
500 considering all ten years of data. Furthermore, our results indicate a potentially low number
501 of core interactions and a vast number of non-core ones. Usually, core microorganisms are
502 defined based on sequence abundances, as those microorganisms (or taxonomical groups)

503 appearing in all samples or habitats being under investigation [74]. Shade & Handelsman
504 [74] suggested that other parameters, including connectivity, should create a more complex
505 portrait of the core microbiome and advance our understanding of the role of key
506 microorganisms and functions within and across ecosystems [74]. Using a temporal network
507 we identified core associations based on recurrence, which contributes to our understanding
508 of key interactions underpinning microbial ecosystem functions. Considering associations
509 within each month, we found more highly-prevalent associations in colder than in warmer
510 months. Our results indicate microbial connectivity is more repeatable (indicating higher
511 predictability) in colder than in warmer waters. On the one hand, the microbial community
512 in colder waters being more recurrent [13] may explain our observations indicating a more
513 robust connectivity during this period. Alternatively, it may be the stronger connectivity what
514 leads to more similar communities in colder waters at the BBMO. Last but not least, the
515 interplay of both species dynamics and interactions may determine community turnover in
516 the studied ecosystem. From a technical viewpoint, our monthly sampling strategy and/or the
517 overall single static network may have not been able to detect interactions appearing solely
518 in summer resulting in smaller monthly subnetworks. For instance, previous work on
519 freshwater lakes constructed season-specific networks and found more associations in
520 summer than in winter [67].

521 Several network-based analyses have been used to particularly study Cyanobacteria
522 associations. For example, in the southern Californian coast, Chow et al. [24] identified 44
523 potential relationships of 12 Cyanobacteria (*Prochlorococcus* and *Synechococcus*) with two
524 potential eukaryote grazers (a ciliate and a dinoflagellate), 39 to other bacteria, and three
525 between Cyanobacteria, which were all positive. Similarly, all cyanobacterial ASVs in our
526 study connected primarily to other bacterial ASVs, and featured mainly positive associations.

527 Furthermore, Cyanobacteria displayed primarily positive associations with other
528 microorganisms in a global ocean network [66]. This suggests that other sampling or
529 computational approaches are needed to detect negative associations involving marine
530 cyanobacteria.

531 Identifying different potential association partners for closely related Cyanobacteria
532 may indicate adaptations to different niches. A recent study found distinct seasonal patterns
533 for closely related bacterial taxa indicating niche partitioning at the BBMO, including
534 *Synechococcus* ASVs [64]. Our approach can complement and further characterize “sub-
535 niches” by providing association partners for different ASVs. Moreover, in contrast to a
536 single static network, temporal networks allow identifying associated partners in time
537 (Supplementary Figure 7). An increase in the abundance of a microorganism may promote
538 the growth of associated partners and a decrease may hinder the growth of partners or cause
539 predators to prey on other microorganisms. Moreover, given the majority of association
540 partners being other bacteria, the growth of Cyanobacteria may affect other bacteria and their
541 growth, which is why it is necessary to identify potential interaction partners [67].

542 Our approach allowed us to disentangle in time the associations captured by a single
543 static network using monthly samples for ten years. Future studies should determine whether
544 higher sampling frequency (e.g., daily samples during a month) can capture other
545 associations not present in our networks. Thus, our results should be considered taking into
546 account the used (monthly) sampling frequency. In addition, certain network metrics may
547 depend on the tool used to infer the single static network, e.g., edge density, and, therefore,
548 should be interpreted with care. An additional consideration is that we disregarded local
549 network patterns by using global network metrics. Future work could use the local-
550 topological metric based on graphlets [75]. Counting the number of graphlets a node is part

551 of quantifies their local connection patterns, which allows inferring seasonal microorganisms
552 through recurring connection patterns in a temporal network.

553

554 **CONCLUSION**

555 Incorporating the temporal dimension in microbial association analysis unveiled multiple
556 patterns that often remain hidden when using single static networks. Investigating a coastal
557 marine microbial ecosystem over ten years revealed a one-year-periodicity in the network
558 topology. The temporal network architecture was not stochastic, but displayed a modest
559 amount of recurrence over time, especially in winter. Future efforts to understand the ocean
560 microbiome should consider the dynamics of microbial interactions as these are likely
561 fundamental for ecosystem functioning.

562

563 **Ethics approval and consent to participate**

564 Not applicable.

565

566 **Consent for publication**

567 Not applicable.

568

569 **Availability of data and material**

570 The BBMO microbial sequence abundances (ASV tables), taxonomic classifications,
571 environmental data including nutrients, networks and R-Markdowns for data analysis
572 including commands to run eLSA and EnDED (environmentally-driven-edge-detection and

573 computing Jaccard index) are publicly available:

574 <https://github.com/InaMariaDeutschmann/TemporalNetworkBBMO>.

575

576 **Competing interests**

577 The authors declare that they have no competing interests.

578

579 **Funding**

580 This project and IMD received funding from the European Union's Horizon 2020 research

581 and innovation program under the Marie Skłodowska-Curie grant agreement no. 675752

582 (ESR2, <http://www.singek.eu>) to RL. RL was supported by a Ramón y Cajal fellowship

583 (RYC-2013-12554, MINECO, Spain). This work was also supported by the projects

584 INTERACTOMICS (CTM2015-69936-P, MINECO, Spain), MicroEcoSystems (240904,

585 RCN, Norway) and MINIME (PID2019-105775RB-I00, AEI, Spain) to RL. FL was

586 supported by the Spanish National Program FPI 2016 (BES-2016-076317, MICINN, Spain).

587 SC was supported by the CNRS MITI through the interdisciplinary program Modélisation

588 du Vivant (GOBITMAP grant). DE and SC were supported by the H2020 project AtlantECO

589 (award number 862923). A range of projects from the EU and the Spanish Ministry of

590 Science funded data collection and ancillary analyses at the BBMO. We acknowledge

591 funding of the Spanish government through the ‘Severo Ochoa Centre of Excellence’

592 accreditation (CEX2019-000928-S).

593

594 **Author’s contributions**

595 The overall project was conceived and designed by RL and AKK. VB, JMG, and RM were

596 responsible for the sampling and contextual data at the BBMO. RL processed the amplicon
597 data from BBMO generating the ASV tables. AKK constructed the initial preliminary
598 network which was the starting point of the present study. IMD developed the conceptual
599 approach and DE, SC, and RL contributed to its finalization. IMD performed the data
600 analysis. ED, DE, SC, FL, AKK, CM, JMG, and RL contributed with the biological
601 interpretation of the results. IMD wrote the original draft. All authors contributed to
602 manuscript revisions and approved the final version of the manuscript.

603

604 **Acknowledgements**

605 We thank all members of the Blanes Bay Microbial Observatory (<http://bbmo.icm.csic.es>)
606 team with the multiple projects funding this collaborative effort. Part of the analyses have
607 been performed at the Marbits bioinformatics core at ICM-CSIC
608 (<https://marbits.icm.csic.es>). We thank L. Felipe Benites for fruitful discussions regarding
609 microorganisms and their interactions.

610

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- 794

795 **FIGURE LEGENDS**

796 **Figure 1:** Estimating a temporal network from a single static network via subnetworks. A)

797 A complete network would contain all possible associations (edges) between microorganism

798 (nodes). B) The single static network inferred with the network construction tool eLSA and

799 the applied filtering strategy considering association significance, the removal of

800 environmentally-driven associations, and associations whose partners appeared in more

801 samples together than alone, i.e., Jaccard index being above 0.5. An association having to be

802 present in the single static network is the first out of the three conditions for an association

803 to be present in a monthly subnetwork. C) In order to determine monthly subnetworks, we

804 established two further conditions for each edge. First, both microorganisms need to be

805 present in the sample taken in the specific month. Second, the month lays within the time

806 window of the association inferred through the network construction tool. Here, three months

807 are indicated as an example. D) Example of monthly subnetworks for the three months. The

808 colored nodes correspond to the abundances depicted in C).

809

810 **Figure 2:** Global (sub)network metrics. A) Number of ASVs (counting an ASV twice if it

811 appears in both size fractions) for each of the 120 months of the Blanes Bay Microbial

812 Observatory time series. There are 1709 ASVs, of which 709 ASVs are connected in the

813 static network. In black, we show the number of nodes connected in the temporal network,

814 and in red the number of nodes that are isolated in the temporal network, i.e., they are

815 connected in the static network and have a sequence abundance above zero for that month

816 ("non-zero"). In dark grey, we show the number of ASVs that are non-zero in a given month

817 but were not connected in the static and subsequently temporal network. In light grey, we

818 show the number of ASVs with zero-abundance in a given month. The sum of connected and
819 isolated nodes and non-zero ASVs represents each month's richness (i.e., number of ASVs).
820 B) By comparing the edges of two consecutive months, i.e., two consecutive monthly
821 subnetworks, we indicate the number of edges that have been lost (red), preserved (black),
822 and those that are gained (blue), compared to the previous month. C) Six selected global
823 network metrics for each sample-specific subnetwork of the temporal network. The colored
824 line indicates the corresponding metric for the static network.

825

826 **Figure 3:** Associations with a monthly prevalence of at least 90%. Bacteria and eukaryotes
827 are separated and ordered alphabetically. We provide in parentheses the number of
828 associations that appeared in at least nine out of ten monthly subnetworks.

829

830 **Figure 4:** *Cyanobacteria* associations. A) Fraction of edges in the temporal network
831 containing at least one *Cyanobacteria* ASV. B) Location of *Cyanobacteria* associations in
832 the temporal network and the single static network. Here we show, as an example, selected
833 months of year 2011. The number and fraction of cyanobacterial edges and total number of
834 edges is listed below each monthly subnetwork and the single static network.

835

836 **TABLES**

837 **Table 1:** Number and fraction of ASVs and reads (total, bacterial and eukaryotic) for the sequence abundance
 838 tables (A, B, and C), the preliminary network with significant edges (D), and the single static network (E) obtained
 839 after removing environmentally-driven edges and edges with association partners appearing more often alone
 840 than with the partner. If an ASV appeared in the nano- and pico-plankton size fractions, it was counted twice.

Count tables	ASVs	Reads	Eukaryote	Eukaryotic reads	Bacteria	Bacterial reads
A	2 924	2 273 548	1 365	1 121 855	1 559	1 151 693
B	1 782	2 155 318	1 009	1 057 599	773	1 097 719
C	1 709	2 062 866	1 007	1 057 263	702	1 005 603
D	754	1 657 885	306	730 025	448	927 860
E	709	1 621 959	294	719 558	415	902 401

Fractions	ASV	Reads	Eukaryote	Eukaryotic reads	Bacteria	Bacterial reads
B/A*100	60.94	94.80	73.92	94.27	49.58	95.31
C/A*100	58.45	90.73	73.77	94.24	45.03	87.32
D/C*100	44.12	80.37	30.39	69.05	69.05	92.27
E/C*100	41.49	78.63	29.20	68.06	59.12	89.74

841 *A – raw sequence abundance table; B – sequence abundance table without rare ASVs; C – sequence abundance table after size-fraction*
 842 *filtering; D – preliminary network with significant edges; E – single static network*

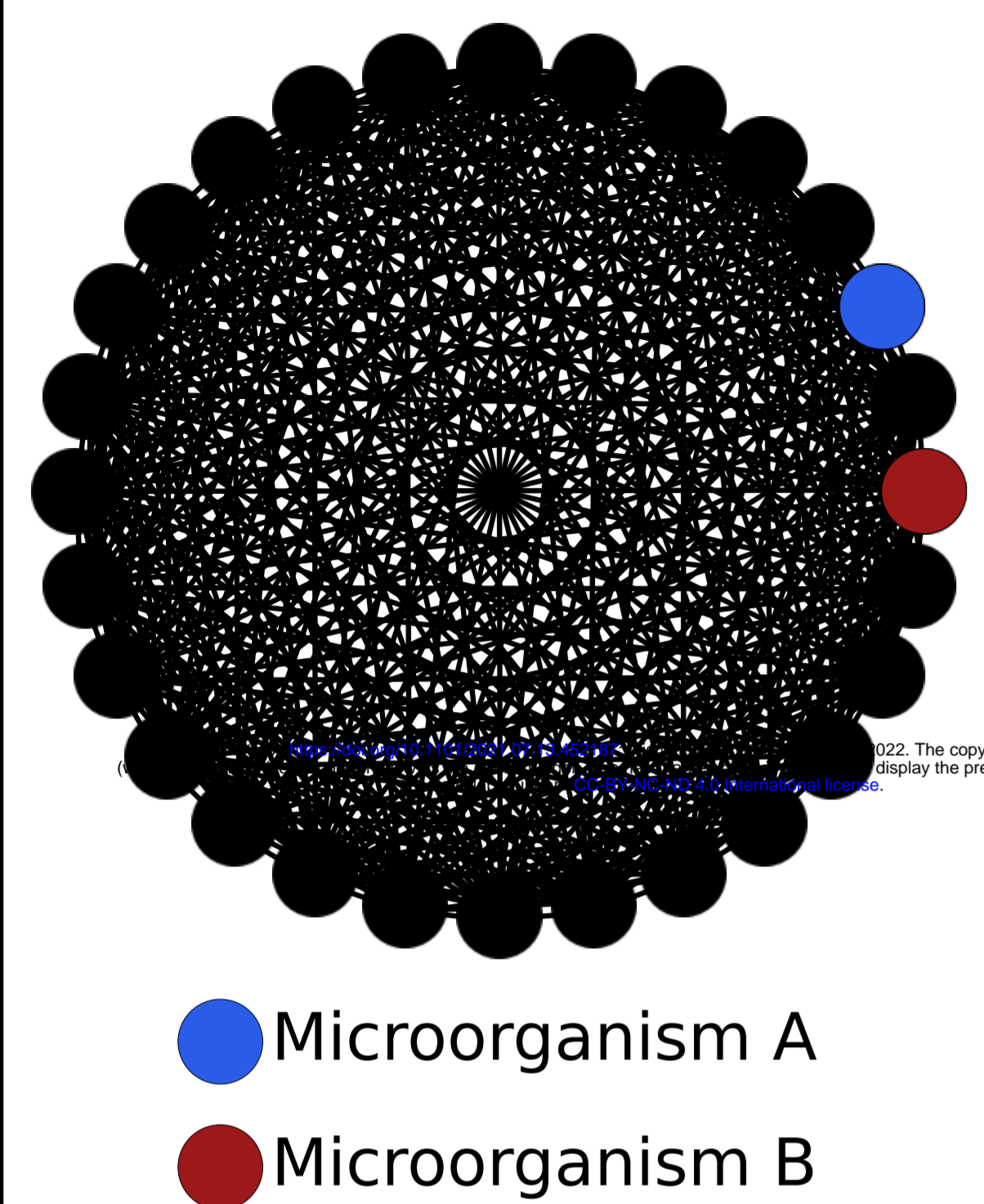
843

844 **Table 2:** Global network metrics of previously described microbial association networks

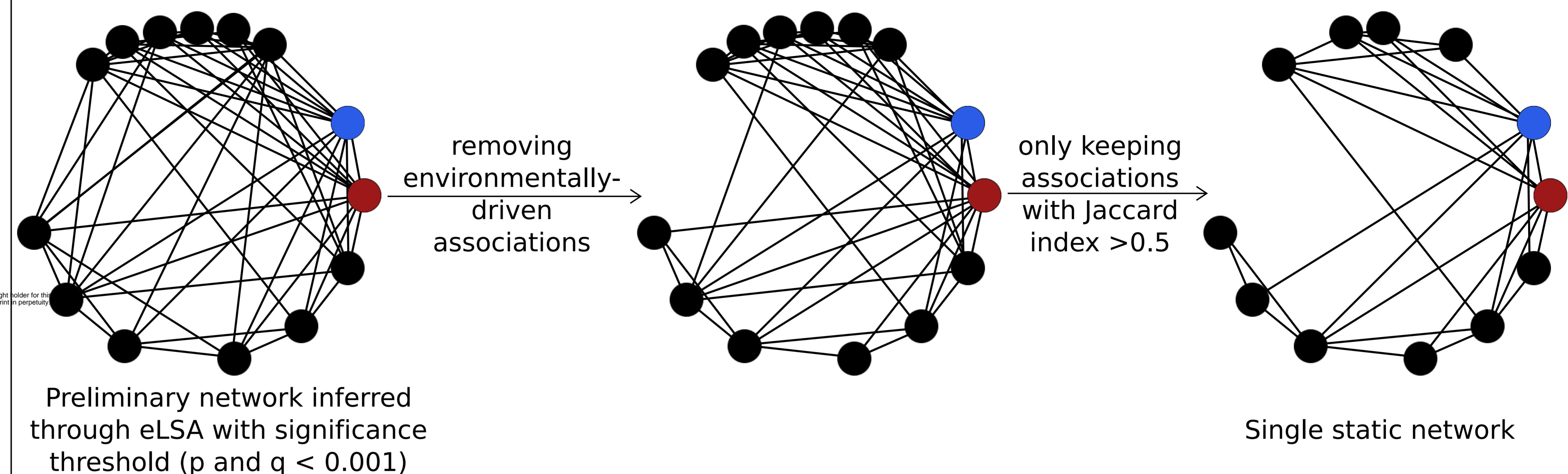
Location & Depth	Edge density	Transitivity	Average path length	Sampling	Domains	Notes	Reference
SPOT (Off the southern California coast); Deep chlorophyll maximum.	0.04	0.26	3.05	Monthly. August 2000 - March 2004	Archaea, bacteria, and eukaryotes	Edge density for microbial network including environmental factors. Transitivity and average path length for microbial network.	[22]
SPOT; Surface ocean and deep chlorophyll maximum	0.14	0.33	1.94	Monthly. August 2000 - January 2011	Free-living bacteria and picoeukaryotes	Metrics from surface layer network.	[23]
SPOT; Surface	0.02	0.24		Monthly. March 2008 - January 2011	Free-living eukaryotes (0.7–20 µm), bacteria (0.22–1 µm) and viruses (30 kDa–0.22 µm)		[24]
SPOT; Five depths (5 m - Surface, the deep chlorophyll maximum layer, 150 m, 500 m and 890 m - just above the sea floor)	0.04	0.28	2.07	Monthly. August 2003 - January 2011	Free-living bacteria	Metrics for 5 m layer network.	[25]
52 samples from freshwater lakes in China; Surface	(0.023) W:0.033, Sp:0.032 , S:0.036, F:0.029	(0.472) W:0.518, Sp:0.480, S:0.475, F:0.573	(4.84) W:2.16, Sp:5.03 S:7.26, F:3.04	Spatial	Bacteria	Metrics for (whole network) and seasonal networks: W: winter, Sp: spring, S: summer, and F: fall	[67]
68 stations from the Tara Oceans expedition across eight oceanic provinces; Surface and Deep chlorophyll maximum	0.005, 0.003, 0.008	0.2, 0.0, 0.43	3.05, 3.02, 2.56	Spatial	Organisms from seven size fractions	Metrics from surface networks including eukaryotes only, eukaryotes and prokaryotes (0.5-5 µm), and prokaryotes only (0.2-1.6 µm)	[66]
115 stations from the Tara Oceans expedition covering all major oceanic provinces from pole to pole; Surface and deep chlorophyll maximum	0.002	0.036		Spatial	Bacteria, archaea, and eukaryotes from six size fractions.	Metrics represent the means of sample-specific subnetworks.	[68]

845

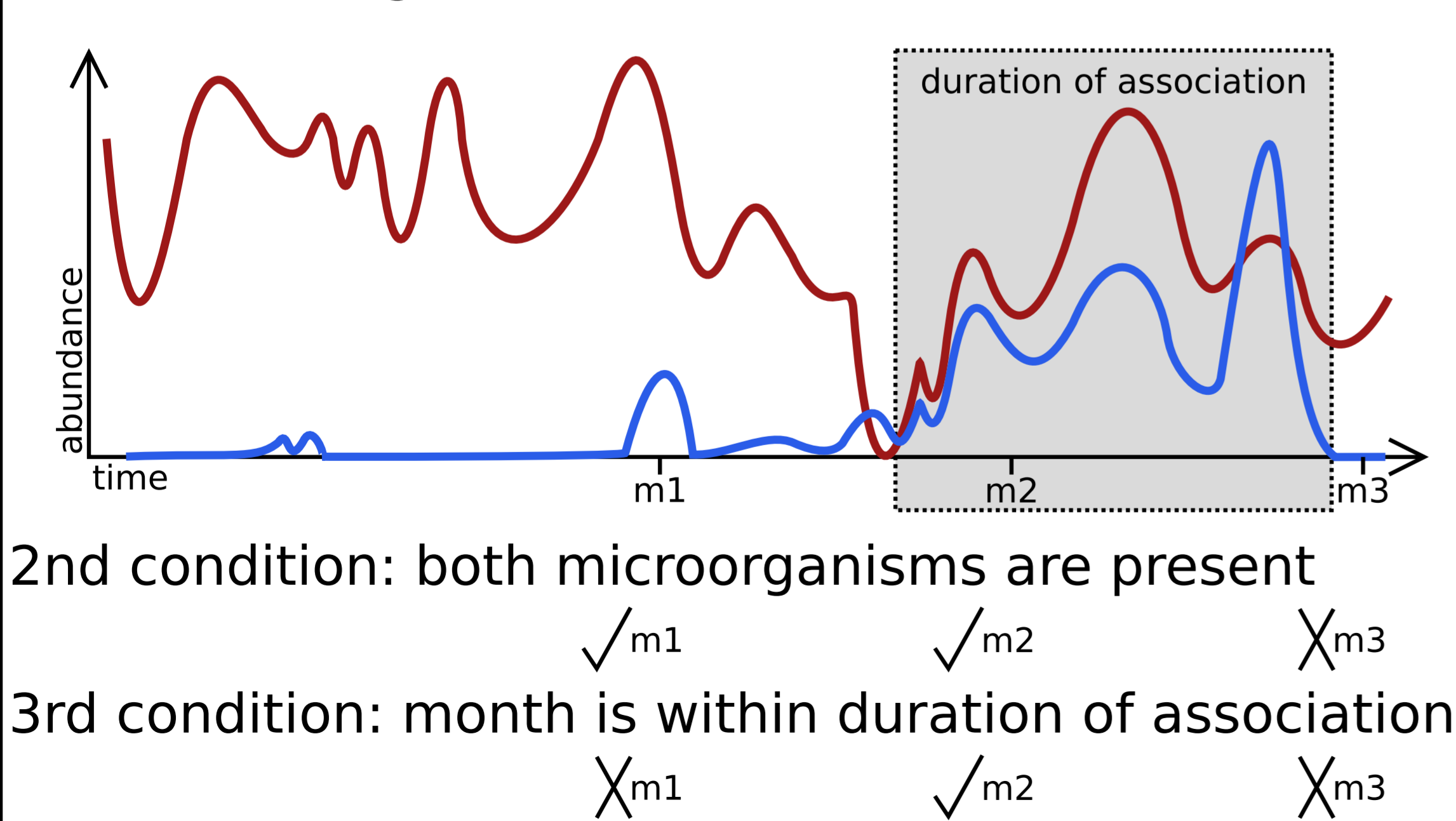
A) all potential associations



B) determining single static network (1st condition)



C) determining subnetworks



D) temporal network constituted from monthly subnetworks

