An INDEL genomic approach to explore population diversity of phytoplankton : *Bathycoccus*, a case study

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Running head : Markers of genetic diversity in *Bathycoccus*

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Abstract

Although metabarcoding has generated large dataset on world-wide phytoplankton species diversity, little is known about the intraspecies diversity underlying adaptation to environmental niches. To gain insight into population diversity, a novel INDEL based method was developed on *Bathycoccus prasinos*. Oxford Nanopore Technology (ONT) sequencing was first used to characterise structural variants (SV) among the genomes of *Bathycoccus* sampled from geographically distinct regions in the world ocean. Markers derived from INDEL were validated by PCR and sequencing in the world-wide strains. These markers were then used to genotype 55 *Bathycoccus* strains isolated during the winter bloom 2018-2019 in the bay of Banyuls-sur-Mer. With five markers, eight Multi Loci Genotypes (MLG) were determined, two of which represented 53% and 29% of the isolates. Physiological studies confirmed that isolates are phenotypically different, cells isolated in February growing better at low temperature than those isolated in December and January. When tested directly on environmental samples, two diversity markers showed a similar allele frequency in sea water as in individual *Bathycoccus* strains isolated at the same period. We conclude that these markers constitute a resource to identify the most abundant variant alleles in a given bloom. A follow-up on three consecutive blooms revealed differences in allele abundance during the course of a bloom, particularly at initiation and between years. This INDEL-based genotyping constitutes a new methodological approach that may be used to assess the population structure and diversity of other species.

39 **Introduction**

40 Marine phytoplankton, including picoeukaryotic algae, is responsible for a large fraction of
41 primary production (Li et al. 1983). In temperate regions, the abundance and diversity of the
42 phytoplankton is often seasonal and occurs in bursts, as algal blooms. Per se, blooms have a
43 large impact on global primary production and therefore the understanding of the genetic
44 basis of phytoplankton adaptation to seasonal niches and the effects of ocean warming on
45 phytoplankton blooms are of the utmost importance.

46 Meta-ribosomal barcoding on the nuclear or plastidial 18/16S rRNA gene has opened the
47 access to massive data in time and space and has accelerated the study of phytoplankton
48 species diversity, interspecies co-occurrence and potential biotrophic interactions in natural
49 communities. However, metabarcoding approaches do not provide information on
50 intraspecies genetic variations. Assessing only interspecies diversity, underestimates the
51 diversity of the populations. Equally, a single isolate cannot represent the diversity of a
52 population. Since natural selection acts on variation among individuals within populations, it
53 is essential to incorporate both intra and interspecies trait variability into community ecology
54 (Violle et al. 2012). Raffard et al. (2019) demonstrated that intraspecies variation has
55 significant ecological effects across a large set of species, confirming a previous estimate
56 based on a more restricted species set (Des Roches et al. 2018). Furthermore, it has been
57 shown that diversity within species is rapidly decreasing, making them more homogenous and
58 highlighting the need to preserve intraspecies variations (Des Roches et al. 2018) since
59 intraspecies diversity reinforces the overall population stability in the face of environmental
60 change.

61 In diatoms, intraspecies variation has been shown to play a key role in the responses of the
62 species to several important environmental factors such as light, salinity, temperature and
63 nutrients (Godhe et al. 2017). Modelling efforts indicate that this variation within species

64 extends bloom periods and likely provides sufficient variability in competitive interactions
65 between species under variable conditions. The intraspecies variation most likely corresponds
66 to optimal fitness in temporary microhabitats. This rich intraspecies genetic diversity allows
67 for the possibility of local adaptation and for differentiation in important physiological
68 characteristics that produces local populations that are exceptionally fit and competitive in
69 their respective local habitat.

70 Several studies suggest that previously recognized cosmopolitan species are actually
71 composed of multiple populations or even multiple species (Kashtan et al. 2014). Genotypes
72 or species can either replace each other temporally (but with overlap) as in the case of the
73 marine diatoms *Pseudo-nitzschia multistriata* (Tesson et al. 2014) and *Skeletonema costatum*
74 (Gallagher, 1980) or co-exist sympatrically as in the freshwater *Asterionella formosa* (Van
75 Den Wyngaert et al. 2015). Furthermore, the frontier between variant genotype and species is
76 thin and insight will be gained by whole genome sequencing of a large number of strains.
77 Read et al. (2013) documented a pan genome of the coccolithophore *Emiliania sp* revealing
78 that what was previously considered a single species, is actually composed of multiple
79 species. Similarly Bentif et al. (2023) with morphological and genomic surveys showed
80 *Gephyrocapsa huxleyi* has evolved to comprise at least three distinct species.

81 Since an assembly of genotypically diverse individuals constitutes a population,
82 methodological approaches have been developed in order to determine the genetic variation
83 among individuals (reviewed for Diatoms in Rynearson et al. 2022). One of the major
84 challenges is the difficulty to isolate individuals in sufficient number for classical diversity
85 analyses.

86 Historically, the intraspecies markers corresponded to small nucleotide repeats such as
87 microsatellite (Srivastava et al. 2019), chloroplastic (Wheeler et al. 2014) and mitochondrial
88 (Galtier et al. 2009) genes and a few nuclear genes that were applied to several hundreds of

89 isolated individuals. Microsatellites have been described in some diatoms (Tesson et al. 2011)
90 but not to date in Mamiellales. The development of PCR for Randomly Amplified
91 Polymorphic DNA (RAPD, Lewis et al. 1997) and more recently Restriction-site-Associated
92 DNA sequencing techniques (RADseq, Andrews et al. 2016) have allowed the discovery and
93 genotyping of thousands of genetic markers for any given species at relatively low-cost. Some
94 of these approaches have been used to analyse the diversity of populations during algal
95 blooms (Rengefors et al. 2017). The recent dramatic increase of the number of sequenced
96 genomes led to large-scale diversity studies with large sets of nuclear genes or whole genome
97 comparisons. However most of these approaches required the isolation of a large number of
98 individuals. As a consequence, intraspecies diversity has been poorly documented in the past
99 in marine phytoplankton.
100 Widely distributed from the equator to arctic and antarctic poles with a marked seasonality in
101 temperate and polar regions (Joli et al. 2017, Tragin et al. 2018, Lambert et al. 2019, Leconte
102 et al. 2020), picoeukaryotes belonging to the order of Mamiellales (*Bathycoccus*,
103 *Ostreococcus and Micromonas*) have a cosmopolitan presence illustrating a high capacity for
104 adaptation to a wide range of contrasting environments. Novel, rapid and cheap sequencing
105 technologies have given access to Mamiellales diversity by metagenomic approaches
106 (Leconte et al. 2020, Da Silva et al. 2022, Richter et al. 2022) or metatranscriptomic
107 approaches (Simmons et al. 2016), however to date, very little information is available on
108 intraspecies diversity of Bathycoccaceae with the exception of *Ostreococcus tauri* (Blanc-
109 Mathieu et al. 2017). Unlike *O. tauri* which is usually not detectable in publicly available
110 metagenomes, *Bathycoccus* is the most cosmopolitan Mamiellophyceae (de Vargas et al.
111 2015). *Bathycoccus* can be divided in two species, the polar and temperate *Bathycoccus*
112 *prasinos* type B1 genome (Moreau et al. 2012, Joli et al. 2017) and the tropical *Bathycoccus*
113 *calidus* type B2 genome (Vannier et al. 2016, Limardo et al. 2017, Bachy et al. 2021). Thus

114 the cosmopolitan nature of *Bathycoccus* from poles to equator might be due to the
115 combination of both B1 and B2 species.
116 In the bay of Banyuls, *Bathycoccus* and *Micromonas* bloom yearly from November to April,
117 *Bathycoccus* being one of the most abundant species (Lambert et al. 2019). The highly
118 reproducible yearly occurrence of *Bathycoccus* in the Banyuls bay during the last decade
119 (Lambert et al. 2019) raises the question of the persistence of a *Bathycoccus* population
120 adapted to the bay or of a variation of the population structure each year. In addition, since
121 outside of the bloom period Mamiellales are virtually absent from the bay, is the *Bathycoccus*
122 bloom initiated by an uptake of resident "resting cells" in the sediment or by a fresh input
123 carried by North western Mediterranean currents along the Gulf of Lion? At present no
124 resting stages that can act as inoculum of subsequent blooms have been described for
125 *Bathycoccus*.
126 To assess the intraspecies diversity of *Bathycoccus* in the Bay of Banyuls, we combined an
127 efficient method to isolate Mamiellales together with whole genome sequencing by Oxford
128 Nanopore Technology (ONT) in order to identify Structural Variants (SV) in the *Bathycoccus*
129 genome. Diversity markers designed from INDEL (insertion or deletion of bases in the
130 genome of an organism) were used to genotype *Bathycoccus* strains and populations from
131 environmental samples. This approach constitutes an unprecedented tool which could be
132 potentially applied to a large variety of species.
133
134 **Materials and Methods**
135 *Algal strains and culture conditions*
136 World-wide *Bathycoccus* strains were obtained at the Roscoff Culture Collection (RCC)
137 centre and renamed as a town or region to be reminiscent of their geographical origin:
138 RCC4222 (named BANYULS), RCC5417 (BAFFIN), RCC1613 (OSLO), RCC685

139 (HELGOLAND), RCC1615 (DIEPPE), RCC1868 (ROSCOFF), RCC4752 (NAPLES)
140 (Supplemental Table 1). RCC1105-REFERENCE from which the current *Bathycoccus*
141 *prasinos* reference genome originated (Moreau et al. 2012) was lost and replaced by
142 RCC4222-BANYULS. The strains were cultivated in 100 mL flasks in filtered artificial
143 seawater (24.55 g/ L NaCl, 0.75 g/L KCl, 4.07 g/L $MgCl_2$ $6H_2O$, 1.47 g/L $CaCl_2$ $2H_2O$, 6.04
144 g/L $MgSO_4$ $7H_2O$, 0.21 g/L $NaHCO_3$, 0.138 g/L NaH_2PO_4 and 0.75 g/L $NaNO_3$)
145 supplemented with trace metals and vitamins (Supplemental data 1). Cultures were
146 maintained under constant gentle agitation in an orbital platform shaker (Heidoph shaker and
147 mixer unimax 1010). Sunlight irradiation curves recreating realistic light regimes at a chosen
148 latitude and period of the year were applied in temperature-controlled incubators (Panasomic
149 MIR-154-PE).

150 *Cell isolation*

151 Surface water was collected at 3 meter depth at SOLA buoy in Banyuls bay, North Western
152 Mediterranean Sea, France (42°31′N, 03°11′E) approximately every week from December 2018
153 to March 2019, November 2019 to March 2020 and October 2020 to April 2021. Two ml aliquots
154 were used to determine the quantity and size of phytoplankton by flow cytometry. For the
155 bloom 2018/2019, 50 ml were filtered through a 1.2-µm pore-size acrodisc (FP 30/1.2 CA-S
156 cat N° 10462260 Whatman GE Healthcare Sciences) and used to inoculate 4 culture flasks
157 with 10 ml of filtrate each. The sea water was supplemented by vitamins, NaH2PO4, NaNO3
158 and metal traces at the same final concentration as artificial sea water (ASW; Supplemental
159 data 1), antibiotics (Streptomycine sulfate and Penicillin at 50 µg/ml) were added to half of
160 the cultures. The cultures were incubated under light and temperature conditions similar to
161 those during sampling date for 3-4 weeks. The presence of picophytoplankton was analysed
162 by a BD accuri C6 flow cytometer. In general, superior results were obtained without
163 antibiotics. Cultures containing at least 90% of picophytoplankton with only residual

164 nanophytoplankton were used for plating on agarose. Colonies appearing after 10 days were
165 hand-picked and further cultured in 2 ml ASW in deepwell plates (Nunc, Perkin Elmer,
166 Hessen, Germany) for 10 days. Cells were cryopreserved at this stage. Circa 500 clones were
167 cryopreserved. At the same time, DNA extraction and PCR were performed in order to
168 identify *Bathycoccus* clones.

169 *DNA extraction, genome sequencing, assembly and PCR amplification*

170 For PCR analysis, total DNA was extracted from 4 ml *Bathycoccus* cell cultures according to
171 the Plant DNA easy Qiagen protocol. For whole genome sequencing by Oxford Nanopore
172 technology (ONT), DNA was extracted by a CTAB method from 100 ml culture principally
173 based on Debladis et al. (2017). ONT libraries were barcoded using the Rapid Barcoding
174 Sequencing (SQK-RBK004) and deposited on R9.4 flow cell. For environmental samples, 5
175 litres of seawater at SOLA 3 meter depth were passed through 3 microns and 0.8 micron pore
176 filters. DNA from cells collected on the 0.8 micron filters were extracted using the Plant DNA
177 easy Qiagen protocol with the addition of a proteinase K treatment in the AP1 buffer. PCR
178 was performed using the Red Taq polymerase Master mix (VWR) with the required primers
179 (Supplemental data 2) and corresponding DNA. For sequencing, the PCR products were
180 purified using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel reference
181 740609.50) and the filtrate was sent to GENEWIZ for Sanger sequencing.

182 Raw ONT Fast5 data were basecalled using Guppy 4.0.5 (https://nanoporetech.com) and the
183 HAC model, and QC performed using NanoPlot 1.38.1 (De Coster et al. 2018). All reads with
184 a QPHRED higher than 8 were retained and subjected to genome assembly using Flye 2.8
185 (Kolmogorov et al. 2019) under standard options. Raw assemblies were then polished with 3
186 turns of standard Racon (Vaser et al. 217) after mapping of raw reads on the previous
187 sequence using minimap2 (-ax map-ont mode; Li et al. 2021). Final scaffolding was
188 performed using Ragoo 1.1 (Alonge et al. 2019) upon the original *B. prasinos* reference

189 genome (GCA_002220235.1, Moreau et al. 2012). Final QC of assemblies was performed
190 using QUAST 5.0 (Mikheenko et al. 2018).

191 *Relative allelic abundance in environmental samples*

192 Amplifications were performed twice with a difference of 2 cycles in order to obtain clear
193 bands on ethidium bromide stained agarose gels for each sample. Similarly, the gels were
194 photographed after different exposure times in order to obtain a non-saturated image for each
195 sample. Relative abundance of each variant within the same DNA sample was performed
196 using ImageJ software Analyse Gel.

197 *Determination of Growth Rates*

198 Cells isolated during December 2018, January and February 2019 in Banyuls bay were used
199 in this experiment. For each culture condition the cell number was determined by flow
200 cytometry daily, for 9 days. The growth rate was determined as Ln(N)/dT, where N is the cell
201 concentration per ml and T the time (days). The maximal growth rate (μmax) was determined
202 according to Guyon et al. (2018) on a graph expressing the neperian logarithm of cell
203 concentration as a function of time of culture. Mmax corresponded to the slope of the linear
204 part of the growth curve (i.e., excluding the lag phase and the stationary phase).

205 $$\mu_{max} = Log(N_{fmax}) - Log(N_0)/Log(2) \times T$$

206 *Measurement of photosynthetic efficiencies*

207 Cultures were acclimated to the temperature and light rhythm and intensity for 7-10 days
208 before subculturing at 10^6 cells /ml in triplicates. After 3-4 days of growth, the photosynthetic
209 activities were recorded with PHYTO-PAM-II (Walz). After 20 min in obscurity, the samples
210 were transferred into the PHYTO-PAM and Fv/Fm, ETRmax and NPQ were measured.

211

212 **Results**

213 ***Search for intraspecies diversity markers***

214 With the aim to differentiate *Bathycoccus* isolates, we undertook a search for genetic
215 determinants of diversity. Since only two Mediterranean strains were available in the Roscoff
216 Culture Collection at the beginning of the project, we examined the world-wide diversity of
217 *Bathycoccus* and selected the most geographically dispersed strains (Supplemental Table 1).
218 The strains align along a latitude gradient from the Baffin bay (67°) to the Mediterranean sea
219 (40°): RCC5417-AFFIN, RCC1613-OSLO, RCC685-HELGOLAND, RCC1615-DIEPPE,
220 RCC1868-ROSCOFF, RCC4222-BANYULS and RCC4752-NAPLES. For simplification,
221 the town close to the site of sampling instead of the RCC number will name strains further on.
222 Oxford Nanopore Technology (ONT) was used to sequence the genome of the selected
223 strains. After *de novo* assembly, each genome was compared to the reference genome
224 (Moreau et al. 2012). There were some large chromosomal rearrangements but for the design
225 of diversity markers, we only considered INDEL inferior or equal to 2 kb within regions
226 mapping on the reference genome. The number and size of INDEL are detailed in Table 1.
227 The goal was to identify INDEL instead of SNP (Single Nucleotide Polymorphism) that could
228 be used to genotype the strains directly by PCR.

229 ***Validation of sequence variations in the genomes of world-wide Bathycoccus***

230 Putative markers were selected on several criteria. The insertion should be found at the same
231 or close location in the genome of at least three strains and of different sizes in at least two
232 genomes, preferably three. In addition, the size of the amplified fragment should be between
233 200 bp and 2 kb (this size restriction reduced the mean number of insertions from 88 to 35,
234 Table 1) and sufficiently different among the genomes of the strains to be unambiguously
235 visualised on agarose gel after amplification with a single set of primers. The aim of this
236 drastic selection was to identify the most divergent markers among the largest available
237 genetic diversity of *Bathycoccus* with the expectation that some of this variation would be
238 found in local communities of *Bathycoccus* in the Banyuls bay. Only five candidate markers

239 met these criteria and were experimentally tested. For two markers targeting variations of the
240 number of amino acid repeats in open reading frames, primers positioned at proximity of the
241 repeats did not produce a single amplimer and these two predictions from ONT sequencing
242 could not be validated nor invalidated. Marker on chromosome 15 (the number of repeats in a
243 zinc finger protein), marker chromosome 3 (variation in repeat number in a flavodoxin-like
244 protein) and marker chromosome 1 (insertion and deletion into the promoter of yrdC gene)
245 were validated (Table 2). To increase the number of markers, the striking insertion of 1.5 kb
246 into the promoter of the clock gene TOC1 (TIMING OF CAB EXPRESSION 1) on
247 chromosome 17 was included even though its diversity was below three (Table 2). The fifth
248 marker was selected as marker of the Big outlier Chromosome (BOC) on chromosome 14, an
249 atypical chromosomal structure found in Mamiellales (Moreau et al. 2012).

250 Typical PCR results for the 5 validated diversity markers are presented in Figure 1.
251 The detailed description of each marker is provided as supplementary data 3.

252 Marker yrdC promoter: Bathy01g04300 encodes a yrdC domain-containing protein of
253 unknown function. In *Escherichia coli*, yrdC binds preferentially to double-stranded RNA,
254 consistent with a role of the protein in translation (Teplova et al. 2000). A diverse
255 organisation was identified in the promoter region of Bathy01g04300 in comparison to the
256 reference genome and this was visualised by PCR amplification (Figure 1, Table 2,
257 Supplemental data 3A). This set of primers showed marked differences in its amplification
258 success among the strains (specially in Baffin) indicating important nucleotide variations.

259 Marker TOC1 promoter: The intergenic region upstream Bathy17g01510 encoding an
260 homolog of TOC1 involved in the control of circadian rhythm was different in some strains.
261 A 2.2 kb insertion was identified in OSLO, DIEPPE and NAPLES, whilst the other strains
262 were similar to BANYULS (Figure 1, Table 2, Supplemental data 3B).

263 Marker flavodoxin-like: Bathy03g02080 encodes a protein containing a flavodoxin-like
264 domain, a flavin mononucleotide (FMN)-binding site and 6 imperfect repeats of 25 amino
265 acids. In comparison to the reference, ONT sequencing revealed a deletion in BAFFIN,
266 insertions of 147 bp in BANYULS, 74 bp in OSLO and 375 bp HELGOLAND, while
267 ROSCOFF and NAPLES were unchanged. These predictions verified by PCR and sequencing
268 confirm that substantial INDEL can also occur within coding regions (Figure 1, Table 2,
269 Supplemental data 3C).
270 Marker Zinc Finger: Bathy15g02320 encodes a protein with Zinc Finger repeats (ZF) of a
271 greater length in BAFFIN (9ZF) and HELGOLAND (7ZF) than in BANYULS (6ZF),
272 differences that were tested by PCR (Figure 1, Table 2, Supplemental data 3D). Since the
273 primers did not amplify a single fragment in BAFFIN and HELGOLAND, the corresponding
274 region was obtained by the ONT data (Table2). Zinc finger C2H2 proteins are numerous (53
275 genes) and highly conserved in the *Bathycoccus* reference genome (Moreau et al. 2012).
276 Marker TIMa: Bathy14g30100 encodes a protein containing a TIM domain found in the
277 protein Timeless involved in circadian rhythm control in Drosophila (Sehgal et al. 1995). This
278 gene is located in the BOC region of chromosome 14. The ORF of TIMa was found
279 conserved among the selected strains (Figure 1, Table 2). This fifth marker was non
280 discriminating among the subset of world-wide *Bathycoccus* strains.
281 ***Accessory genes in Bathycoccus prasinos genome***
282 The large insertions found in the promoters of yrdC and TOC1 were sequenced and analysed
283 in detail (supplementary data 3A-B).
284 In yrdC promoter, a gene encoding a protein of unknown function possessing ANK repeats
285 similar to the gene products of Bathy01g04610 (68% amino acid identity), Bathy01g04570
286 (67%) and Bathy11g02720 (57%) was inserted between an Evening Element-like (EEL) *cis*
287 element and the start of Bathy01g04300 in BAFFIN, OSLO, HELGOLAND and NAPLES. In

288 order to gain information on this additional gene, the Ocean Gene Atlas (OGA) website
289 (http://tara-oceans.mio.osupytheas.fr/ocean-gene-atlas/) (Villar et al., 2018, Vernette et al.
290 2022) was interrogated with the additional protein and its three homologs. The only sequence
291 retrieved from OGA shared significant similarity with the 4 proteins but was not identical. So
292 the geographical distribution that we obtained is not the one corresponding to the accessory
293 ANK gene.
294 In TOC1 promoter, the 2.2 kb insertion identified in OSLO, DIEPPE and NAPLES encodes a
295 Methyltransferase-like protein (AdoMTase *METTL24* IPR026913) of 320 aa with 41.7%
296 amino acids identity to the predicted *Ostreococcus lucimarinus* CCE9901 protein
297 (XP_001422352). Searches were performed at high stringency in OGA (Expect threshold 1e-
298 300) so that only the presence of the near-identical sequences was retrieved. TOC1 and
299 AdoMTase sequences were not strictly co-occuring. At one station located near Chile (arrow
300 Figure 2), TOC1 sequences were abundant but no AdoMTase was recorded which suggests
301 that in this particular area the *Bathycoccus* genomes are almost devoid of AdoMTase.
302 Surprisingly, it was possible to also find AdoMTase sequences not associated to *Bathycoccus*
303 TOC1 sequences. Many AdoMTase hits were found near the equator at temperatures higher
304 than 25°C, temperatures too high for *Bathycoccus prasinos* growth, indicating that this
305 accessory gene can be found in other unknown microorganisms suggesting possible
306 horizontal gene transfer. As a control, TOC1 and CCA1 (CIRCADIAN CLOCK
307 ASSOCIATED 1, Bathy06g4380) sequences were fully co-occurrent (Supplemental Figure
308 1). Transcription of the gene encoding this AdoMTase was confirmed in the metaT database
309 at OGA (data not shown).
310 In conclusion, two large INDEL comprised additional or duplicated genes and these should be
311 considered as structural rearrangements rather than simple INDEL. These accessory genes
312 could potentially be beneficial for adaptation.

313 ***Isolation of Bathycoccus during the 2018/2019 winter bloom in Banyuls bay***

314 Surface water was collected weekly from December 3rd to March 19th. During this period, the
315 sea temperature rose to 15.87°C in December and did not descend below 10.68°C in February
316 (Figure 3). According to the 10-year study at the same location (SOLA Buoy in Banyuls bay)
317 for 2007-2015 (Lambert et al. 2019) and 2015-2017, this period can be considered as an
318 average climatic year in term of temperature (Lambert et al. 2021). The presence and
319 abundance of pico- and nano-phytoplankton <3 μm were determined by flow cytometry
320 (Figure 3). Whilst cyanobacteria were the most abundant at all time with a peak at the end of
321 February, picophytoplankton was the second most abundant category with a first peak in
322 December and a second in February (Supplemental Figure 2). At each sampling date,
323 collected seawater was also filtered through 1.2 μm pore-size and transferred to culture flasks.
324 This pore size allows the passage of most *Bathycoccus* cells (which size is estimated at 1,5
325 μm) and most importantly eliminate the potential larger predators.

326 After a period of acclimation of two weeks in supplemented sea water, cells were isolated by
327 plating on agarose. Light green-yellow coloured colonies were picked and sub-cultured. In
328 order to accelerate the identification of *Bathycoccus* among the isolated cells, amplifications
329 of a fragment of the *LOV-HK* (Bathy10g02360) gene were performed. These primers were
330 specific to the *Bathycoccus prasinos* genome and did not amplify the homologous gene in
331 *Ostreococcus* or *Micromonas* nor in any other species. The identity of these clones was
332 further confirmed by ribotyping (amplification of a 2kb ribosomal DNA fragment followed
333 by sequencing). In total, 55 *Bathycoccus prasinos* isolates were recovered at nine sampling
334 dates (Supplemental Table2).

335 ***Identification of dominant Bathycoccus Multi Loci Genotypes in Banyuls bay in 2018/2019***

336 The five diversity markers were used in a combination of PCR and sequencing in order to
337 distinguish the different isolates of *Bathycoccus* sampled during the 2018/2019 winter bloom.

338 For the *yrdC* promoter, the inserted gene encoding a protein with ANK repeats was not
339 detected in the Banyuls samples. In one isolate only (F1), the yrdC promoter was near
340 identical to the one in RCC4222-BANYULS (99% identity in 332 bp, Supplemental data 3A).
341 In 54 other isolates, a fragment of 200 bp similar in size to that in ROSCOFF was amplified
342 and sequenced (100% identity, Supplemental data 3A). The insertion present in the *TOC1*
343 promoter was more prevalent in the Banyuls isolates (91%) than in the world-wide strains
344 (43%) (Table 2 and 3). A high degree of similarity (98.6%) was observed over the entire
345 insertion whilst the core promoter of *TOC1* containing the essential Evening Element-Like
346 (EEL) *cis* element had a minimum of 71.6% nucleotide identity in 400 bp. A phylogenetic
347 tree was constructed with these sequences (Supplemental data 3B). The 2 kb inserted
348 sequence was not included in the study but its occurrence is indicated between brackets for
349 each isolate. Most of the Mediterranean isolates were found in the main clade which divided
350 further into two subclades Ia and Ib. Subclade Ia contained all the Mediterranean reference
351 type and HELGOLAND, whereas subclade Ib most of the Mediterranean 2kb type and
352 DIEPPE. OSLO and NAPLES lie in a separate clade II. ROSCOFF and BAFFIN contained
353 the most divergent sequences in clade III. As a consequence of the 2kb insertion, the *TOC1*
354 promoter activity is most likely not abolished since the crucial EEL *cis* element is still present
355 but the analysis of the core promoter sequences indicates that its activity could differ between
356 the clades. Thus the *TOC1* promoter could constitute a functional marker as well as a
357 diversity marker.
358 The gene encoding a flavodoxin-like protein was more diverse in size than were the intergenic
359 regions (Supplemental data 3C, Table 2, 3). The maximum size difference increases to 245
360 amino acids between F1 (February Banyuls isolate) and BAFFIN. The function related to this
361 sequence variation is unknown. It forms a coiled-coil structure of several alpha-helices.
362 Coiled-coil domains have been identified in enzymes where they function as molecular

363 spacers positioning catalytic activities. Thus the variable length of the repeats could influence
364 the activity of the flavodoxin-like protein.
365 The genome of most Banyuls isolates encoded a Zinc finger C2H2 protein with 6 ZF motifs
366 (Bathy15g02320, Supplemental data 3D, Table 3). Only three isolates from February
367 sampling were similar to HELGOLAND with an additional ZF motif.
368 Amplification of a fragment of 530 bp of TIM (TIMa) was observed in 66% (approximately
369 two thirds) of the isolates (Table 3). For the isolates for which there was no amplification, we
370 designed an additional primer in order to amplify a variant of TIM, TIMb. The alignment of
371 the two predicted variant proteins TIMa in RCCB4222-BANYULS and TIMb in A8 isolate
372 showed that one third of the protein is well conserved while two thirds were more variable
373 (Supplemental data 3E). A phylogenetic tree confirmed this dichotomy (Figure 4A). OGA
374 metagenomic database was interrogated with TIMa and TIMb and each retrieved a single hit,
375 respectively OGATIMa and OGATIMb confirming the existence of the 2 isoforms of TIM
376 world-wide. OGATIMb has a marked abundance in high latitudes and cold temperatures in
377 the Northern hemisphere while OGATIMa is more widely distributed (Figure 4B).
378 Based on the results described above, the Banyuls isolates were classified in eight MLG
379 (Table 3). MLG 1 and MLG 2 represent respectively 53% and 29% of the population. Since
380 the number of isolates from each sampling date was not identical (Supplemental Table 2), this
381 percentage may not be entirely representative. However, the presence of MLG 1 and 2 in five
382 out of nine independent samplings rules out a bias due to experimental cloning during
383 isolation (Table 3). We can thus confidently state that these two MLG were dominant in
384 Banyuls bay during the 2018/2019 bloom. No isolate with a MLG identical to RCC4222-
385 BANYULS was found.
386 ***Determination of major allelic variants in environmental samples: a three year follow-up***

387 The identification of dominant MLG during the bloom 2018/2019 raises the question of their
388 yearly or occasional prevalence in Banyuls bay. Since isolating strains is highly time
389 consuming, an alternative approach was developed to estimate local diversity. Five litres of
390 seawater were sampled once a week and filtered between 3 and 0.8 μm. DNA extracted from
391 0.8 μm filters was used as template for PCR analysis using our set of diversity markers.
392 Samplings were performed during 3 successive blooms from 2018 to 2021 (Figure 3,
393 Supplemental Figure 2).
394 Variations in the yrdC and TOC1 promoters and TIM ORF sequences were analysed on these
395 environmental DNA samples. For markers Flavodoxin-like and zinc finger, despite the high
396 specificity of the primers on DNA of individual *Bathycoccus* (Table3), they could not be used
397 on complex environmental DNA samples due to the presence of high background.
398 The initiation of the bloom was analysed during two consecutive years (Figure 5). In 2019,
399 the presence of *Bathycoccus* was detected in the third week of November with a clear
400 predominance of the 200 bp allele of yrdC promoter and the presence of both TIMa and
401 TIMb. In 2020, *Bathycoccus* was detected in October, was barely detected or absent in
402 November and reappeared in December, consistent with the decrease of abundance of
403 picophytoplankton by flow cytometry (Supplemental Figure 2). In 2020, the allelic ratios of
404 200/400 bp of the yrdC promoter were clearly different from those in 2019 and TIMb was not
405 detected. We conclude that the onsets of the bloom were different both their chronology and
406 their population diversity.
407 The diversity of populations was also assessed during the bloom (Figure 5, Figure 3). The
408 abundance of alleles of yrdC promoter and TIM were clearly different in November-
409 December compared to February.
410 In summary, the study of three successive blooms showed changes in the diversity of
411 *Bathycoccus* populations within and between blooms.

412 ***Physiological characteristic of isolated Bathycoccus strains***

413 Since abundance of alleles showed that November-December and February populations are
414 different and because temperature and light intensity are significantly different in December
415 (15.9°C, 9h15 light, maximum intensity 540 μE/m^2/s) and February (11.8°C, 10h30 light,
416 maximum intensity 830 μE/m^2/s), the physiological parameters of the December (D,
417 03/12/2018), January (J, 28/01/2019) and February (F1 F2, 25/02/2019; F3-F4, 26/02/2019)
418 isolates were determined. Cells were grown at 13°C or 16°C under December or February
419 illumination. In the 4 conditions, F1-F4 isolates grew better than D and J cells (Figure 6),
420 although the difference was reduced at 16°C.

421 Photosynthetic parameters were determined by PAM fluorometry. For most isolates, no
422 significant differences in indicators of photosynthesis parameters were observed with the
423 exception of F1 with the unique MLG 7 (Supplemental Figure 4).

424 Together, the results showed a clear difference in the growth curve of the *Bathycoccus*
425 isolated in December and February mainly due their capacity of adaptation to low
426 temperature.

427 **Discussion**

428 ***Contribution of ONT sequencing to the identification of* Bathycoccus *INDEL markers***

429 *Bathycoccus* has a small nuclear genome of approximately 15 Mb distributed among 19
430 chromosomes and has only been found in a haploid phase (Moreau et al. 2012). This
431 organisation makes it suitable for Oxford Nanopore Technology Rapid barcoding libraries
432 and sequencing. On a single flow cell, it was possible to obtain sufficient coverage for the
433 genome of up to 4 strains. The main difficulty was to obtain good quality genomic DNA for
434 each strain, a criterion particularly important when pooling barcoded libraries. With the
435 exception of RCC1615-DIEPPE, all ONT data were superior to 10 times the size of the
436 *Bathycoccus* genome (Table 1). When comparing ONT data from RCC4222-BANYULS to

437 the clonal RCC1105 reference genome, 19 INDEL were found. Two main reasons can be
438 proposed to explain this variation. Firstly, RCC14222-BANYULS is not strictly identical to
439 RCC1105. Secondly, ONT is particularly suitable to identify structural variations previously
440 undetected by conventional sequencing method (Michael et al. 2018, Mantere et al. 2019).
441 Therefore, most of the INDEL identified between RCC1105 and RCC4222-BANYULS
442 genomes could result from the use of different sequencing techniques. To design diversity
443 markers, INDEL in intergenic regions (promoters) as well as ORF encoding repeat amino
444 acids sequences were selected. Essentially all predictions were accurate and validated by PCR
445 and sequencing.

446 ***Identification of Bathycoccus local diversity using INDEL markers***

447 The 55 freshly isolated Mediterranean *Bathycoccus* were genotyped using five INDEL
448 markers that were based on ONT sequencing of strains originating from contrasted
449 geographic locations between arctic and temperate regions. The presence of a marker type
450 was quite different between the world-wide strains and the Banyuls strains (Table 2 and 3).
451 For example, the insertion of a gene encoding ANK repeats was found in four out of seven
452 world strains but was absent from Banyuls strains, whereas the insertion of the AdoMTase
453 sequence into the TOC1 promoter was much more prevalent in Banyuls strains. In general,
454 the genomes of Banyuls *Bathycoccus* isolates possess common allelic variants (65-98%,
455 Table 3) representing the dominant MLG 1 and 2. Remarkably, these three dominant
456 INDEL/rearrangements (200 bp yrdC, 2.2 Kb TOC1, 1.2 Kb Flavodoxin-like) were not found
457 in RCC4222-BANYULS that was isolated in the Banyuls bay in 2006. In addition, none of
458 the 2018-2019 isolates share the same five marker types with BANYULS (2006) or NAPLES
459 that was isolated in 1986 (Table 3). Thus, it is clear that RCC4222-BANYULS isolated in
460 Banyuls bay in 2006 was certainly not abundant and probably not present during the bloom

461 2018/2019. Finally, we did not observe any obvious consistent pattern of occurrence between
462 specific INDEL markers and the geographic origin of the world-wide strains (Table 2 and 3).
463 The observation of eight MLG in *Bathycoccus* is probably an underestimation. As an example
464 in diatoms (although not directly comparable since microsatellite markers have a higher
465 mutation rate than other region of the genome), more than 600 individuals have been
466 genotyped using microsatellite markers, it was estimated that the blooming population was
467 comprised of at least 2400 different genotypes (Rynearson and Armbrust 2005). With
468 additional markers, the number of MLG would probably increase. However, the dominance of
469 a few MLG among *Bathycoccus* isolates is probably representative, despite being based on a
470 small number of isolates and loci.
471 The existence of major MLG highlights an apparent paradox: how can blooms be diverse,
472 given that the best genotype should prevail? Blooms are predicted to quickly become
473 dominated by a few particularly well-adapted genotypes (De Meester 1996). Nevertheless,
474 most studies describing genetic diversity of blooming phytoplankton populations report high
475 intraspecies variation (Rynearson and Armbrust 2005, Alpermann et al. 2009, Lebret et al.
476 2012, Dia et al. 2014). Indeed, our results revealed such diversity by the detection of six
477 minor MLG in addition to the two major ones. Remarkably, the different growth rates
478 observed between December/January and February 2018/2019 isolates correlate with
479 fluctuations in allelic frequencies in population at onset of a bloom and during the course of a
480 bloom as determined on seawater samples, suggesting that best seasonal MLG may become
481 dominant at specific times of the year. Similarly, temporal succession of two genetically
482 distinct sub-populations was observed during the bloom of the haploid *Alexandrium*
483 *dinoflagellate* in Gulf of Maine (Erdner et al., 2011).
484 ***Structural variants as markers to follow intraspecies diversity in environmental samples***

485 We aimed to develop a rapid and cost effective alternative which did not rely on isolated
486 individuals since it is very challenging and time consuming to isolate marine microalgae from
487 complex microbial communities. Compared to short read sequencings, the recent ONT and
488 PACBIO sequencing technology provided information on structural variants, in particular on
489 relatively large INDEL and on repeated/low complexity sequences, with some of which
490 previously overlooked (Wellenreuther et al. 2019). This knowledge was particularly useful to
491 develop a novel type of marker for assessing intraspecies diversity that will rely only on PCR
492 amplification of variable size fragment without the need of sequencing. Furthermore, with
493 INDEL based on ONT sequencing we are reaching a higher level of population structure
494 compared to microsatellite or SNP markers. Structural variants are expected to be less neutral
495 and more stable than microsatellite markers (Mérot et al. 2020). Microsatellite markers can
496 change in clonal strains of *P. multistriata* in the laboratory over several months (Ruggiero et
497 al. 2018), while our structural markers are still identical in the reference genome published in
498 2012 and its clonal strain RCC4222-BANYULS sequenced in 2018. Thus INDEL markers
499 can identify large subpopulations rather than small groups of individuals. Our studies on three
500 successive blooms in Banyuls showed that INDEL markers are capable of determining the
501 dominant allelic variants and their perennial occurrence. Similarly, INDEL markers could be
502 used as query on metagenomic databases for a wider analysis of *Bathycoccus* populations.

503 Variations in allele frequencies were observed for three consecutive years, raising the
504 question of the nature of the highly reproducible yearly occurrence of *Bathycoccus* in the bay
505 of Banyuls. Seasonal blooms may result either from re-activation of "dormant/survivor" cells
506 from the water column (whose genetic fingerprint will determine the genetic profile of the
507 next bloom) or by yearly *de novo* seeding by cells carried by the north Mediterranean current
508 along the gulf of Lion. At first glance, our preliminary results are in favour of the introduction
509 of a new population rather than "resurrection" of cells from the previous bloom since allelic

510 frequencies are distinct between the end of a bloom and the onset of the next (Figure 5). By
511 monitoring the temporal population structures of the dinoflagellate *Alexandrium minutum* in
512 two estuaries in France, Dia et al. (2014) showed that interannual genetic differentiation was
513 greater than intra-bloom differentiation. Alternation of genotypes/populations has also been
514 observed with diatoms in the dominance of one of the two sympatric populations of
515 *Pseudonitzschia multistriata* which could be due either to environmental factors favouring
516 one population over the other or intrinsic factors coupled to the obligate sexual life cycle of *P.*
517 *multistriata* (D'Alelio et al. 2010). Thus the observed fluctuations in allele frequencies could
518 equally be the result of new inoculum from currents or sexual reproduction. Even though
519 sexual reproduction has not been demonstrated in *Bathycoccus*, there is genomic evidence
520 that it may occur (Benites et al. 2021). Sexual recombination generates new combinations of
521 alleles, whereas clonality favours the spread of the fittest genotype through the entire
522 population (Dia et al. 2014). Erdner et al. (2011) propose for *A. fundyense* that mitosis is the
523 primary mode of multiplication during blooms whereas mating is triggered presumably in
524 response to unfavourable conditions at the end of blooms, with vegetative cells not
525 overwintering in the water column. In Banyuls bay, the abundance during the bloom is
526 followed by severe bottlenecks in which *Bathycoccus* are hardly detected in the water column
527 (Lambert et al. 2019).
528 Knowing that (1) *Bathycoccus* blooms are followed by severe bottlenecks between one bloom
529 and the next, (2) allelic frequencies were different at the end of one bloom and at the onset of
530 the next and (3) structural markers are very stable in mitotic dividing cells, the hypothesis of
531 rare vegetative cells remaining in the water column between the blooms is unlikely except if
532 those remaining cells were produced by sexual reproduction. The alternative hypothesis of
533 new strains brought by current is equally probable.
534 ***Structural variants versus functional variants***

535 Our principal interest was to identify markers of intraspecies diversity in order to follow the
536 dynamics of *Bathycoccus* population during annual blooms in the bay of Banyuls. However
537 structural variants are probably not neutral unlike microsatellite markers. Most of the selected
538 markers could also represent functionally significant variants such as an additional gene
539 function or a modified promoter activity or protein function, that could correspond to an
540 adaptation to contrasted intra- and inter-annual variations in environmental parameters in the
541 Banyuls Bay (Lambert et al., 2021).
542 Remarkably in just five world-wide INDEL, we discovered two additional genes in the
543 genome of *Bathycoccus prasinos* and a particular protein structure. The additional ANK
544 repeat encoding gene in chromosome 1 has probably arisen by gene duplication or gene loss
545 since it belongs to a multigenic family. The origin of the AdoMTase could be the result of
546 Horizontal Gene Transfer. The Flavodoxin-like protein has an organisation specific to
547 *Bathycoccus* with a coiled-coil domain of variable size with similarity to Eukaryotes parasites
548 and toxic bacteria proteins and a flavodoxin domain found in the 7 other *Bathycoccus*
549 flavodoxin-like proteins. *Bathycoccus* culture strains do not possess a flavodoxin per se while
550 it was found in uncultured *Bathycoccus* (Pierella Karlusich et al. 2015). This peculiar
551 flavodoxin-like protein could represent a case of neofunctionalisation in *Bathycoccus*. The
552 core promoter of the central circadian clock *TOC1* gene has a conserved evening element like
553 box (EEL box) that has been experimentally demonstrated as essential in the central oscillator
554 of *Ostreococcus tauri* (Corellou et al. 2009). Although the EEL box is found in all the strains
555 sequenced, the distance between the *cis* element and the initiation codon is variable. In
556 addition, the phylogenetic tree of the core promoter sequences clearly discriminated BAFFIN
557 and ROSCOFF and to a lesser extent, OSLO and NAPLES (Suppl. data 3B). An insertion of
558 the AdoMTase was found about 100 bp upstream the EEL box. This insertion could
559 potentially modify the promoter activity and ultimately the expression pattern of TOC1. Such

560 a natural variation of promoter length modulates the photoperiodic response of FLOWERING
561 LOCUS T by differentially spacing two interdependent regulatory regions (Liu et al. 2014).
562 Although the presence of the AdoMTase was not correlated with the latitude or the
563 temperature in the Ocean Gene Atlas (Figure 2), it could still be associated with a seasonal
564 niche. Less information is available for the promoter and function of the yrdC gene. The
565 rearrangements are more drastic, especially with the displacement of the EEL box by insertion
566 or deletion, and could lead to the inactivation of the promoter. The most striking feature
567 concerns the TIM protein where only one third of the protein is conserved between TIMa and
568 TIMb. Due to its position in the BOC of chromosome 14 putatively involved in mating, this
569 raises the question of the mating types of cells with genome containing a TIMa or a TIMb.
570 Taken together, these data suggest that some INDEL markers identified in this study may be
571 potentially involved in adaptation to changing environmental conditions.

572 **Conclusions and perspectives**

573 In this paper we described the conception and construction of a new type of diversity marker
574 based on genomic structural variants. After validation on freshly isolated individuals, INDEL
575 markers were used *in situ* on environmental samples. This pioneer study on *Bathycoccus*
576 diversity in the bay of Banyuls now paves the way to an in depth analysis of multiple markers
577 present in more than a decade of bimonthly sampled metagenomic data at a discrete location
578 (Lambert et al. 2019; Lambert et al. 2021). The sequencing of the whole genomes of the
579 different MLG, together with the assessment of their physiological performances will bring
580 additional information contributing to the local diversity of *Bathycoccus* and provide insight
581 into their seasonal pattern of abundance. In addition, the INDEL markers represent an
582 essential tool for grasping the maximum diversity of newly isolated *Bathycoccus* and to
583 identify putative molecular mechanisms involved in adaptation to environmental niches of
584 this cosmopolitan genus.

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Data accessibility

Strains isolated in Banyuls have been sent to Roscoff Collection Centre and will be publicly available after curation by the Centre. For whole genome sequences, basecalled reads and

801 RaGOO outputs of the 7 RCC strains used in this study are available at Zenodo

802 https://doi.org/10.5281/zenodo.7594933

803

804 **Author contribution**

805 MD, FYB and FS conceived the work and acquired funding. MD extracted high molecular

806 weight genomic DNA. ONT sequencing was performed by CM and MD and sequence

807 analysis by LD and FS. Diversity markers were designed and validated by MD. PS analysed

808 seawater samplings by flow cytometry. MD and VV isolated Banyuls strains during the

809 winter bloom, genotyped by MD and JCL. MD determined diversity of seawater. MD and

810 FYB wrote the article and all authors participated in critical revisions and approved the final

811 version for submission.

812

Table 1. Number and sizes of INDEL

Strain	Name	Insertion		Insert 0.2-2kb	deletion		Coverage
		number	size range	number	number	size range	
RCC5417	BAFFIN	101	36-15979 bp	36	80	50-19025bp	x9
RCC1613	OSLO	149	51-18972 bp	37	67	50-13730 bp	x239
RCC685	HELGOLAND	116	51-16349 bp	60	69	51-19026 bp	x52
RCC1615	DIEPPE	62	72-4620 bp	23	69	73-13730 bp	x4
RCC1868	ROSCOFF	49	50-5509 bp	22	43	51-13732 bp	x19
RCC4222	BANYULS	14	76-3011 bp	11	5	51-3999 bp	x30
RCC4752	NAPLES	53	50-15411 bp	34	60	50-13730 bp	x14
mean*		88		35			
*without RCC4222							
INDEL within aligment							

Table 2. Distribution of diversity markers in world-wide strains

RCC	**Name**	**yrcD prom chr1**	**TOC1 prom chr17**	**Flavodoxin chr3**	**Zinc finger chr15**	**TIMa chr14**
primers		MDB33+MDB34	MDB7+MDB9	MDB40+MDB41	MDB68+MDB69	MDB57+MDB58
RCC4222	BANYULS	400 bp	700 bp	800 bp	730 bp	530bp
RCC5417	BAFFIN	1400 bp	700 bp	600 bp	900 bp	530bp
RCC1613	OSLO	1400 bp	2.2 kb	1000 bp	730 bp	530bp
RCC685	HELGOLAND	1400 bp	700 bp	1200 bp	820 bp	530bp
RCC1615	DIEPPE	400 bp	2.2 kb	1400 bp	730 bp	530bp
RCC1868	ROSCOFF	200 bp	700 bp	800 bp	730 bp	530bp
RCC4752	NAPLES	1400 bp	2.2 kb	1200 bp	730 bp	530bp
		3 size variants	2 size variants	5 size variants	3 size variants	1 size variant
		14.28% (200 bp)	42.86% (2.2 kb)	28.57% (1.2 kb)	71.5% (730 bp)	100% (530 bp)

Table 3. Multi Loci Genotypes (MLG) of Banyuls isolates

Strains	**INDEL marker**							
	Chr1	Chr17	Chr3	Chr15	chr14			
	yrcD prom	TOC1 prom	flavodoxin	zinc finger	TIMa			
4222-BANYULS	400bp	700bp	800bp	730 bp	530bp			
4752-NAPLES	1.4kb	2.2kb	1200bp	730 bp	530bp			
						Numer of	Occurance in	Percentage
Banyuls 18/19						isolates	samplings	of isolates
MLG 1	200bp	2.2 kb	1200bp	730 bp	530bp	29	5/9 samplings	53%
MLG 2	200bp	2.2kb	1200bp	730 bp	0	16	5/9 samplings	29%
MLG 3	200bp	2.2 kb	800bp	730 bp	530bp	4	3/9 samplings	8%
MLG 4	200bp	700 bp	1200bp	730 bp	530bp	2	2/9 samplings	4%
MLG 5	200bp	2.2kb	1200bp	820 bp	0	1	1/9 samplings	2%
MLG 6	200bp	700 bp	1200bp	820 bp	0	1	1/9 samplings	2%
MLG 7	400bp	700 bp	1400bp	820 bp	530bp	1	1/9 samplings	2%
MLG 8	200bp	2.2kb	1600bp	730 bp	530bp	1	1/9 samplings	2%
Mediterranean								
Size number	2*	2	4	2	2			
Frequency	98% (200bp)	91% (2.2 kb)	89% (1.2kb)	95% (730 bp)	66% (530 bp)			
World-wide								
Size number	3	2	4	3	1			
Frequency	14.28% (200 bp)	42.86% (2.2 kb)	14.28% (1.2 kb)	71.5% (730 bp)	100% (530 bp)			
* no insertion of ANK gene tested with MDB33+MDB35								
similar to RCC4222-BANYULS								
similar to RCC 4752-NAPLES								

815 **Figure 1. Diversity markers among world-wide strains**

816 Samples are arranged on a latitudinal gradient from Baffin to Naples. Arrows indicate the size
817 of the various fragments. A: Size variation in intergenic region in the promoters of yrdC on
818 chromosome 1 and TOC1 on chromosome 17. B: size variation in nucleotidic sequences
819 encoding amino acid repeats in a Flavodoxin-like gene on chromosome 3 and a zinc-finger
820 protein on chromosome 15. C: detection of the nucleotidic sequence encoding the specific C
821 terminal region of TIMa in world-wide strains on the left and in seven *Bathycoccus* isolates
822 from Banyuls during winter 2018/2019 (B1-B7) on the right.

823 **Figure 2. Geographical distribution of TOC1 and AdoMTase proteins**

824 The protein sequences of TOC1 and AdoMTase were used as a query at high stringency in
825 OGA. Their abundance was expressed as percent of total reads. Under these conditions, a
826 single hit was found and its presence and abundance are represented on the world map at
827 surface water (SRF) and deep chlorophyll maximum layer (DCM) and in relation to latitude
828 and temperature. The arrows point to a station at the Chilean coast where TOC1 is present
829 but not AdoMTase.

830 **Figure 3. Abundance of phytoplankton during three successive blooms**

831 Seawater at SOLA buoy (Banyuls) was sampled at a depth of 3 meter. After passage on 3 μm
832 filter, the flow through was analysed by flow cytometry. At each sampling time, the
833 phytoplankton was categorised and quantified in function of cell size (pico- and nano-
834 phytoplankton, cyano-bacteria) with indication of the seawater temperature. The main peak of
835 picophytoplankton abundance was in December 3th 2018 and February 19th 2019, January 7th
836 and February 11th 2020, January 27th and March 2nd 2021. The most striking difference
837 between the three years was the sudden abundance in nanophytoplankton in March 2021.

838 **Figure 4. Distribution and abundance of TIM variant proteins in MetaG database**

839 A. Phylogenetic tree of TIM proteins from world-wide and Banyuls isolates presenting two
840 main clades each containing one TARA OGA hit, OGATIMa or OGATIMb. OGATIMa was
841 retrieved after a query with the TIM protein from RCC4222 and OGATIMb with the variant
842 TIM protein in the A8 Banyuls isolate.
843 B. Geographical presence of TIM variants in OGA. The presence and abundance (percent of
844 total reads) of each variant is represented in SRF and DCM samples with the temperature and
845 latitude parameters.
846 **Figure 5**. Diversity markers in seawater
847 Results the PCR amplification of marker yrdC (chr1) and TIM (chr14) from natural sea water.
848 For clarity, only a subset of the analysis is presented in this Figure, the complete dataset is
849 presented in Supplemental Figure 3 and Supplemental Table 3.
850 Seawater was filtered in autumn from end of November in 2019 and from October 2020.
851 The time of sampling is indicated as week of the month (e.g. Oct-01 = 1st week of October) to
852 facilitate the comparison between years. The relative abundance of the 2 allelic variants of
853 yrdC (200 bp, a deletion or 400 bp, the reference type) and TIMa and b (absence or presence)
854 were recorded. ND: not determined.
855 **Figure 6.** Growth curves and rates of Banyuls isolates
856 The growth curves and rates of the December (D), January (J) and February (F1-F4)
857 *Bathycoccus* isolates were determined under 4 different conditions by sampling every day for
858 9 days. Cell concentration was determined by flow cytometry and is expressed as 10^6
859 cells/ml.
860 **Table 1.** Number and sizes of INDEL
861 **Table 2.** Distribution of diversity markers in world-wide strains
862 **Table 3.** Multi Loci Genotypes of Banyuls isolates
863 **Supplemental Table1.** Strains used in this study

864 **Supplemental Table 2.** Isolation of *Bathycoccus* strains during 2018/ 2019 winter bloom in
865 the Banyuls bay
866 **Supplemental Table 3.** Relative abundance of diversity markers in sea water
867 **Supplemental data 1.** Sequences and Alignments
868 **Supplemental data 2.** Primers used in this study
869 **Supplemental Figure 1.** Geographical distribution of TOC1 and CCA1 (Bathy05g02420)
870 proteins
871 **Supplemental Figure 2.** Abundance of picophytoplankton during winter blooms
872 **Supplemental Figure 3.** Images of sea water PCR amplification for yrdC, TOC1 and TIM
873 used for Supplemental Table 3
874 **Supplemental Figure 4.** Photosynthesis parameters of Mediterranean *Bathycoccus* strains
875

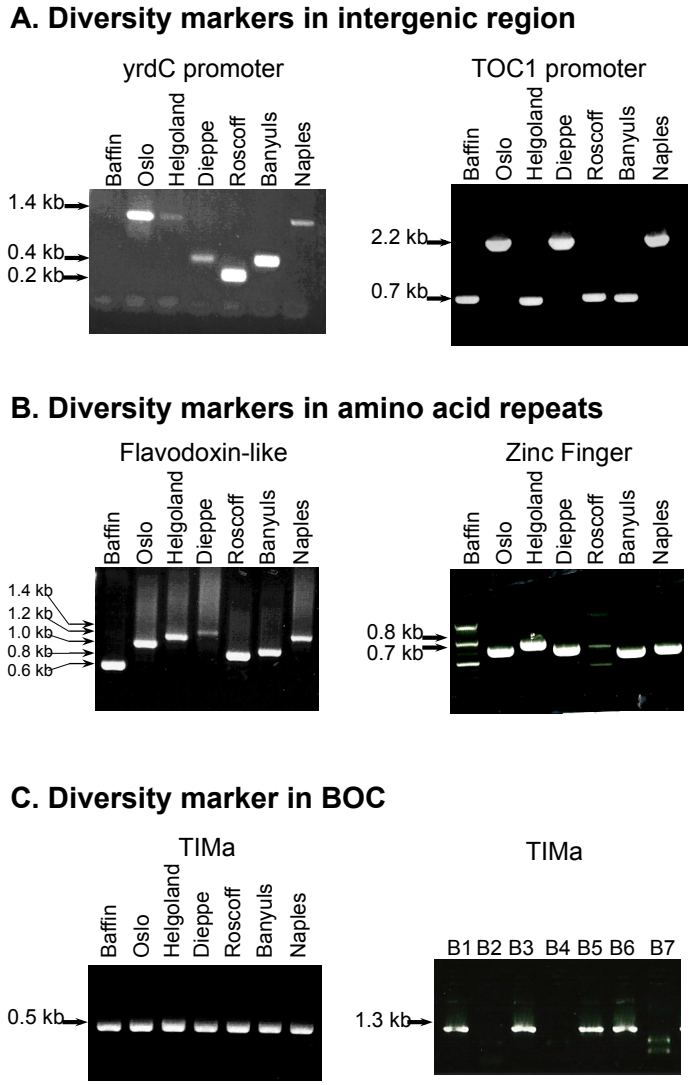

Figure 1. Diversity markers world-wide strains

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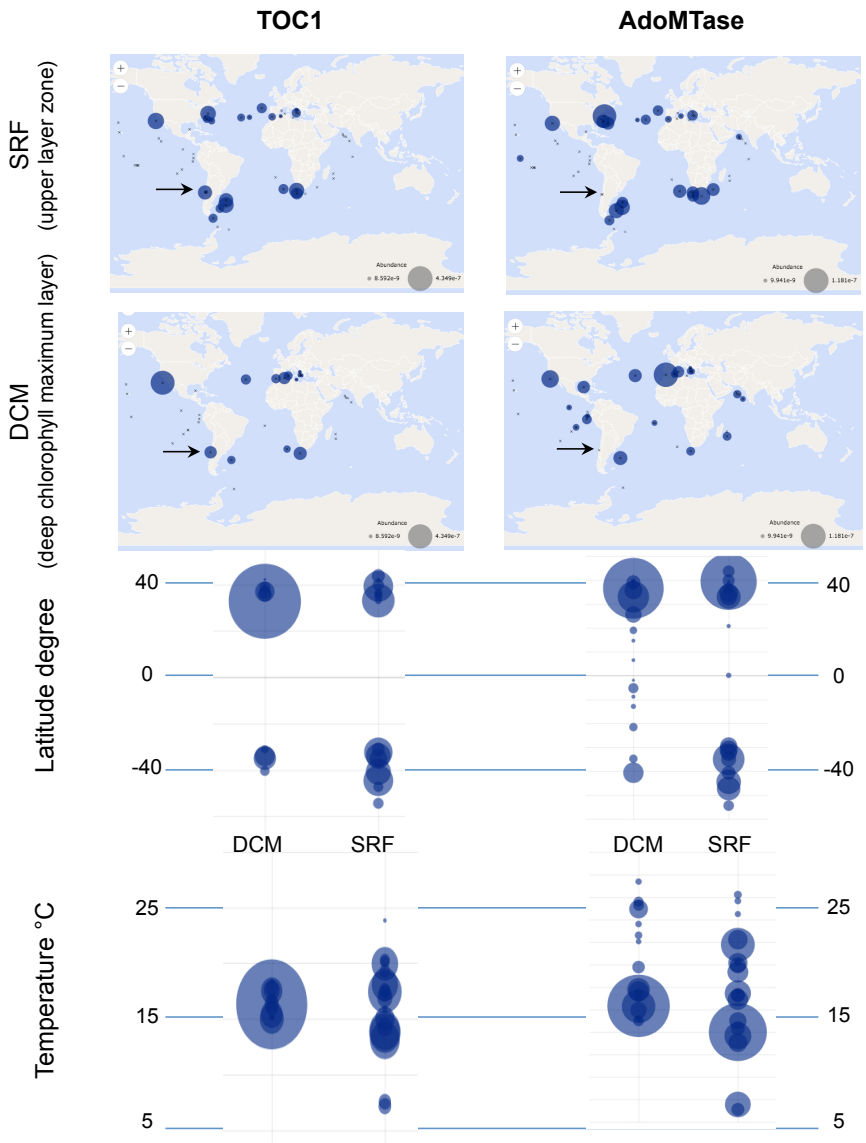

Figure 2 : Geographical distribution of TOC1 and AdoMTase proteins

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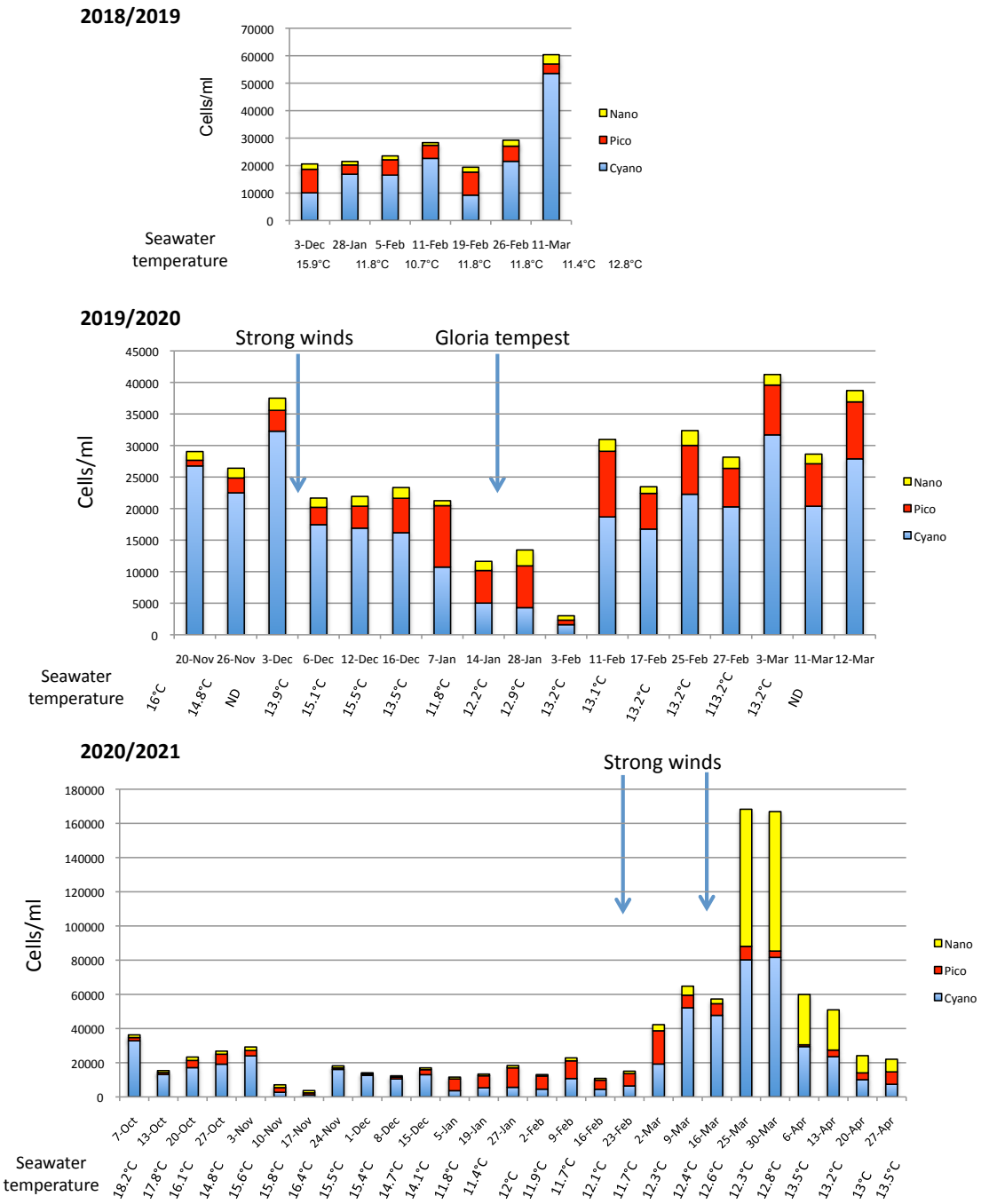

Figure 3. Abundance of phytoplankton during three successive blooms

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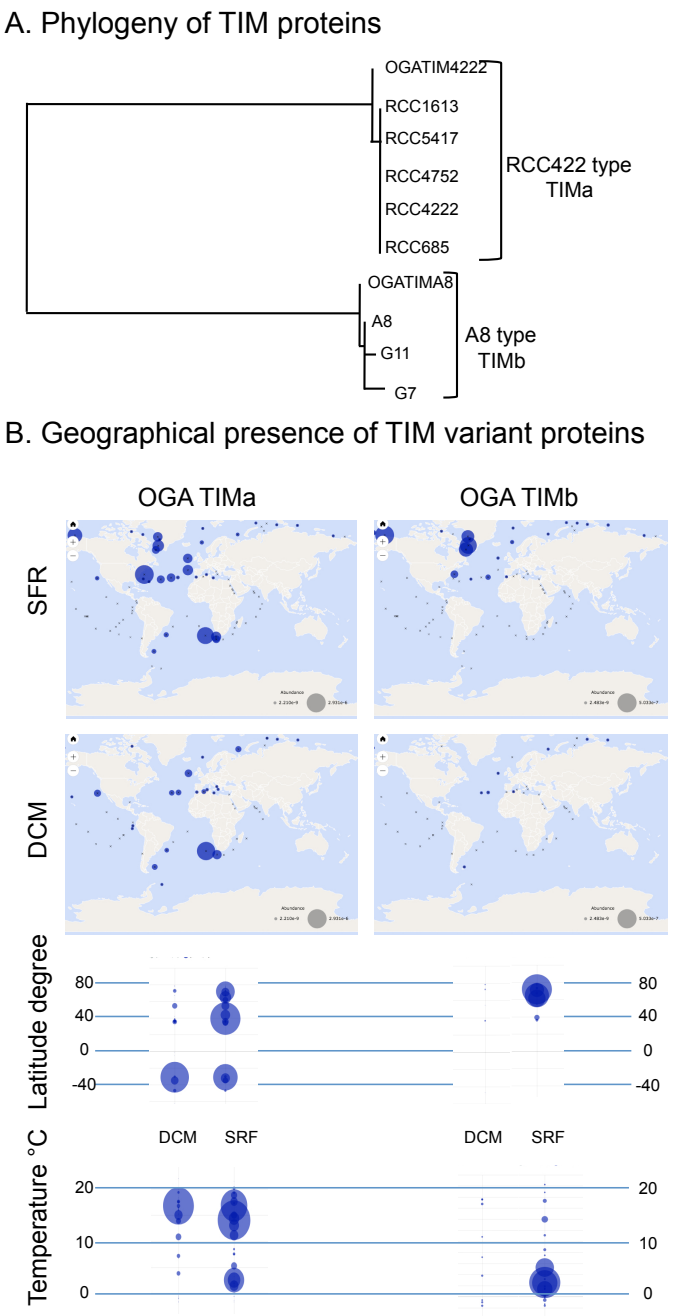
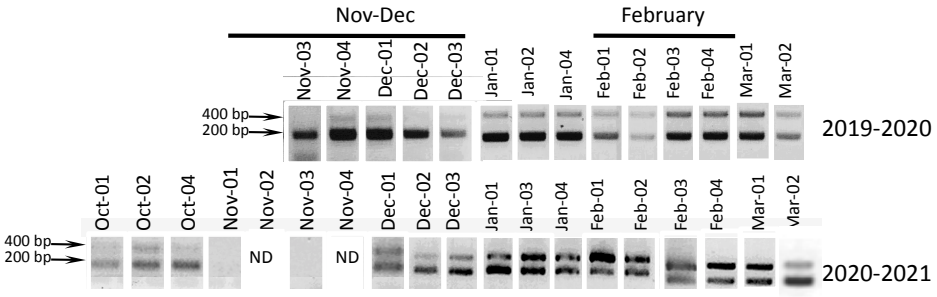


Figure 4. Distribution and abundance of TIM variant proteins in MetaG database

878

Marker chromosome 1 : yrdC promoter



Marker chromosome 14 : TIM

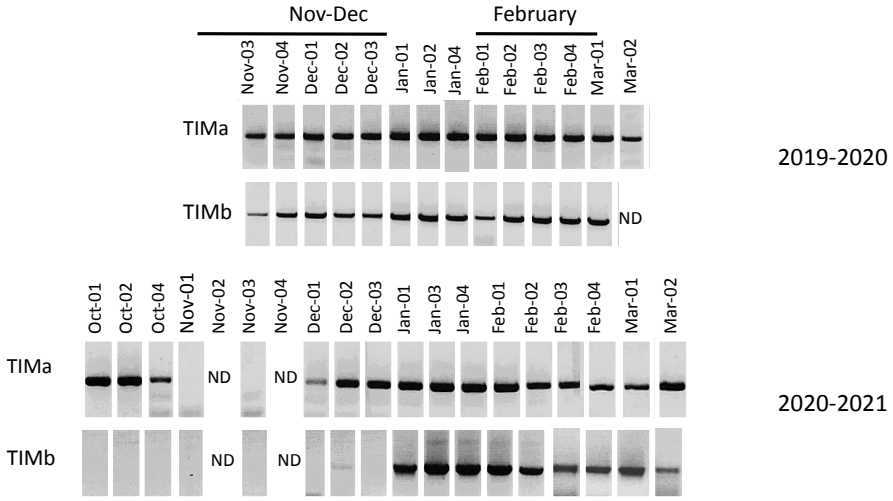


Figure 5. Diversity markers in seawater

879

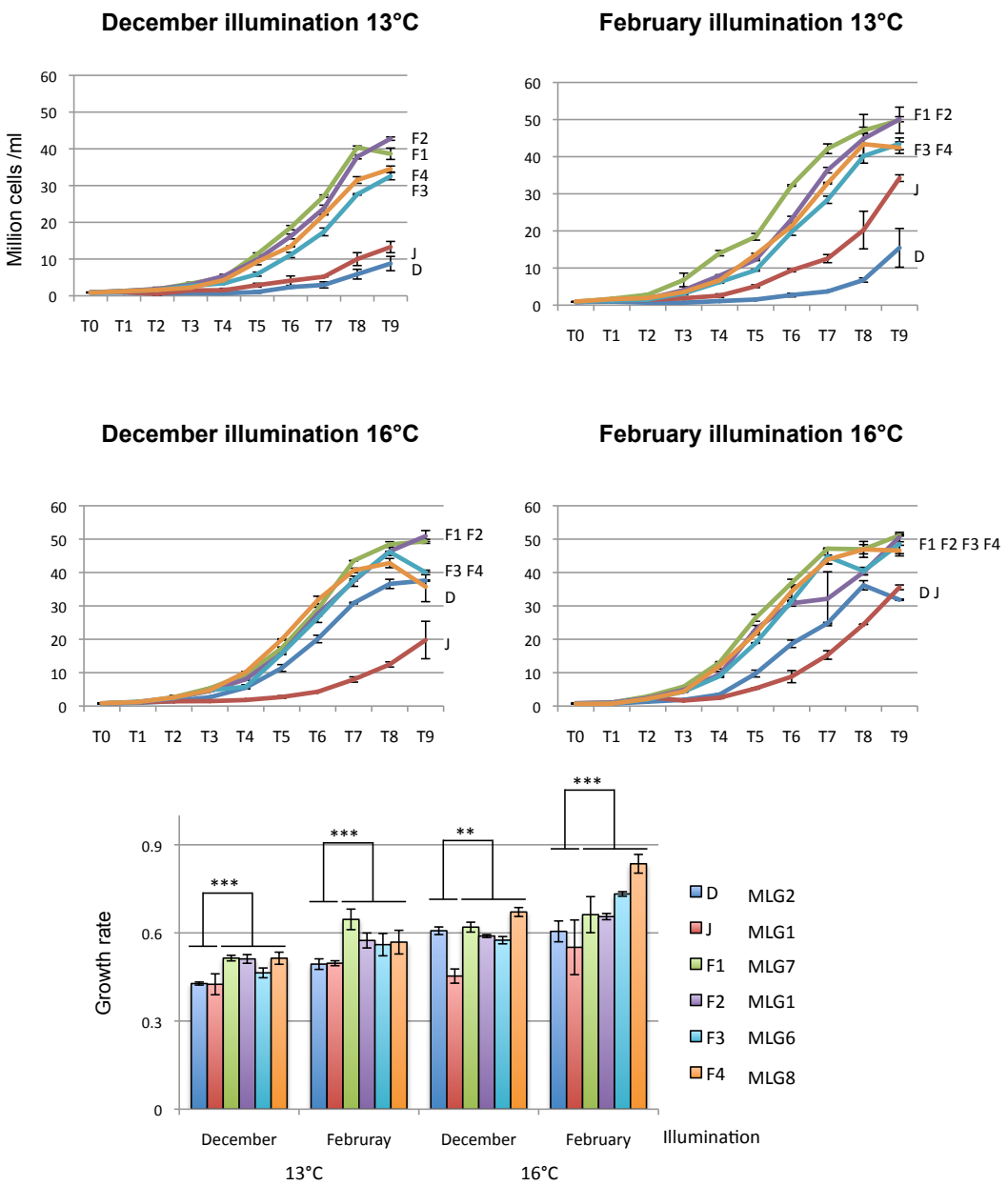


Figure 6 : Growth curves and rates of Banyuls isolates

880