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1 DIFFERENTIAL INFLUENCE OF LIFE CYCLE ON GROWTH AND TOXIN PRODUCTION
2 OF THREE *PSEUDO-NITZSCHIA* SPECIES (BACILLARIOPHYCEAE)¹
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34 Condensed running title: Life cycle and *Pseudo-nitzschia* toxin

35 ABSTRACT

36

37 We used a multi-strain approach to study the intra- and interspecific variability of the growth rates
38 of three *Pseudo-nitzschia* species – *P. australis*, *P. fraudulenta*, and *P. pungens* – and of their
39 domoic acid (DA) production. We carried out mating and batch experiments to investigate the
40 respective effects of strain age and cell size, and thus the influence of their life cycle on the
41 physiology of these species. The cell size - life cycle relationship was characteristic of each species.
42 The influence of age and cell size on the intraspecific variability of growth rates suggests that these
43 characteristics should be considered cautiously for the strains used in physiological studies on
44 *Pseudo-nitzschia* species. The results from all three species do not support the hypothesis of a
45 decrease in DA production with time since isolation from natural populations. In *P. australis*, the
46 cellular DA content was rather a function of cell size. More particularly, cells at the gametangia
47 stage of their life cycle contained up to six times more DA than smaller or larger cells incapable of
48 sexual reproduction. These findings reveal a link between *P. australis* life cycle and cell toxicity.
49 This suggest that life cycle dynamics in *Pseudo-nitzschia* natural populations may influence bloom
50 toxicity.

51

52 KEY WORDS: domoic acid, cell size, growth, life cycle, *Pseudo-nitzschia* strains

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54 ABBREVIATIONS: DA, domoic acid; cDA, cellular domoic acid; dDA, extracellular dissolved
55 domoic acid.

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69 INTRODUCTION

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71 Diatoms are one of the most common eukaryotic phytoplankton groups in aquatic
72 environments, with approximately 100,000 different species recorded (Not et al. 2012, Mann and
73 Vanormelingen 2013). This ecological success has previously been related to different
74 characteristics of diatoms: the presence of their unique siliceous cell wall, the frustule (Hamm et al.
75 2003, Armstrong et al. 2009), some metabolic particularities (Allen et al. 2011, Bailleul et al. 2015),
76 important intraspecific diversity (Godhe and Rynearson 2017), and also their unique life cycle
77 (Lewis 1984, 1987). This life cycle is strongly related to variations in cell size, and characterized by
78 two phases: long periods of vegetative multiplication, and short sexual events. During the
79 vegetative phase, cell size decreases over the generations until reaching a minimum non-viable size.
80 This decrease in size is one of the consequences of the presence of the silicified rigid cell wall
81 called a frustule, which is typical of diatoms. While size decreases, vegetative diatom cells are only
82 capable of sexual reproduction within a specific size range corresponding to the gametangia stage.
83 Sexual reproduction then leads production of gametes through meiosis followed by fusion to
84 produce a zygote; then the zygote enlarges to produce a specialized structure called an auxospore
85 allowing for the regeneration of an initial cells of maximum size (Mann 1999, Chepurnov et al.
86 2004, Kaczmarska et al. 2013). The control of sexual reproduction by cell size affects the
87 frequency, the timing, and the importance of sexual reproduction events (D'Alelio et al. 2010,
88 Hense and Beckmann 2015). These in turn probably affect bloom dynamics in natural populations.
89 The link between cell size and life cycle is thus an important characteristic to be explored in
90 diatoms. Furthermore, as far as cell metabolism is concerned, according to allometric laws small
91 cells are generally considered more competent physiologically than larger cells (*e.g.* thanks to better
92 growth or nutrient acquisition; Edwards et al. 2012, Marañón et al. 2013, Otero et al. 2018), even if
93 some authors reported a decrease in growth rate for smaller cells linked to certain life cycle stages
94 (Chisholm and Costello 1980, Von Dassow et al. 2006). However, the influence of cell size
95 reduction on diatom physiology as related to the life cycle is still poorly documented.

96 The heterothallic pennate diatoms of the genus *Pseudo-nitzschia* are cosmopolitan. Fifty
97 species of *Pseudo-nitzschia* are currently described, out of which 24 are considered to be toxic, *i.e.*
98 capable of producing domoic acid (DA), a neurotoxin that will accumulate in marine food webs and
99 cause amnesic shellfish poisoning (ASP) events (Lelong et al. 2012 and references herein, Lim et al.
100 2013, Orive et al. 2013, Fernandes et al. 2014, Teng et al. 2014, 2016, Li et al. 2017, Ajani et al.
101 2018, Frøsig Gai et al. 2018). According to the literature on different *Pseudo-nitzschia* species,
102 important interspecific differences in DA cellular content exist (*e.g.* Pan et al. 1996a and 1996b,

103 Fehling et al. 2004, Howard et al. 2007, Lelong et al. 2012, Thorel et al. 2014, Martin-Jézéquel et
104 al. 2015, Radan and Cochlan 2018). However, a few authors studied several strains *per* species and
105 also reported significant intraspecific diversity (*e.g.* Garrison et al. 1992, Villac et al. 1993, Álvarez
106 et al. 2009). Intraspecific diversity in DA production was even higher than interspecific diversity in
107 *P. multiseriata*, *P. calliantha*, and *P. fraudulenta* from USA coastal waters (Thessen et al. 2009). In
108 contrast, interspecific differences in DA production were greater than intraspecific variability in
109 three *P. australis*, *P. pungens* and *P. fraudulenta* strains from the French coastal waters (Lema et al.
110 2017). These authors also pointed out that the time spent in culture since isolation might influence
111 *Pseudo-nitzschia* species physiology, including DA production, as previously reported by Lelong et
112 al. (2012). In addition, previous studies concluded that cell size reduction induced a decrease in DA
113 production in several *Pseudo-nitzschia* species (Bates et al. 1999, Mafra et al. 2009, Amato et al.
114 2010).

115 The objective of this study was therefore to investigate the inter- and intraspecific variability
116 of growth and domoic acid production in three *Pseudo-nitzschia* species from the French coastal
117 waters, namely *P. australis*, *P. pungens*, and *P. fraudulenta*. We applied a multi-strain approach to
118 grasp intraspecific variability, by studying at least nine strains *per* species. In addition, we made a
119 special effort to explore the influence of the life cycle on the physiology of each species by studying
120 strains of different cell sizes and the same strains at different sizes during the cell size reduction
121 process. This also allowed us to investigate the influence of strain age on growth and toxin
122 production. Improving knowledge of intraspecific variability in *Pseudo-nitzschia* species is required
123 for an accurate characterization of the physiology of each species, and thus a better understanding
124 of their harmful bloom dynamics.

125

126 MATERIALS AND METHODS

127

128 *Strains*

129

130 Some *Pseudo-nitzschia* spp. strains were isolated from natural populations: from the west coast of
131 Brittany and in Arcachon Bay (Atlantic coast, France) for *P. australis*, and from the Bay of Seine
132 (English Channel, France) for *P. pungens* and *P. fraudulenta*. Other strains were obtained by
133 isolating initial cells produced during sexual reproduction experiments (Table 1). Single cells or
134 short chains were isolated using a micropipette, washed three times with filter-sterilized (0.2 µm)
135 seawater, and incubated in 4-well culture plates in K/2-medium (Keller et al. 1987) enriched in Si
136 (54 µM) at a temperature of 16°C, an irradiance of 30 µmol photons.m⁻².s⁻¹, and a 14:10 L:D light

137 cycle. When the clonal culture was established, it was maintained in 15 ml ventilated flasks in K/2-
138 medium + Si in the same conditions. Cultures were not axenic, but they were periodically checked
139 for bacterial development by optical microscopy observations, and hardly any bacteria were
140 detected. Most *Pseudo-nitzschia* species were identified from measurements of frustule properties
141 by transmission electron microscopy (TEM) and some by molecular sequencing of the entire
142 internal transcribed spacer region (ITS1-5.8S-ITS2) of the ribosomal RNA (see Lema et al. 2017).
143 For TEM observations, samples were cleaned to remove organic material according to Thorel et al.
144 (2017). To characterize the cell size range of each species, the cell size of each strain was measured
145 every month. The minimum cell size was characterized as the minimum viable size in four *P.*
146 *australis* strains, six *P. fraudulenta* strains, and six *P. pungens* strains. The strains were observed
147 under a Nikon Eclipse 80i light microscope equipped with a Nikon DS-Ri2 camera, and 20 cells
148 were measured (length and width) using NIS-Elements Imaging Software. Cell apical length (called
149 cell size here) was calculated as the mean \pm standard deviation of each batch of 20 cells (Lundholm
150 et al. 2002).

151

152 *Experiment 1: Mating experiments*

153

154 The main objective of these experiments was to link *Pseudo-nitzschia* spp. cell size to shifts in life
155 cycle stages, especially by defining the gametangia and the initial cell size ranges. Mating
156 experiments were carried out monthly with *P. australis*, *P. pungens*, and *P. fraudulenta* strains in
157 the course of their cell size reduction process for one year. Strains were considered at the
158 gametangia stage when they were capable of sexual reproduction. These experiments also provided
159 large-size strains from initial cells (F1 strains). Before carrying out the experiments, the cultures
160 (Table 1) were acclimated to the experimental conditions (16°C, 100 $\mu\text{mol photons.m}^{-2}.\text{s}^{-1}$ and
161 14:10 L:D light cycle), and 20 cells from each culture were measured. Mating experiments were
162 performed in 6-well culture plates in 5 ml of K/2-medium + Si, with an initial concentration of
163 5,000 cells.ml⁻¹ for each of the two strains of the compatible mating type. The mating type was
164 assessed by crossing the strains with reference strains of known mating types. The strain that bore
165 the auxospores was defined as “PN-”, and the other one as “PN+” (Kaczmarska et al. 2013). Plates
166 were incubated in growth chambers at 16°C, 100 $\mu\text{mol photons.m}^{-2}.\text{s}^{-1}$ and 14:10 L:D light cycle
167 (Economic Delux, Snijders Scientific B. V., UK). The crosses were checked daily for the formation
168 of sexual stages (gametes, zygotes, auxospores, or initial cells) under an inverted light microscope
169 (Leica DMIL LED, Wetzlar, Germany). Initial cells were sampled from the successful crosses, and

170 20 cells were measured (length and width) under a Nikon Eclipse 80i light microscope equipped
171 with a Nikon DS-Ri2 camera using NIS-Elements Imaging Software.

172

173 *Experiment 2: Batch experiments*

174

175 Batch experiments were performed to study two physiological indices, *i.e.* the growth rate and
176 domoic acid production (cellular domoic acid – cDA – and extracellular dissolved DA – dDA –
177 concentrations). The growth rate parameter was estimated during the exponential growth phase, and
178 DA concentrations were measured on the second day of the stationary phase because DA
179 production is higher during this phase before the culture starts declining (Cusack et al. 2002,
180 Fehling et al. 2004, Thessen et al. 2009).

181

182 Batch experiments were carried out with *P. australis*, *P. pungens*, and *P. fraudulenta* strains (Table
183 1). Before each experiment, each strain was acclimated to experimental conditions *i.e.* 16°C, 100
184 $\mu\text{mol photons.m}^{-2}.\text{s}^{-1}$, and 14:10 L:D light cycle in growth chambers (Economic Delux, Snijders
185 Scientific B.V., UK). The experiments were carried out in 250 ml filter flasks (NEST™) with 100
186 ml of K/2 + Si. Si(OH)_4^- and PO_4^{2-} concentrations were modified to obtain phosphate or silicate
187 limitation in the stationary phase because DA production is obtained in *Pseudo-nitzschia* cultures
188 after an exponential growth phase followed by quasi-exhaustion of P or Si which induces growth
189 arrest (Pan et al. 1996a, 1996b, 1998, Fehling et al. 2004, Amato et al. 2010). Nitrogen, phosphorus,
190 and silicon concentrations in the culture medium were as follows: 400 $\mu\text{M NaNO}_3$, 5 $\mu\text{M KH}_2\text{PO}_4$,
191 and 125 $\mu\text{M Na}_2\text{SiO}_3$ for P limitation, and 400 $\mu\text{M NaNO}_3$, 25 $\mu\text{M KH}_2\text{PO}_4$, and 25 $\mu\text{M Na}_2\text{SiO}_3$
192 for Si limitation. At the beginning of each experiment, an acclimated culture in the exponential
193 growth phase was centrifuged (10 min, 800 g, 16°C) in order to add only cells to the culture
194 medium without affecting nutrient concentrations. The initial cell concentration for all experiments
195 was around $2.5 \cdot 10^3 \text{ cells.ml}^{-1}$ ($\pm 0.25 \cdot 10^3$). Samples were collected once a day in the early afternoon
196 to monitor cell concentrations using a Nageotte counting chamber. At the end of the experiment,
197 each flask still contained at least half of the initial volume of culture.

198

199 The growth rate was calculated using the following equation:

200

$$201 \mu = \frac{\ln(X_2) - \ln(X_1)}{t_2 - t_1}$$

202

203 Where t is time in days, and X_1 and X_2 are cell concentrations (cells.ml^{-1}) at t_1 and t_2 , respectively.

204

205 On the second day of the stationary phase, samples were taken for DA measurements. A 10 ml
206 aliquot was taken from each flask, centrifuged, and the supernatant was recovered for extracellular
207 dissolved DA (dDA) measurements. A second 10-ml aliquot was sonicated on ice with a sonication
208 probe (Bioblock Scientific Vibracell 72442 ultrasons) for 4 minutes to disrupt cell membranes and
209 release DA from the cells, and filtered on a 0.2- μ m filter (33 mm, cellulose acetate membrane).
210 This fraction was used to measure total DA (tDA). The samples were frozen at -20°C prior to
211 analysis. DA quantification was performed using an ASP ELISA kit (Biosense Laboratories,
212 Bergen, Norway) following the manufacturer's instructions. Each sample was analyzed in duplicate
213 for quality control purposes. The absorbance was measured using a microplate spectrophotometer
214 (iMark™ Microplate Absorbance Reader, Bio-Rad Laboratories, Inc) equipped with a 450 nm
215 filter. According to the manufacturer, the calibrated range of the assay is 10 – 300 pg.ml⁻¹.

216

217 The cellular domoic acid content (cDA, pg.cell⁻¹) was calculated from tDA and dDA (in pg.ml⁻¹)
218 normalized to the cell concentration (cells.ml⁻¹) as follows:

219

$$220 \quad cDA = \frac{tDA - dDA}{cell \text{ concentration}}$$

221

222 To characterize interspecific and intraspecific variability in growth and DA production, these batch
223 experiments were performed on multiple strains for each of the three species, *i.e.* nine *P. australis*
224 strains, thirteen *P. pungens* strains, and twelve *P. fraudulenta* strains (Table 1). Potential
225 intraspecific variability may originate from genetic variability among strains, but also from the
226 influence of cell size linked to the life cycle or the influence of the time spent in culture. Therefore,
227 we calculated the age of strains isolated from natural populations as the time spent in culture
228 between the date of isolation and the date of each experiment. It is worth noting that we did not take
229 into account F1 strains when we studied the effect of strain age because they were not isolated from
230 natural population. Some strains (four *P. australis*, three *P. pungens*, and four *P. fraudulenta*
231 strains) were also studied at different cell sizes in the course of the size reduction process to
232 estimate the influence of cell size or time in culture (Table 1). Furthermore, we also performed
233 batch experiments on F1 strains and their parent strains to compare their growth rate and DA
234 production and test whether these were inherited characters or influenced by changes in cell size.

235 *Statistics*

236

237 After testing the normality and homoscedasticity of the data, ANOVA was used to test differences
238 in initial size, growth rate, cDA and dDA concentrations among the three species using the “car”
239 package in R version 3.5.1. Linear regressions were used to study the effect of cell size or age on
240 the growth rates of *P. australis*, *P. pungens*, and *P. fraudulenta* strains, and cDA or dDA in *P.*
241 *pungens* and *P. fraudulenta* cells larger or smaller than 60 μm , using the “lm” package in R.
242 Multiple linear regression analyses were performed to study the combined effect of cell size and age
243 on growth rate, using the “lm” package in R. Statistical significance was set at $\alpha = 0.05$ in all tests.
244

245 RESULTS

246 *Life cycle characteristics*

247 We characterized the minimum and maximum (initial cells) cell size for each species, along
248 with the size range within which the cells were capable of sexual reproduction – the gametangia
249 size range – to study the influence of the life cycle on *Pseudo-nitzschia* physiology.
250

251 The sizes of the initial cells issued from the mating experiments significantly differed
252 among the three species ($P < 0.001$). The initial cell sizes ranged from 134 μm to 167 μm (144 μm
253 on average) for *P. australis*, from 149 μm to 183 μm (162 μm on average) for *P. pungens*, and from
254 100 μm to 128 μm (113 μm on average) for *P. fraudulenta* (Fig. 1). Globally speaking, the multiple
255 crosses showed that initial cell size was not dependent on parental cell size whatever the species
256 (Fig. 1). The minimum cell size for a species was estimated as the minimum cell size for which
257 cells grew in culture. This mean minimum cell size was $29.5 \mu\text{m} \pm 1.7$ for *P. australis*, $32.6 \mu\text{m} \pm$
258 1.4 for *P. pungens*, and $22.3 \mu\text{m} \pm 0.8$ for *P. fraudulenta* (Fig. 2). These values corresponded to
259 $17.6 \% \pm 1.0$, $17.8 \% \pm 0.8$, and $17.5 \% \pm 0.6$ of the maximum initial cell size, respectively.
260 Microscopic observations revealed abnormal valve shapes in small-sized cells (Supplementary Fig.
261 1).
262

263 Gametangia sizes ranged from 55 to 85 μm , 43 to 75 μm , and 29 to 90 μm in *P. australis*, *P.*
264 *pungens*, and *P. fraudulenta*, respectively. These sizes corresponded to 33 - 51 %, 23 - 41 %, and 27
265 – 70 % of the maximum initial cell sizes, respectively (Fig. 2). These results mean that *P.*
266 *fraudulenta* cells were at the gametangia stage for 57.5 % of their life cycle, *P. australis* for only
267 21.6 %, and *P. pungens* 21.3 %, even if these evaluations may have been influenced by variation in
268 division rate with cell size (D’Alelio et al. 2009). These size ranges corresponded to gametangia
269
270

271 capable of producing new initial cells through sexual reproduction. However, the mating
272 experiments revealed that slightly smaller or larger cells were also able to produce gametes that
273 fused to form zygotes; yet, in these parental cell sizes, the resulting auxospores did not fully
274 develop and therefore did not produce initial cells (Fig. 2).

275

276 *Growth rate: influence of strain age and cell size*

277

278 The growth rate of *P. australis*, *P. pungens* and *P. fraudulenta* spanned from 0.31 to 0.82
279 day⁻¹, 0.15 to 0.75 day⁻¹ and 0.19 to 0.68 day⁻¹, respectively (Fig. 3B). These results show great
280 intraspecific variability in growth rate in *Pseudo-nitzschia* spp., while no significant difference
281 among the three species emerged. The high diversity of the strains in terms of cell size and age
282 allowed us to explore the relative influence of these two parameters on the growth rate. Even if
283 globally older strains tended to be characterized by smaller cells, there was no simple and direct
284 relationship between the cell size and the age when considering all strains used for one species.
285 Since all strains were not isolated at the same cell size, some strains of the same age exhibited different
286 cell sizes (Supplementary Fig. 2).

287

288 In *P. australis*, *P. pungens* and *P. fraudulenta*, the growth rate decreased significantly with
289 age ($P < 0.001$, Fig. 3A). However, in *P. pungens* and *P. fraudulenta*, we did not study strains of all
290 ages, but rather two groups corresponding to two sampling periods (2011-2012 and 2016, Table 1).
291 Therefore, some strains were more than 40 months old, and others were less than 10 months old.
292 For these two species, although globally growth rate decreased with age, it is worth nothing that no
293 tendency of growth rate evolution as a function of age emerged in either of these two groups taken
294 separately (Fig. 3A).

295

296 Globally, for the three species, growth rates were always lower for cell size below 60 μm
297 (Fig. 3B). However, beyond this general tendency, the relationship between cell size and growth
298 rates were species specific. There was no relationship between cell size and growth rate in *P.*
299 *fraudulenta*, except the difference below and above this threshold of 60 μm . In *P. pungens*, the
300 growth rate decreased linearly with cell sizes above 60 μm , but below this size there was no
301 significant tendency of growth evolution with cell size except that growth rates were the lowest.
302 Finally, in *P. australis*, the growth rate significantly decreased with cell size ($P < 0.001$ above and
303 below 60 μm). However, the decrease was more pronounced when cell sizes were below 60 μm
304 (Fig. 3B).

305

306 In each strain cell size decreased with age, as in all diatom cultures, so we used multiple
307 linear regression analyses to discriminate the effect of age from the effect of cell size on the
308 measured growth rates (equations 1A, 1B, 2, and 3).

309

310 (1A) *P. australis*, size < 60 µm: $\mu = -0.5113 + 0.0225 \cdot \text{Size}$ (P < 0.001)

311 (1B) *P. australis*, size > 60 µm: $\mu = 0.3847 + 0.0028 \cdot \text{Size}$ (P < 0.001)

312 (2) *P. pungens*: $\mu = 0.4563 - 0.0051 \cdot \text{Age} + 0.0014 \cdot \text{Size}$ (P < 0.001)

313 (3) *P. fraudulenta*: $\mu = 0.6093 - 0.00481 \cdot \text{Age}$ (P < 0.001)

314

315 Even if *P. australis*, growth rate decreased with age and cell size taken separately, the
316 results of this statistical approach show that *P. australis* growth rate could be predicted from cell
317 size alone. *P. australis* strains of different ages exhibited similar growth rates (e.g. around 0.60 day⁻¹
318 for 9-month-old or 21-month-old strains), while strains of the same age grew at different rates
319 (e.g. 0.60 or 0.30 day⁻¹ for 20 month-old strains, Fig. 3A). In contrast, the largest *P. australis* strains
320 (> 60 µm) had the highest growth rates (> 0.60 day⁻¹), and the strains whose cell size was below 45
321 µm (< 0.45 day⁻¹, Fig. 3B) had the lowest. In *P. pungens*, the growth rate was a function of both cell
322 size and age. The growth rate decreased from around 0.60 to 0.15-0.38 day⁻¹ between young (less
323 than 10 months old) and older *P. pungens* strains (more than 40 months old). But these changes in
324 growth rate in *P. pungens* were also linked to changes in cell size: the strains whose cell size was
325 above 60 µm exhibited the highest growth rates, while smaller strains grew slower (Fig. 3B). In
326 contrast, the growth rate of *P. fraudulenta* was only a function of the strain age. For example, the
327 growth rate was between 0.52 and 0.59 day⁻¹ for strains less than 10 months old, but between 0.19
328 and 0.44 day⁻¹ for more than 50-month-old *P. fraudulenta* (Fig. 3A).

329

330 *The life cycle influences toxicity*

331

332 All the strains of all three species produced domoic acid under our experimental conditions.
333 Whatever the species, there was no significant difference in domoic acid cellular content (cDA)
334 between phosphate limitation and silicate limitation (Supplementary Fig. 3). Even if, for each strain
335 considered individually, cDA could be higher under Si or P limitation (data not shown), there was
336 no global tendency for one limitation to promote higher DA production. In contrast, we measured
337 significant differences among species, with *P. pungens* maximum cDA one order of magnitude

338 above *P. fraudulenta* maximum cDA, and *P. australis* maximum cDA also one order of magnitude
339 above *P. pungens* maximum cDA ($P < 0.001$, Supplementary Fig. 3).

340

341 Figures 4 and 5 show cDA and dDA for the three species, respectively. In *P. australis*, *P. pungens*,
342 and *P. fraudulenta*, cDA was between 12 and 645 fg.cell⁻¹, 0.2 and 50 fg.cell⁻¹, and 0.03 and 5.6
343 fg.cell⁻¹, respectively (Fig. 4A and 4B). In these three species, dDA was between 0.022 and 8 ng.ml⁻¹
344 ¹, 0.04 and 0.395 ng.ml⁻¹, and 0.03 and 0.215 ng.ml⁻¹, respectively (data not shown). When
345 considering the biomass in the cultures, dDA concentrations corresponded to 1-5,970 fg.cell⁻¹ for *P.*
346 *australis*, 4-90 fg.cell⁻¹ for *P. pungens*, and 0.05-10 fg.cell⁻¹ for *P. fraudulenta* (Fig. 5A and 5B).
347 The measured dDA concentrations significantly differed among the three species ($P < 0.001$). *P.*
348 *australis* produced the highest amounts of dDA, followed by *P. pungens*, and then *P. fraudulenta*
349 (Fig. 5A and 5B).

350 cDA and dDA concentrations were not significantly influenced by strain age in *P. pungens*
351 and *P. fraudulenta*, even if we measured a few higher cDA concentrations in some of the young
352 strains and if no data is available for age between 6 and 40 months (Supplementary Fig. 4B, Fig 5A,
353 and 5B). In *P. australis*, cDA concentrations increased when the strains were between 5 and 12
354 months old (Supplementary Fig. 4A). However, this increase in cDA seemed to be rather related to
355 *P. australis* cell size (Fig. 4A). The cell size did not affect cDA concentrations in *P. fraudulenta*
356 (Fig. 4B), while dDA concentrations in this species were significantly higher (although very low) in
357 some strains with cell sizes above 75 μm ($P < 0.05$, Fig. 5B). Measured dDA concentrations in *P.*
358 *pungens* were not related to cell size (Fig. 5B). There was no clear relationship between cDA
359 concentrations and *P. pungens* cell size (Fig. 4B). However, we recorded the highest cDA
360 concentrations for this species ($> 20 \text{ fg.cell}^{-1}$) only in the large strains ($> 70 \mu\text{m}$), although some
361 large-size strains also exhibited very low cDA concentrations. Furthermore, no clear relationship
362 appeared between the cDA or dDA concentrations in F1 strains as compared to their parental strains
363 whatever the species (data not shown).

364

365 There was a strong relationship between DA and cell size in *P. australis*. Measured cDA
366 concentrations varied with cell size following a Gaussian model ($P < 0.001$, Fig. 4A):

367

$$368 \quad (4) \quad cDA = 524 * e^{(-0,5 * (\frac{Size - 71}{18})^2)}$$

369

370 cDA concentrations increased from 130 to 425 fg.cell⁻¹ as cell size decreased from ~130 to 80 μm .
371 Maximum cDA concentrations were between 600 and 645 fg.cell⁻¹ for cell sizes between 62.7 and

372 69.1 μm . The Gaussian model gave a maximum cDA concentration for 71- μm cells. Below 60 μm ,
373 cDA concentrations decreased down to 10 $\text{fg}\cdot\text{cell}^{-1}$ (Fig. 4A). These results were confirmed by
374 monitoring four *P. australis* strains whose cDA concentrations followed the Gaussian model over
375 time while the cells were decreasing in size (Fig. 6). These results show that large (100 - 130 μm)
376 and small (40 - 30 μm) *P. australis* strains could present cDA concentrations comparable to those of
377 *P. pungens* strains (Fig. 4). Therefore, the difference in cDA between *P. australis* and the other two
378 species was greater in the size range around 50-100 μm . Furthermore, in *P. australis*, measured
379 dDA concentrations also increased in 70 -100 μm strains (Fig. 5A). It is interesting to note that the
380 cell size range for which cDA concentrations were higher in *P. australis* coincided with the size
381 range characteristic of gametangial cells capable of sexual reproduction for this species (55-85 μm ,
382 Fig. 2). dDA concentrations were also higher in the upper part of the gametangial cell size range
383 (70-85 μm).

384

385 DISCUSSION

386

387 Using a multi-strain approach for three *Pseudo-nitzschia* species, we studied the intra- and
388 interspecific variability of growth rates and toxicity. This approach made it possible to disentangle
389 the respective effects of strain age and cell size. Furthermore, taking into account cell size offered a
390 unique perspective on the influence of the life cycle on the physiology of *Pseudo-nitzschia* species.

391

392 *Cellular and dissolved domoic acid production in P. australis, P. pungens, and P. fraudulenta*

393

394 *Pseudo-nitzschia* species are not all systematically toxic, and even among toxic species
395 some strains may not be able to produce DA (Lelong et al. 2012). Previous studies on strains of
396 different geographic origins reported great variability in cDA concentrations in *P. pungens* and *P.*
397 *fraudulenta*. Although *P. fraudulenta* has sometimes been reported to be a non-producer of DA
398 (Hasle 2002, Thessen et al. 2009, Quijano-Scheggia et al. 2010), our *P. fraudulenta* strains
399 exhibited cDA concentrations between 0.03 and 5.6 $\text{fg}\cdot\text{cell}^{-1}$. These concentrations are similar to
400 those reported in the review by Trainer et al. (2012, up to 30 $\text{fg}\cdot\text{cell}^{-1}$) and in Lema et al. (2017, up
401 to 55 $\text{fg}\cdot\text{cell}^{-1}$), though in the lower range of concentrations. For *P. pungens*, in contrast with studies
402 reporting non-toxigenic strains (Villac et al. 1993, Bates et al. 1998 and references therein), each of
403 our strains produced DA, even if the measured cDA concentrations (0.2 - 50 $\text{fg}\cdot\text{cell}^{-1}$) were also in
404 the lower range compared with previous studies (Bates et al. 1998, Baugh et al. 2006, Calu et al.
405 2009, Rhodes et al. 2013, Lema et al. 2017). *P. pungens* and *P. fraudulenta* also consistently

406 produced dissolved domoic acid. To our knowledge, this is the first report of dDA production by *P.*
407 *fraudulenta*, in contrast with Baugh et al. (2006) and Lema et al. (2017) who did not detect dDA
408 production by this species. As for cDA, our results confirm variability in dDA production among *P.*
409 *pungens* strains, in agreement with previous studies. Lema et al. (2017) did not detect any dDA in
410 their *P. pungens* cultures, while Baugh et al. (2006) measured dDA levels similar to the lowest
411 concentrations measured in the present study (at similar cell concentrations).

412

413 *P. australis* produced the highest cDA concentrations (between 12 and 645 fg.cell⁻¹), in the
414 same range as those already measured in strains isolated on French coasts (maximum between 30
415 and 700 fg.cell⁻¹, Thorel et al. 2014, Martin-Jézéquel et al. 2015, Lema et al. 2017), but much lower
416 than in strains isolated in Ireland (up to 26,000 fg.cell⁻¹, Cusack et al. 2002) and in the East Pacific
417 coastal waters (up to 1,740 fg.cell⁻¹ in Chile, Álvarez et al. 2009; up to 1,870 fg.cell⁻¹ in Baja
418 California, Santiago-Morales and García-Mendoza 2011; and up to 37,000 fg.cell⁻¹ in California,
419 Garrison et al. 1992). These differences in toxicity may point out the existence of different *P.*
420 *australis* ecotypes with contrasting capacities of DA production, as previously shown for other
421 *Pseudo-nitzschia* species by Thessen et al. (2009). In the present study, *P. australis* also produced
422 the highest dDA concentrations, in accordance with previous reports by Maldonado et al. (2002, up
423 to 7.6 ng.ml⁻¹), Martin-Jézéquel et al. (2015, up to 20.1 ng.ml⁻¹), and Lema et al. (2017, 430 fg.cell⁻¹).
424

425

426 These results confirm the high interspecific variability in DA production already highlighted
427 by Lelong et al. (2012). More particularly, the gradation in toxicity between *P. fraudulenta*, *P.*
428 *pungens*, and *P. australis* already observed by Lema et al. (2017) on strains from the same area
429 confirms the hypotheses from an *in situ* study in the English Channel relating bloom toxicity to
430 *Pseudo-nitzschia* species diversity (Thorel et al. 2017). However, our results show that the most
431 toxic *P. pungens* strains are as toxic as some *P. australis* strains. Therefore *P. pungens* also has to
432 be considered when dealing with DA toxic events observed in French coastal waters, particularly in
433 the English Channel where *P. australis* is mainly pointed out (Klein et al. 2010, Thorel et al. 2017).

434 Only few studies present dDA measurements during blooms (*e.g.* Bargu et al. 2008), while
435 impacts on marine organisms have already been documented (Liu et al. 2007). All our strains
436 produced dDA at significant concentrations, even if the extrapolation of dDA concentrations
437 obtained in culture to *in situ* bloom situations is uncertain. Increased knowledge regarding this
438 parameter is required to grasp its impact on marine ecosystems.

439

440 *Life cycle characteristics in relation to cell size and consequences on sexual reproduction in natural*
441 *populations*

442

443 We characterized the link between cell size and life cycle stages in *P. australis*, *P. pungens*,
444 and *P. fraudulenta* by determining initial cell size, the vegetative cell size range, and the
445 gametangia size range. As reported in the literature (Chepurnov et al. 2004, Kaczmarska et al.
446 2013), these cardinal points of the diatom life cycle were clearly species-specific. The initial cell
447 sizes were between 134 μm and 167 μm for *P. australis*, 149 μm and 183 μm for *P. pungens*, and
448 100 μm and 128 μm for *P. fraudulenta*. To our knowledge, this is the first report of initial cell size
449 for *P. fraudulenta*. However, the initial cell size range can be even larger in this species: Cusack et
450 al. (2004) reported vegetative cells greater than 128 μm . *P. pungens* initial cell sizes are in
451 agreement with those found by Chepurnov et al. (2005). Moreover, in *P. australis* and *P. pungens*,
452 these sizes are similar to the initial cell sizes measured during a sexual reproduction event observed
453 *in situ* during a bloom in Washington coastal waters (Holtermann et al. 2010). The relationship
454 between the size of parental cells and the initial cell size has been studied in different pennate and
455 centric diatoms. Some authors report a linear relationship both in centric (Jewson 1992) and pennate
456 diatoms (Davidovich 1994, Edlund and Bixby 2001, Davidovich et al. 2010). However, Armbrust
457 and Chisholm (1992), Davidovich (1994) and Fuchs et al. (2013) observed no relationship between
458 parental size and initial cell sizes in *Thalassiosira weissflogii*, *Fragilariopsis kerguelensis*, and
459 *Synedra tabulata*, respectively. In the present study, the initial cell size was also independent of the
460 parent cell size in all three *Pseudo-nitzschia* species within the studied size range.

461

462 Minimum sizes were 28 μm , 31 μm , and 22 μm in *P. australis*, *P. pungens*, and *P.*
463 *fraudulenta*, respectively. These values are close to those obtained for *P. pungens* (25-30 μm) by
464 Chepurnov et al. (2005) or for *P. arenysensis* (~18 μm) in a laboratory study (Amato et al. 2005)
465 and with a model of size reduction (Schwarz et al. 2009). Interestingly, the minimum cell size
466 corresponded to 17.6 % \pm 0.8 of the maximum initial size for the three species. It could be
467 interesting to explore if this threshold of 17 % is valid for other diatom species. These very small
468 cells are rarely observed *in situ* and may not be representative of natural populations for
469 physiological studies.

470

471 The gametangia size ranges observed in this study are in accordance with the size range
472 favorable to sexual reproduction usually admitted for diatoms (~30-75 %, Mann et al. 2003,
473 Chepurnov et al. 2004, Von Dassow et al. 2006, Davidovich et al. 2012, Fuchs et al. 2013,
474 Vanormelingen et al. 2013). The three species presented closed gametangia size ranges, since the

475 cells lost their ability to reproduce sexually before reaching the minimum viable cell size
476 (Chepurnov et al. 2004). These gametangia size ranges fall within previous ranges measured for
477 *Pseudo-nitzschia* species: 20-90 % for *P. arenysensis* (Amato et al. 2005), 23-70 % for *P.*
478 *multiseries* (Hiltz et al. 2000), 39-71 % for *P. multistriata* (D'Alelio et al. 2009), although *P.*
479 *australis* and *P. pungens* presented narrower gametangia size ranges. For example, Chepurnov et al.
480 (2005) found that their *P. pungens* strains could mate in a wider size range (20-60 % of initial cell
481 size, according to their data), even if their strains belonged to the same clade as ours (clade I, Lim et
482 al. 2014). When gametangia size ranges are narrower, it can be hypothesized that a smaller portion
483 of the population is capable of sexual reproduction in natural populations. This may affect
484 encounter rates during blooms, and in turn reproductive success. However, the fact that only a small
485 portion of the population can sexually reproduce may also be an ecological advantage regarding
486 competition with the rest of the phytoplankton community because most of the *Pseudo-nitzschia*
487 population would carry on with vegetative multiplication during a sexual reproduction event (Lewis
488 1984). Furthermore, in *P. australis* and *P. pungens*, the narrower gametangia size range is linked to
489 the fact that vegetative cells need to decrease more in size to reach the gametangia stage. These life
490 cycle characteristics probably affect the frequency and the timing of sexual reproduction events in
491 natural populations (Lewis 1984). The difference in gametangia size range observed in the three
492 species therefore suggests that their natural populations could present different sexual reproduction
493 dynamics. This in turn could influence the general population and bloom dynamics (Jewson 1992,
494 Edlund and Stoermer 1997, D'Alelio et al. 2010).

495
496 This study highlights an intermediate stage of the *Pseudo-nitzschia* life cycle. Our
497 experiments revealed a size range wider than that of gametangia, within which cells were capable of
498 gametogenesis and fertilization but could not produce initial cells. This second size range observed
499 for the first time in this study suggests that the metabolic changes linked to the transition from
500 vegetative cells to gametangia occur progressively during the decrease in cell size allowing cells to
501 acquire the ability to reproduce sexually. When entering the larger size range, cells seem to acquire
502 the physiological abilities required for pairing, gametogenesis, and fertilization. This may include
503 production of pheromones for the recognition of complementary sexual types (Sato et al. 2011,
504 Gillard et al. 2013, Frenkel et al. 2014), and mobility abilities for active pairing. In addition, in the
505 larger size range, cells must be physiologically ready for meiosis which leads to gametogenesis
506 (diatom are diplonts). Then, as the cells enter the narrower size range, they probably acquire
507 supplementary physiological abilities that enable them to ensure complete auxosporulation. This
508 process represents a high metabolic cost for the cells: they probably stop most syntheses during at

509 least the first steps of sexual reproduction that lasts two to four days on average in *Pseudo-nitzschia*
510 species (Davidovich and Bates 1998; Sauvey unpublished data). The zygote also needs lots of
511 storage to restore a new large initial cell (Chepurnov et al. 2005). Therefore, in contrast to cells in
512 the larger size range, gametangial cells in the narrower range may present a more efficient
513 metabolism with higher storage capabilities – especially for silicium – to be able to synthesize the
514 frustule of the new initial cell. These results stress the fact that gametangial cells probably present
515 particular metabolic characteristics as compared to vegetative cells.

516

517 *Differential influence of life cycle stages and strain age on growth and DA production in Pseudo-nitzschia*
518 *species*

519

520 The time spent in culture seemed to influence *P. fraudulenta* and *P. pungens* growth since
521 growth rates decreased with increasing strain age. However, the strains had been isolated during
522 two distant sampling periods, so it is difficult to tell whether this difference was a consequence of
523 cell adaptation to natural environmental conditions (*i.e.* the difference reflected the period when the
524 population from which the strains were isolated) or a physiological change during the culturing
525 period (*i.e.* the difference reflected strain age) (Lakeman et al. 2009). Further studies with strains of
526 complementary ages are needed to state on this point. However, these results highlight that it is
527 important to take into account the fact that strains in culture can evolve, so that ecophysiological
528 studies should be performed as soon as possible (≤ 1 or 2 years) after *Pseudo-nitzschia* strains have
529 been isolated.

530

531 In *P. australis*, the link between growth rate and strain age was probably the outcome of the
532 weak relationship between age and cell size since cell size was sufficient to predict the growth rate.
533 The same relationship was observed for the three species, with a sharp decrease in growth rate
534 below a cell size threshold of 60 μm . Above this size, growth rates were similar in all *P.*
535 *fraudulenta* strains whatever the cell size. However, above 60 μm in *P. pungens* and *P. australis*,
536 the growth rate also decreased with cell size, although more slowly. Altogether, the growth rate
537 decreased with decreasing cell size. This result is in contradiction with allometric rules generally
538 accepted for phytoplankton populations. These rules predict an increase of growth rates with
539 decreasing cell size due to higher surface-to-volume ratios that, for example, favor higher nutrient
540 uptake (Amato et al. 2005, Von Dassow et al. 2006a, Edwards et al. 2012, Marañón et al. 2013,
541 Otero et al. 2018). Our results therefore show that allometric rules do not apply when dealing with
542 intraspecific diversity in *Pseudo-nitzschia* species. The distinctive feature of the size-growth
543 relationship for our three *Pseudo-nitzschia* species is the sharp decrease of the growth rate in small

544 cells. Similar results have been reported for *Thalassiosira* species (Chisholm and Costello 1980;
545 Von Dassow et al. 2006). These authors related the decrease of the growth rate in small cells to
546 sexual reproduction. In contrast, in the present study, the lowest growth rates did not coincide with
547 gametangia size ranges, and might rather represent a decrease in metabolism efficiency when cells
548 approached their minimum viable size. Emphasizing this point, we often observed cells studied here
549 displayed abnormal valve shapes in the smallest-size strains (data not shown), suggesting deficient
550 valve deposition as reported by Von Dassow et al. (2006). In natural populations, the fact that small
551 cells may be physiologically less efficient could explain why these size ranges are rarely observed:
552 small cells may not be competitive enough to outcompete larger cells and survive. Consequently, it
553 is important to pay attention to cell size in addition to strain age. Strains of large to medium cell
554 sizes (\geq gametangia sizes) should be used for ecophysiological studies on *Pseudo-nitzschia* species.
555 Furthermore, choosing strains of similar cell sizes appears to be an important prerequisite for
556 comparative (inter- or intraspecific) studies.

557

558 As far as domoic acid (cDA and dDA) production is concerned, strain age had no significant
559 influence in the three species. However, a few young *P. pungens* strains presented higher cDA
560 concentrations than the others, whatever their age. Beyond inter-specific differences, this could
561 explain why a decrease in cDA with time in culture was previously reported in the literature
562 (Lelong et al. 2012 and references herein), in contrast with the present results.

563

564 As for the influence of cell size, we found the highest dDA concentrations in some large *P.*
565 *fraudulenta* F1 strains, and the highest cDA concentrations in some large *P. pungens* strains.
566 However, there was no clear evidence of an influence of cell size on *P. fraudulenta* and *P. pungens*
567 DA production since large strains of these two species also exhibited very low cDA and dDA
568 concentrations. These results do not confirm that DA production systematically decreases with
569 decreasing cell size, as reported in *P. multiseriata* and *P. multistriata* (Bates et al. 1999, Mafra et al.
570 2009, Amato et al. 2010). However, they are not completely in disagreement for some strains,
571 stressing once more the high intraspecific variability characteristic of *Pseudo-nitzschia* species. This
572 great variability was also highlighted by the cDA and dDA concentrations measured in the F1
573 strains, which were not related to concentrations in parental strains. This result is in agreement with
574 Amato et al. (2010), who studied *P. multistriata* and concluded that the inheritance of the ability to
575 produce DA was not a simple Mendelian process.

576

577 Our results revealed that cDA concentrations could be predicted from cell size by a
578 Gaussian model in *P. australis*. This is not completely in contradiction with previous results
579 reporting a decrease in cDA with decreasing cell size (Bates et al. 1999, Mafra et al. 2009, Amato et
580 al. 2010). Our cell size range was greater than in any other study because we took into account very
581 large strains issued from initial cells obtained in mating experiments. In the literature, the decrease
582 in cDA corresponds to sizes ranging from medium-sized to small cells (Bates et al. 1999, Mafra et
583 al. 2009, Amato et al. 2010). We also observed a decrease in cDA with decreasing cell size in *P.*
584 *australis* within that same size range. In addition, we brought supplementary information to
585 currently available data about the relationship showing that showing that medium-sized cells are the
586 most toxic ones and large cells issued from sexual reproduction present cDA concentrations
587 comparable to small cells. These observations show that cDA concentrations are not linearly related
588 to cell size in *P. australis*. Furthermore, the evolution of cDA and dDA concentrations suggests that
589 they are not characteristic of any given strain because DA production is not stable over a whole life
590 time in *P. australis*, in contrast to results reported for *P. multistriata* (Amato et al. 2010). This
591 discrepancy highlights the fact that (i) the relationship between cell size and DA production is
592 species-specific, and (ii) it is important to study the whole size range of a species before drawing
593 conclusions on this relationship.

594

595 The striking result of this study is that the increase in cDA in *P. australis* coincided with the
596 size range within which the cells reached the gametangia stage, while dDA also increased in the
597 larger gametangial cells. We can therefore hypothesize that the physiological changes occurring
598 when vegetative cells become sexualized also result in an increase of DA production. During the
599 sexual reproduction process (gametogenesis, fertilization, and auxosporulation), the cells cannot
600 carry out photosynthesis and probably other metabolic pathways any more. Preparing vegetative
601 cells for this step probably includes increasing cell metabolism efficiency and/or modifications of
602 metabolic pathways to increase cellular quotas of/ the required cellular metabolites (Pan et al.
603 1998). This probable reorientation of the metabolism may also favor DA synthesis pathways. Bates
604 et al. (1999) hypothesized that the ability of cells to produce domoic acid was not related to cell size
605 but to general physiological changes. Our results suggest that these physiological changes can be
606 linked to the life cycle and especially the sexualization of vegetative cells, at least for *P. australis*.
607 Domoic acid synthesis pathways involve the Krebs cycle and the formation of derived compounds,
608 *e.g.* glutamate and geranyl pyrophosphate (Pan et al. 1998, Brunson et al. 2018). We can
609 hypothesize that in *P. australis* the change in metabolism that the cells undergo for their
610 sexualization affects these metabolic pathways, resulting in an increase in DA production.

611 Therefore, the increase in DA cellular content in gametangia cells might be a “physiological
612 coincidence”. However, the role of this toxin still remains to be deciphered, so we can also wonder
613 if DA could play a role in *P. australis* sexual reproduction. In pennate diatoms, interactions between
614 sexualized cells are needed to trigger gametogenesis. The increased production of DA (and
615 especially dissolved DA) therefore raises questions on the potential role of this toxin as a
616 pheromone (previously mentioned by Lelong et al. 2012). Interestingly, the only pheromone
617 identified in a pennate diatom up to now is a cyclic dipeptide derived from proline, whose synthesis
618 is thus linked to amino acid biosynthesis (Guillard et al. 2013, Frenkel et al. 2014). It is however
619 surprising that a similar link between DA production and life cycle was not found in the two other
620 *Pseudo-nitzschia* species which present the same sexual reproduction pattern. In addition, cells may
621 be more vulnerable during sexual reproduction, so domoic acid could act as a grazer deterrent: its
622 production has been shown to increase in the presence of copepods (Harðardóttir et al. 2015,
623 Tammilehto et al. 2015). Finally, different studies also showed an allelopathic effect of *Pseudo-*
624 *nitzschia* or DA addition on different phytoplankton species (Lundholm et al. 2005, Smeti et al.
625 2015, Sobrinho et al. 2017, Van Meerssche et al. 2018). Higher DA production may therefore also
626 represent an ecological advantage that could offset the growth arrest/decrease during sexual events
627 in *Pseudo-nitzschia* blooms.

628

629 The link between DA and cell size, and therefore DA and life cycle stages, suggests that *P.*
630 *australis* life cycle probably impacts bloom toxicity. First, *P. australis* populations can be more or
631 less toxic depending on their cell size spectra. Cell size distribution varies across years in natural
632 populations (D’Alelio et al. 2010), this might partly explain inter-annual variations in bloom
633 toxicity. Furthermore, when a sexual event occurs in a natural population, it results in the
634 production of initial cells and thus in a shift in size distribution towards large cells (Holtermann et
635 al. 2010). During a *P. australis* bloom, this process is expected to result in a decrease in DA
636 concentration in the population, especially as the large cells with slightly higher growth rates will
637 progressively outnumber smaller cells in this population (Armbrust and Chisholm 1992). Life cycle
638 events could therefore also impact DA concentrations in natural populations at the scale of a bloom.

639

640 CONCLUSION

641

642 This study characterizes the toxicity and cell size - life cycle relationship in three *Pseudo-*
643 *nitzschia* species. Even if we evidenced species-specific characteristics, the importance of intra-
644 specific variability proves that the number of studied strains *per* species greatly influences the

645 conclusions on *Pseudo-nitzschia* physiology. Furthermore, our results advocate for the use of
646 *Pseudo-nitzschia* strains not more than one or two years after their isolation, and of cell sizes at or
647 above gametangia size when studying cellular metabolism. However, as far as toxicity is concerned,
648 our results do not support the hypothesis of a decrease in DA production with time in culture in
649 these three species. Most importantly, this study shows that *P. australis* cDA can be predicted from
650 cell size by a non-linear relationship. In this species, cDA is maximum in medium-sized cells at the
651 gametangia stage of their life cycle. These results suggest that either the metabolic changes
652 occurring in gametangia cells favor DA production as compared to vegetative cells, or that DA is
653 involved in sexual reproduction. The *Pseudo-nitzschia* life cycle may therefore influence the
654 toxicity of blooms. Furthermore, the cell size – cDA relationship revealed by our results may be
655 useful to predict bloom toxicity based on the cell size spectra of a *P. australis* population. Finally, a
656 comparative understanding of the cellular metabolism of *P. australis* at different life cycle stages
657 may help to identify the DA biosynthesis pathway.

658

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660

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671

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951 *Table 1. Strains of P. australis, P. pungens, and P. fraudulenta used for mating and batch culture*
 952 *experiments. (A) for strains isolated from the Atlantic and (EC) for strains isolated from the English*
 953 *Channel. The age of the strain (in months, calculated from the date of isolation) and the cell size*
 954 *length (cell size) are indicated for each experiment.*

955

Species	Strain information			Age of strain (month)	Cell size length (µm)	
	Collection reference	Origin (Sampling zone or crossing)	Isolation date			
<i>P. australis</i>	IFR-PAU-010	Ouessant (A)	07/2015	5	50	
	PNaus P1D2	Camaret-sur-Mer (A)	03/2014	21	44	
	PNaus P3B2	Plouzané (A)	03/2014	21	49	
	PNaus 02T	Arcachon Bay (A)	04/2016	9 - 12 - 14 - 16	69 - 63 - 60 - 57	
	PNaus P6B3	Plouzané (A)	04/2014	20 - 26	42 - 35	
	PNaus F1-1A	P6B3 x P2B1			83	
	PNaus F1-4A	P6B3 x P6C1			73	
	PNaus F1-5	P6B3 x P1A3			97 - 82 - 65 - 61 - 57	
	PNaus F2-1B	F1-5 x F1-1A			127 - 106	
<i>P. pungens</i>	PNpun 47	Cabourg (EC)	08/2011	59	48	
	PNpun 66	Ouistreham (EC)	09/2011	51 - 58	41 - 47	
	PNpun 79	Cabourg (EC)	05/2012	50	52	
	PNpun 88	Cabourg (EC)	07/2012	48	47	
	PNpun 89	Cabourg (EC)	07/2012	41 - 48	42 - 49	
	PNpun 129	Luc-sur-mer (EC)	07/2016	2	75	
	PNpun 130	Luc-sur-mer (EC)	07/2016	2	95	
	PNpun 133	Luc-sur-mer (EC)	07/2016	3	109	
	PNpun 134	Luc-sur-mer (EC)	07/2016	3	102	
	PNpun 136	Saint-Vaast-la-Hougue (EC)	07/2016	3	85	
		PNpun F1-7A	66 x 89			157 - 175
		PNpun F1-7B	66 x 89			153
		PNpun F1-11	66 x 47			153
<i>P. fraudulenta</i>	PNfra 1	Cabourg (EC)	08/2011	62	27	
	PNfra 10	Cabourg (EC)	08/2011	51 - 59	45 - 39	
	PNfra 30	Cabourg (EC)	10/2011	49 - 57	41 - 36	
	PNfra 31	COMOR 41 (EC)	07/2011	64	31	
	PNfra 126	Luc-sur-mer (EC)	11/2015	3	65	
	PNfra 132	Luc-sur-mer (EC)	10/2015	3	68	
	PNfra 162	Saint-Vaast-la-Hougue (EC)	05/2016	5	69	
		PNfra F1-8D	30 x 31			107 - 87
		PNfra F1-9A	10 x 31			78
		PNfra F1-9B	10 x 31			113 - 92
		PNfra F1-14A	10 x 1			110
		PNfra F1-14B	10 x 1			113

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965 FIGURES LEGENDS

966

967 *Figure 1.* Mean size of initial cells as a function of mean size of parent strains in *P. australis*, *P.*
968 *pungens*, and *P. fraudulenta*. Standard deviations represent the minimum and maximum sizes
969 measured for initial cells and represent the variance for parental cells.

970

971 *Figure 2.* Size range of vegetative cells (white) and gametangia (grey and hatched pattern) in *P.*
972 *australis*, *P. pungens*, and *P. fraudulenta*. Crosses and dots correspond to cell sizes for which
973 mating experiments were performed. Crosses: no sexual reproduction observed. Hatched pattern
974 and white dots: incomplete sexual reproduction observed (without initial cell production). Light
975 grey and dark grey dots: complete sexual reproduction observed (with initial cell production). The
976 size percentage was calculated based on the maximal initial size. 85 mating experiments were made
977 to define the gametangia size range for *P. australis*, 40 for *P. pungens*, and 89 for *P. fraudulenta*.
978 193 cells were measured to determine the vegetative cell size range for *P. australis*, 334 cells for *P.*
979 *pungens*, and 551 cells for *P. fraudulenta*.

980

981 *Figure 3.* (A) Growth rate (μ , day⁻¹) as a function of the age of the strains (months) since their
982 isolation from natural populations in *P. australis* (n = 9), *P. pungens* (n = 12), and *P. fraudulenta* (n
983 = 9) (F1 strains from sexual reproduction are not present in this graph). (B) Growth rate (μ , day⁻¹)
984 as a function of the cell size (μ m) for all strains studied in *P. australis* (n = 18), *P. pungens* (n = 16),
985 and *P. fraudulenta* (n = 16).

986

987 *Figure 4.* Cellular domoic acid content (cDA, fg.cell⁻¹) as a function of cell size (μ m): (A) in *P.*
988 *australis* and (B) in *P. pungens* and *P. fraudulenta*. Data come from the cDA measurements made
989 in the batch experiments on the second day of the stationary phase linked to a phosphate or silicate
990 limitation.

991

992 *Figure 5.* Dissolved domoic acid (dDA, fg.cell⁻¹) as a function of cell size (μ m): (A) in *P. australis*
993 and (B) in *P. pungens* and *P. fraudulenta*. Data come from the dDA measurements made in the
994 batch experiments on the second day of the stationary phase linked to a phosphate or silicate
995 limitation.

996

997 *Figure 6.* Monitoring of the cellular domoic acid content (cDA, fg.cell⁻¹) of four strains of *P.*
998 *australis* during their decrease in cell size.

999

1000 SUPPLEMENTARY FIGURES LEGENDS

1001

1002 *Figure S1.* Optical microscopy observation of *Pseudo-nitzschia australis* strain (PNaus P6B3) with
1003 abnormal valve shapes in small cells.

1004

1005 *Figure S2.* Relationship between cell size and strain age (month) since their isolation from the
1006 natural environment in *P. australis* (n = 9), *P. pungens* (n = 12), and *P. fraudulenta* (n = 9) (F1
1007 strains are not taken into account). Linear regressions are not significant ($P > 0.05$).

1008

1009 *Figure S3.* Cellular domoic acid content (cDA, fg.cell⁻¹, mean ± SD) under phosphate limitation
1010 (black bars) or silicate limitation (grey bars) in *P. australis* (n = 18), *P. pungens* (n = 16), and *P.*
1011 *fraudulenta* (n = 16). The letters a, b and c indicate the significant differences ($P < 0.001$).

1012

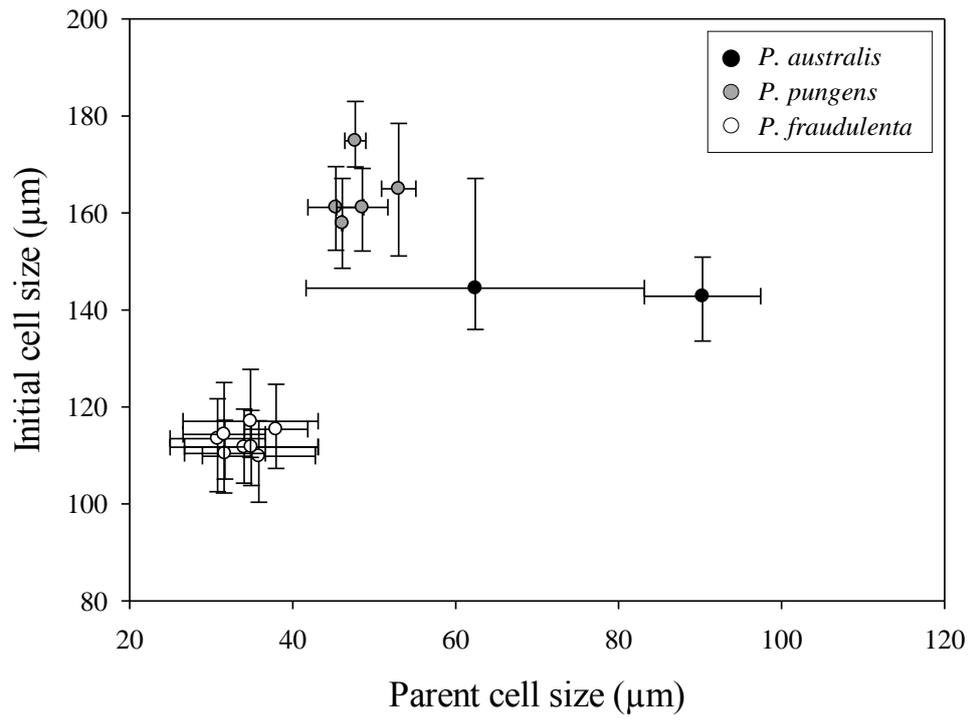
1013 *Figure S4.* Cellular domoic acid content (cDA, fg.cell⁻¹) as a function of the age of the strain
1014 (months) since their isolation from natural populations: (A) in *P. australis* (n = 9) and (B) in *P.*
1015 *pungens* (n = 12) and *P. fraudulenta* (n = 9).

1016

1017 *Figure S5.* Dissolved domoic acid (dDA, fg.cell⁻¹) as a function of the age of the strain (months)
1018 since their isolation from natural populations: (A) *P. australis* (n = 9) and (B) in *P. pungens* (n =
1019 12) and *P. fraudulenta* (n = 9).

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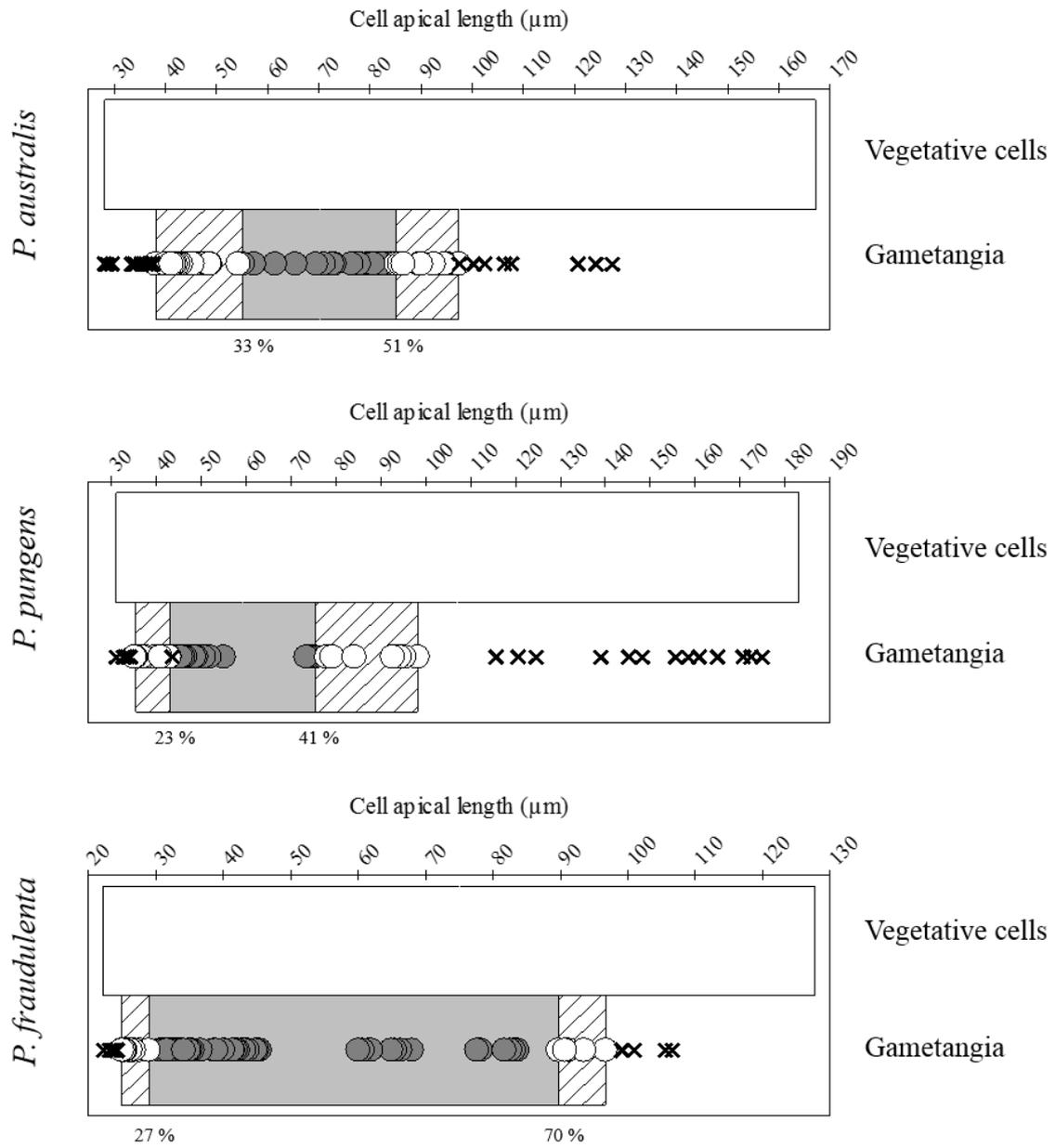
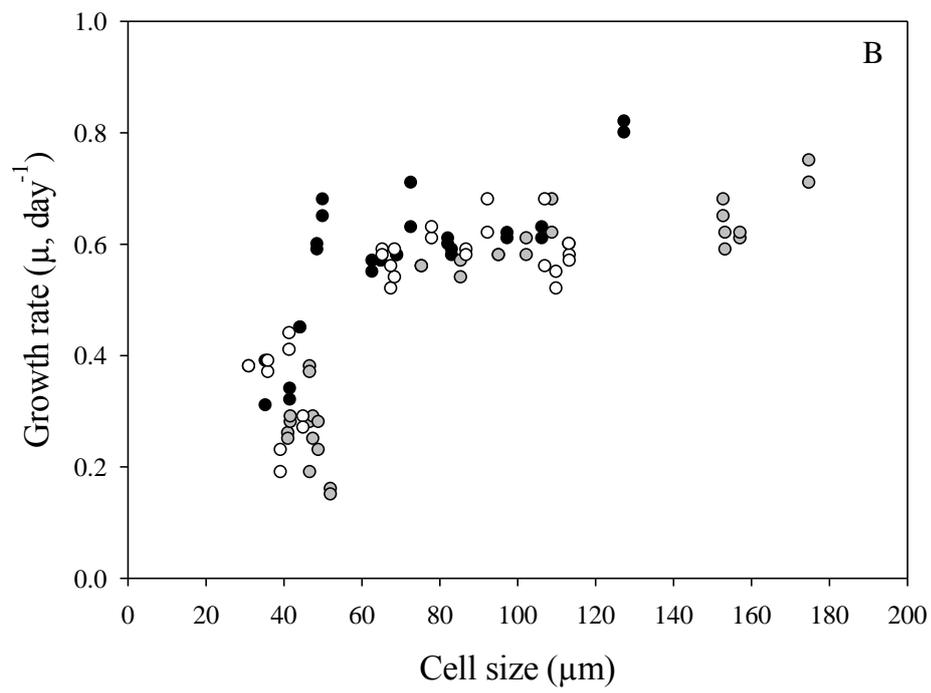
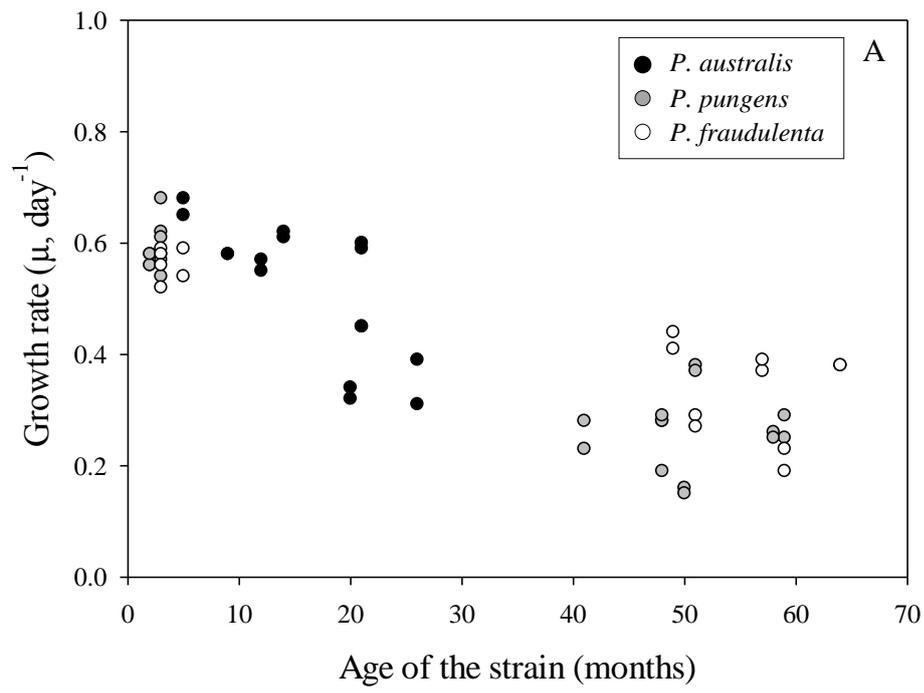
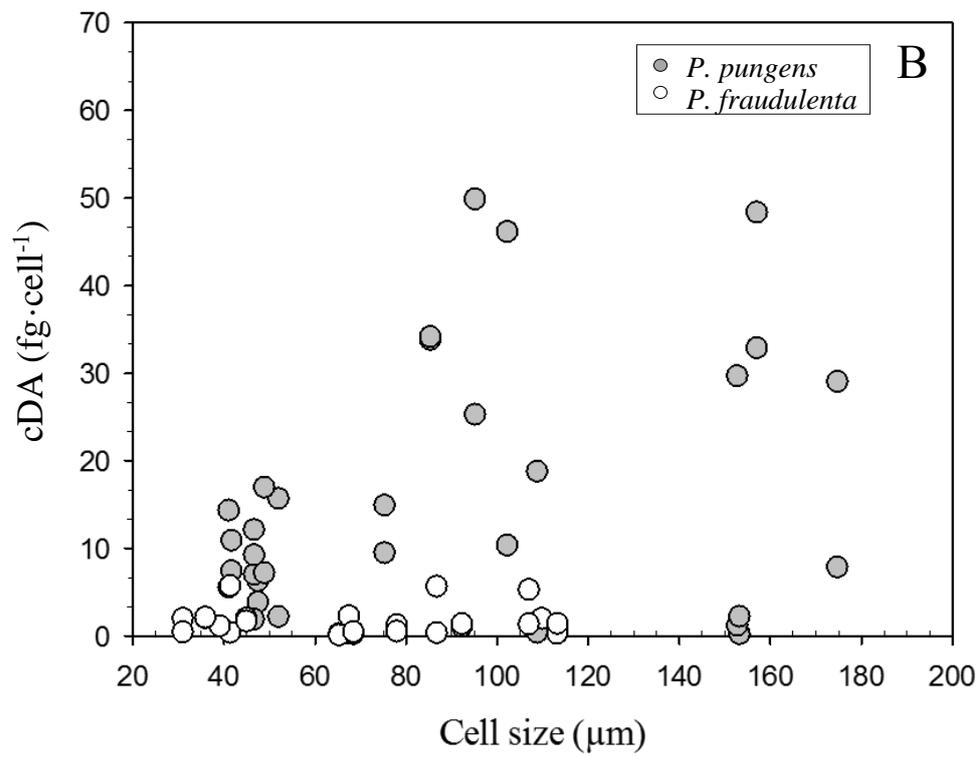
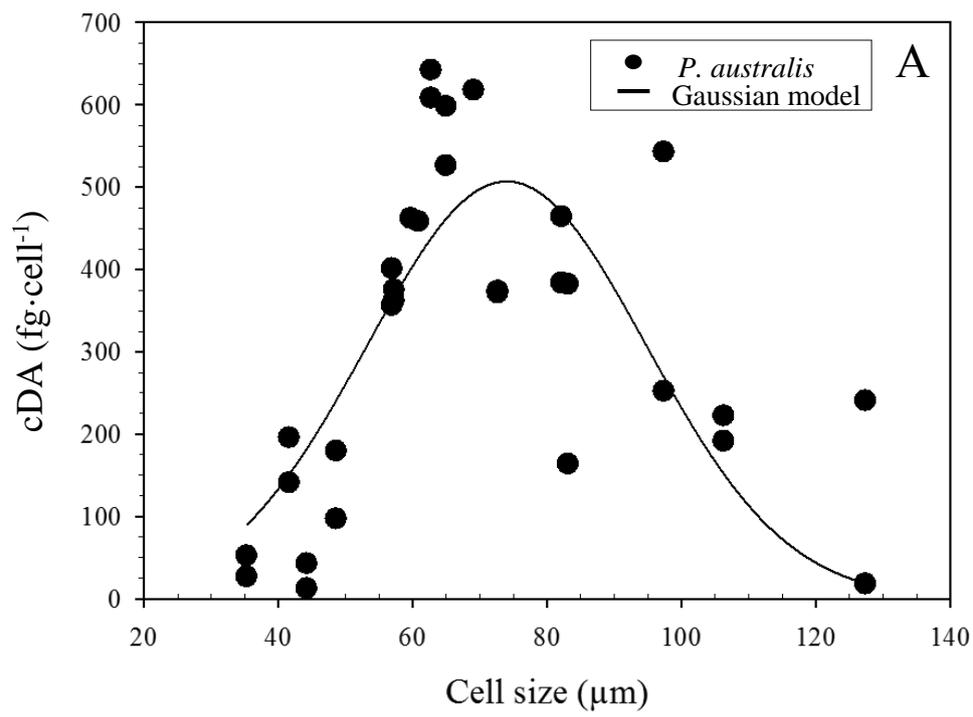
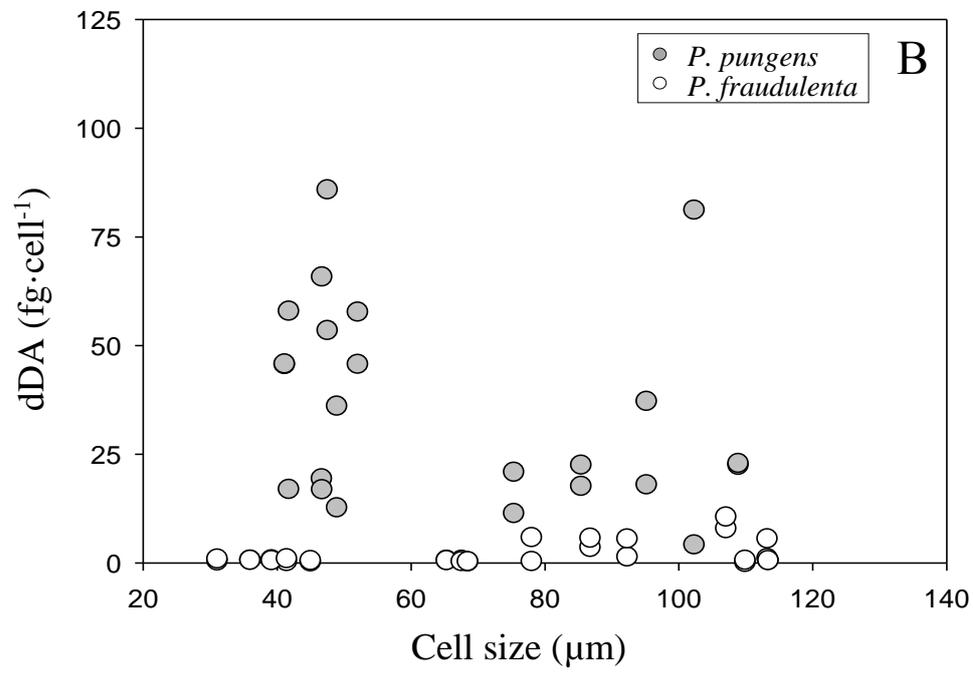
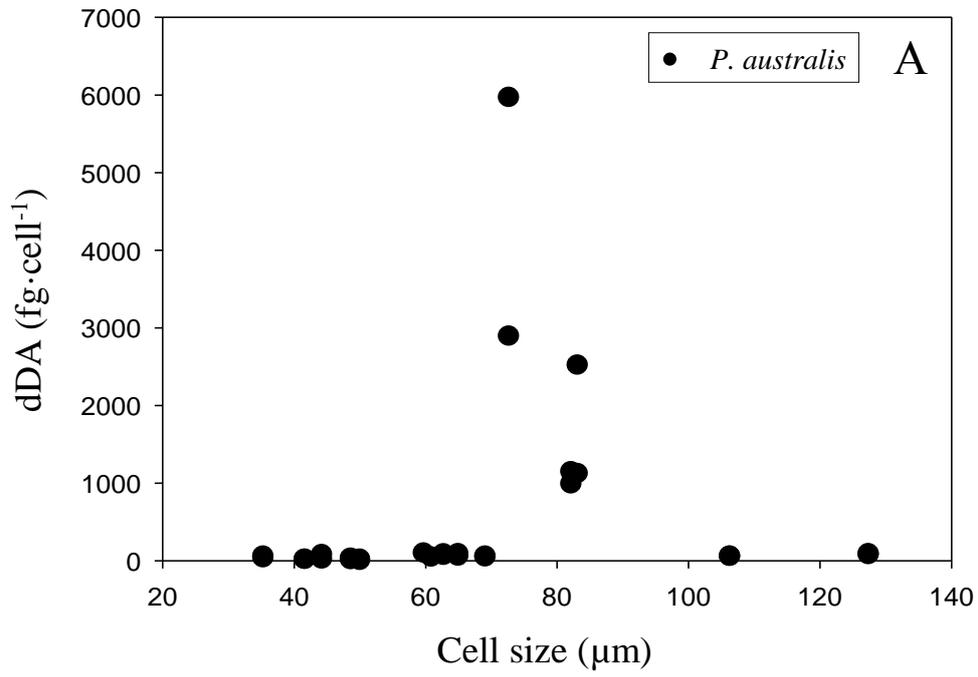
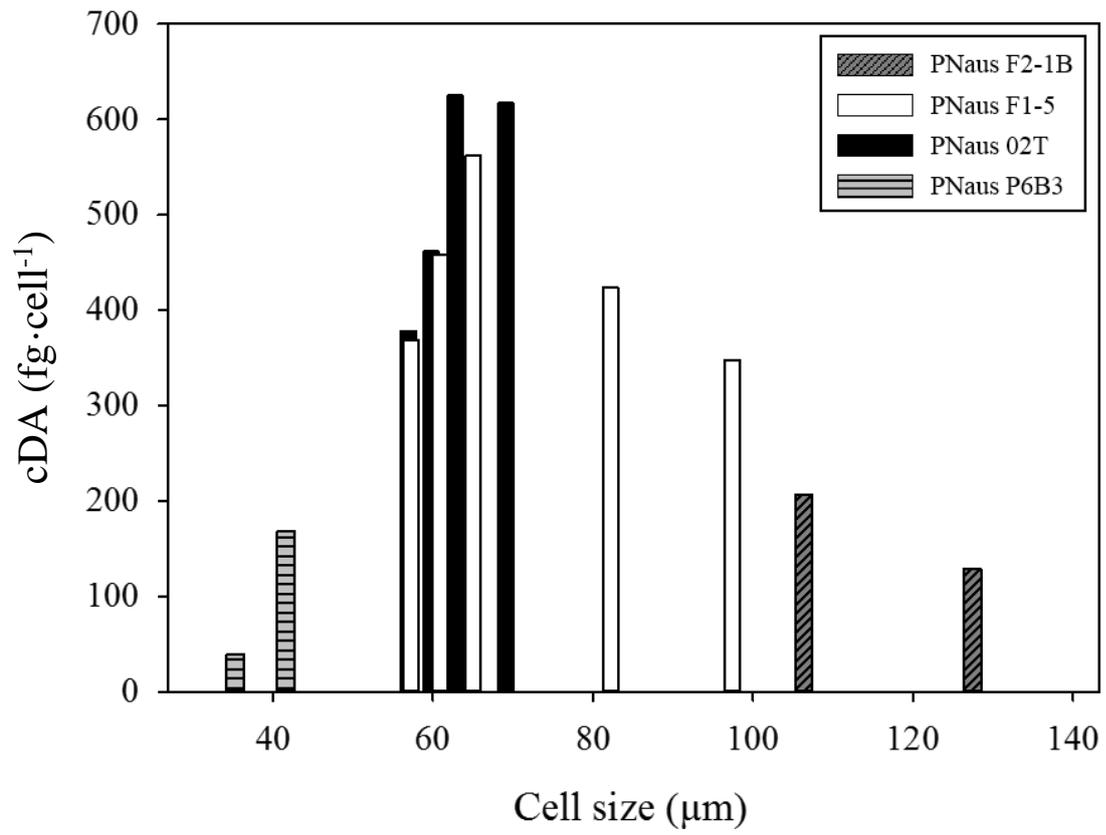


Figure 2











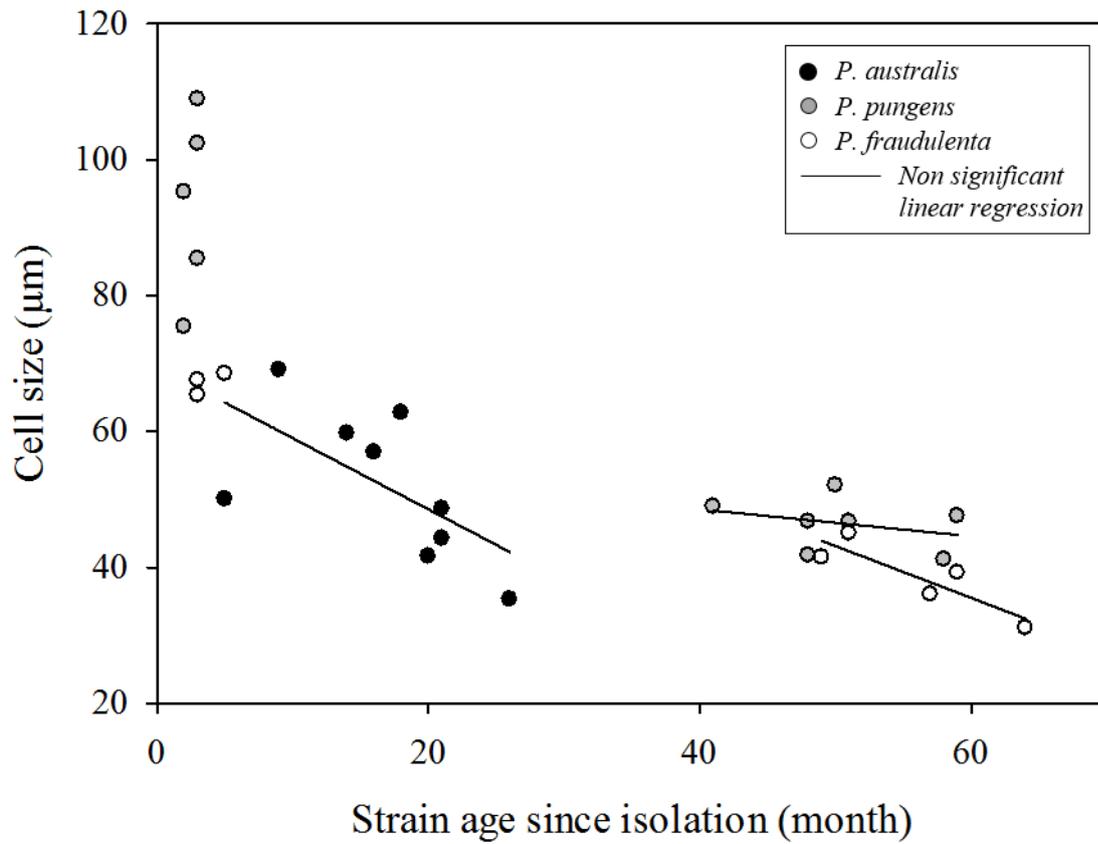


Figure S1. Relationship between cell size and strain age (month) since their isolation from the natural environment in *P. australis*, *P. pungens* and *P. fraudulenta* (F1 strains are not taken into account). Linear regressions are not significant ($P > 0.05$).

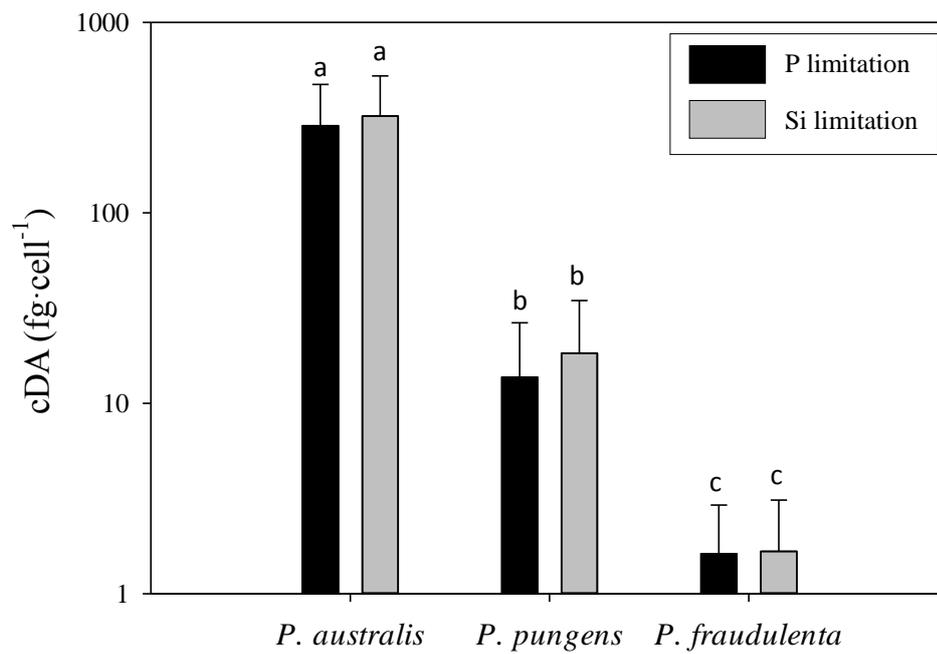


Figure S3. Cellular domoic acid content (cDA, fg·cell⁻¹, mean ± SD) under phosphate limitation (black bars) or silicate limitation (grey bars) in *P. australis* (n = 18), *P. pungens* (n = 16), and *P. fraudulenta* (n = 16). The letters a, b and c indicate the significant differences (P < 0.001).

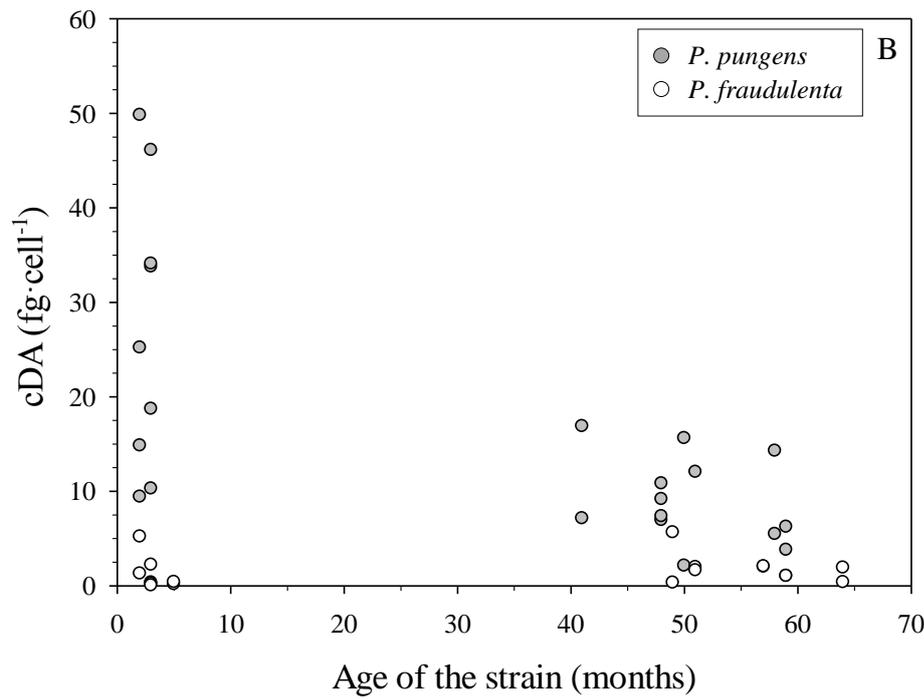
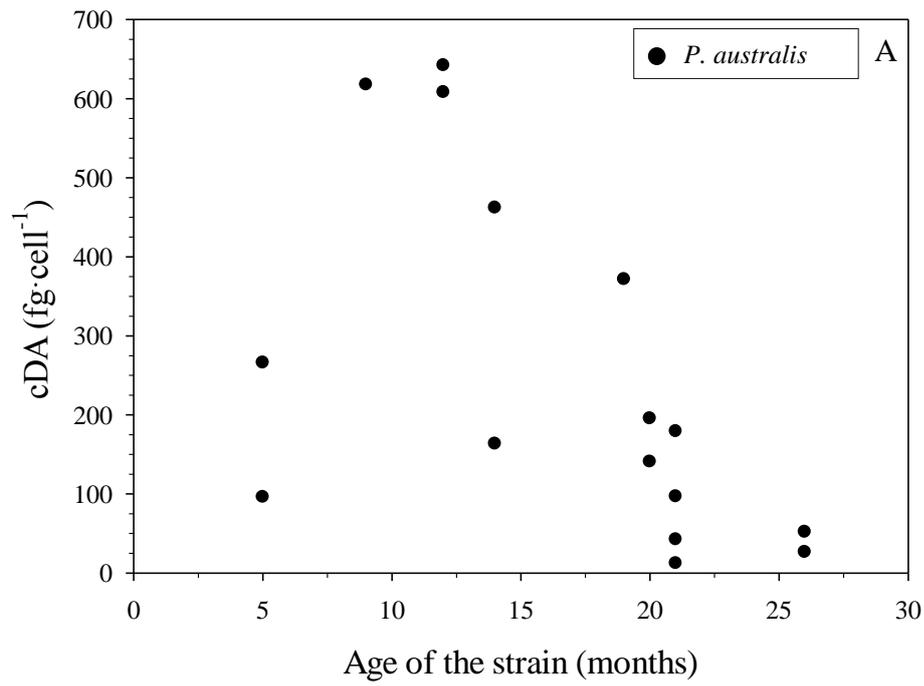


Figure S4. Cellular domoic acid content (cDA, fg·cell⁻¹) as a function of the age of the strain (months) since their isolation from natural populations: (A) in *P. australis* (n = 9) and (B) in *P. pungens* (n = 12) and *P. fraudulenta* (n = 9).

1 FIGURES LEGENDS

2

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4 *P. pungens*, and *P. fraudulenta*. Standard deviations represent the minimum and maximum
5 sizes measured for initial cells and represent the variance for parental cells.

6

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8 *P. australis*, *P. pungens*, and *P. fraudulenta*. Crosses and dots correspond to cell sizes for
9 which mating experiments were performed. Crosses: no sexual reproduction observed.

10 Hatched pattern and white dots: incomplete sexual reproduction observed (without initial cell
11 production). Light grey and dark grey dots: complete sexual reproduction observed (with
12 initial cell production). The size percentage was calculated based on the maximal initial size.
13 85 mating experiments were made to define the gametangia size range for *P. australis*, 40 for
14 *P. pungens*, and 89 for *P. fraudulenta*. 193 cells were measured to determine the vegetative
15 cell size range for *P. australis*, 334 cells for *P. pungens*, and 551 cells for *P. fraudulenta*.

16

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18 isolation from natural populations in *P. australis* (n = 9), *P. pungens* (n = 12), and *P.*
19 *fraudulenta* (n = 9) (F1 strains from sexual reproduction are not present in this graph). (B)
20 Growth rate (μ , day⁻¹) as a function of the cell size (μm) for all strains studied in *P. australis*
21 (n = 18), *P. pungens* (n = 16), and *P. fraudulenta* (n = 16).

22

23 *Figure 4.* Cellular domoic acid content (cDA, fg.cell⁻¹) as a function of cell size (μm): (A) in
24 *P. australis* and (B) in *P. pungens* and *P. fraudulenta*. Data come from the cDA
25 measurements made in the batch experiments on the second day of the stationary phase linked
26 to a phosphate or silicate limitation.

27

28 *Figure 5.* Dissolved domoic acid (dDA, fg.cell⁻¹) as a function of cell size (μm): (A) in *P.*
29 *australis* and (B) in *P. pungens* and *P. fraudulenta*. Data come from the dDA measurements
30 made in the batch experiments on the second day of the stationary phase linked to a phosphate
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32

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35

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39 with abnormal valve shapes in small cells.

40

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42 natural environment in *P. australis* (n = 9), *P. pungens* (n = 12), and *P. fraudulenta* (n = 9)
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44

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46 limitation (black bars) or silicate limitation (grey bars) in *P. australis* (n = 18), *P. pungens* (n
47 = 16), and *P. fraudulenta* (n = 16). The letters a, b and c indicate the significant differences (P
48 < 0.001).

49

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51 (months) since their isolation from natural populations: (A) in *P. australis* (n = 9) and (B) in
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53

54 *Figure S5*. Dissolved domoic acid (dDA, fg.cell⁻¹) as a function of the age of the strain
55 (months) since their isolation from natural populations: (A) *P. australis* (n = 9) and (B) in *P.*
56 *pungens* (n = 12) and *P. fraudulenta* (n = 9).

57

Species	Strain information			Age of strain (month)	Cell apical length (µm)	
	Collection reference	Origin (Sampling zone or crossing)	Isolation date			
<i>P. australis</i>	IFR-PAU-010	Ouessant (A)	07/2015	5	50	
	PNaus F1D2	Camaret-sur-Mer (A)	03/2014	21	44	
	PNaus P3B2	Plouzané (A)	03/2014	21	49	
	IFR-PAU-16.2	Arcachon Bay (A)	05/2016	9 - 12 - 14 - 16	69 - 63 - 60 - 57	
	PNaus P6B3	Plouzané (A)	04/2014	20 - 26	42 - 35	
	PNaus F1-1A	P6B3 x P2B1			83	
	PNaus F1-4A	P6B3 x P6C1			73	
	PNaus F1-5	P6B3 x P1A3			97 - 82 - 65 - 61 - 57	
	PNaus F2-1B	F1-5 x F1-1A			127 - 106	
<i>P. pungens</i>	PNpun 47	Cabourg (EC)	08/2011	59	48	
	PNpun 66	Ouistreham (EC)	09/2011	51 - 58	41 - 47	
	PNpun 79	Cabourg (EC)	05/2012	50	52	
	PNpun 88	Cabourg (EC)	07/2012	48	47	
	PNpun 89	Cabourg (EC)	07/2012	41 - 48	42 - 49	
	PNpun 129	Luc-sur-mer (EC)	07/2016	2	75	
	PNpun 130	Luc-sur-mer (EC)	07/2016	2	95	
	PNpun 133	Luc-sur-mer (EC)	07/2016	3	109	
	PNpun 134	Luc-sur-mer (EC)	07/2016	3	102	
	PNpun 136	Saint-Vaast-la-Hougue (EC)	07/2016	3	85	
		PNpun F1-7A	66 x 89			157 - 175
		PNpun F1-7B	66 x 89			153
		PNpun F1-11	66 x 47			153
<i>P. fraudulenta</i>	PNfra 1	Cabourg (EC)	08/2011	62	27	
	PNfra 10	Cabourg (EC)	08/2011	51 - 59	45 - 39	
	PNfra 30	Cabourg (EC)	10/2011	49 - 57	41 - 36	
	PNfra 31	COMOR 41 (EC)	07/2011	64	31	
	PNfra 126	Luc-sur-mer (EC)	11/2015	3	65	
	PNfra 132	Luc-sur-mer (EC)	10/2015	3	68	
	PNfra 162	Saint-Vaast-la-Hougue (EC)	05/2016	5	69	
		PNfra F1-8D	30 x 31			107 - 87
		PNfra F1-9A	10 x 31			78
		PNfra F1-9B	10 x 31			113 - 92
		PNfra F1-14A	10 x 1			110
		PNfra F1-14B	10 x 1			113