This document is a postprint version of an article published in Harmful Algae, copyright © Elsevier after peer review. To access the final edited and published work see http://dx.doi.org/10.1016/j.hal.2012.06.001

1 **Protein phosphatase inhibition assays for okadaic acid detection in**
2 **shellfish: matrix effects, applicability and comparison with LC-MS/MS**
3 **analysis**
4
5 Diana Garibo[1], Esther Dàmaso[1], Helena Eixarch[1], Pablo de la Iglesia[1],
6 Margarita Fernández-Tejedor[1], Jorge Diogène[1], Yolanda Pazos[2], Mònica
7 Campàs[1]*
8
9 [1] IRTA, Carretera de Poble Nou, km 5.5, 43540 Sant Carles de la Ràpita, Spain
10 [2] INTECMAR, Peirao de Vilaxoán, s/n, 36611 Vilagarcía de Arousa, Spain
11
12
13 **Corresponding author:** Mònica Campàs
14
15 IRTA
16 Carretera de Poble Nou, km 5.5
17 43540 Sant Carles de la Ràpita, Spain
18 Tel.: +34 902 789 449 (ext. 1842); Fax: +34 977 744 138
19 E-mail address: monica.campas@irta.cat
20
21
22
23
24
25

26 **Abstract**

27 The applicability of the protein phosphatase inhibition assay (PPIA) to the
28 determination of okadaic acid (OA) and its acyl derivatives in shellfish samples
29 has been investigated, using a recombinant PP2A and a commercial one.
30 Mediterranean mussel, wedge clam, Pacific oyster and flat oyster have been
31 chosen as model species. Shellfish matrix charge limits for the PPIA have been
32 established, according to the shellfish species and the enzyme source. A
33 synergistic inhibitory effect has been observed in the presence of OA and
34 shellfish matrix, which has been overcome by the application of a correction
35 factor (0.48). Finally, Mediterranean mussel samples obtained from Ría de
36 Arousa during a DSP closure associated to *Dinophysis acuminata*, determined
37 as positive by the mouse bioassay, have been analysed with the PPIAs. The
38 OA equivalent contents provided by the PPIAs correlate satisfactorily with those
39 obtained by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

40

41 ***Keywords:*** recombinant protein phosphatase 2A (PP2A) catalytic subunit,
42 protein phosphatase inhibition assay (PPIA), okadaic acid (OA), liquid
43 chromatography-tandem mass spectrometry (LC-MS/MS), shellfish.

44

45

46

47

48

49

50

51 ## 1. Introduction

52 Okadaic acid (OA) and the dinophysistoxins (DTXs) derivatives are lipophilic
53 marine toxins produced by microalgae of the *Dinophysis* and *Prorocentrum*
54 genera (Steidinger, 1993), usually found in shellfish. The consumption of
55 shellfish contaminated by OA and some DTXs causes diarrheic shellfish
56 poisoning (DSP), human illness characterised by gastro-intestinal symptoms
57 including diarrhoea, nausea and vomiting (Yasumoto and Murata, 1993).
58 Diarrheic lipophilic toxins are known to inhibit the activity of several
59 serine/threonine protein phosphatases (PPs) (Bialojan and Takai, 1988). These
60 toxins bind to PP in the hydrophobic region near to the active site, blocking their
61 activity. As a consequence, hyperphosphorylation of the proteins that control
62 sodium secretion by intestinal cells and of cytoskeletal or junctional moieties
63 that regulate solute permeability is favoured, causing a sodium release and a
64 subsequent passive loss of fluids, responsible for the diarrheic symptoms.
65 DSP toxic episodes are recurrent in coastal waters of European countries.
66 Specifically in Spain, Galicia and Catalonia are very affected regions (Reguera
67 et al., 2012). In order to protect public health, the Commission Regulation (EC)
68 No 853/2004 has established a maximum permitted level (MPL) of 160 µg of
69 OA equivalents/kg in bivalve molluscs. Until recently, the official control method
70 was the mouse bioassay (MBA) (Yasumoto et al., 1978). This method has been
71 successful for the management of shellfish controls because it gives an
72 indication of the total toxicity of a sample. However, because of its low
73 specificity and the controversy of their application, the Commission Regulation
74 (EC) No 15/2011 has recently approved a liquid chromatography-tandem mass
75 spectrometry (LC-MS/MS) method as the reference method for the detection of

76 lipophilic toxins in live bivalve molluscs. In Europe, this new regulation is applied
77 since 1st July 2011 ant the use of the MBA is possible until 31st December 2014.
78 This Commission Regulation also allows a series of methods, such as other
79 chromatographic techniques with appropriate detection, immunoassays and
80 functional (e.g. phosphatase inhibition) assays, as alternatives or
81 supplementary to the LC-MS/MS method, provided that either alone or
82 combined they can detect the required analogues, that they fulfil the
83 corresponding method performance criteria, and that their implementation
84 provides an equivalent level of public health protection.
85 Based on the OA mechanism of action, protein phosphatase inhibition assays
86 (PPIAs) for the determination of DSP toxins have been developed. Although
87 radioactivity (Honkanen et al., 1996) and fluorescence detection has been used
88 (Tsuchiya et al., 1997; Vieytes et al., 1997; Mountfort et al., 1999; Leira et al.,
89 2000; Mountfort et al., 2001; González et al., 2002), the colorimetric detection
90 has received more acceptance (Tubaro et al. 1996; Della Loggia et al. 2000;
91 Campàs and Marty, 2007; Albano et al., Rossini, 2009; Caillaud et al., 2010;
92 Cañete et al., 2010).
93 This functional method has gained much interest because its advantages in
94 terms of simplicity of the protocol, multiple-sample analysis, enough sensitivity
95 and limit of detection (LOD) of the assay, and short analysis time. As a
96 consequence, researchers are trying to improve and refine PPIAs from different
97 perspectives. Undesirable shellfish and microalgae matrix effects have been
98 diminished by the use of sample purification strategies, such us a previous
99 chromatographic fractionation protocol (Caillaud et al., 2010; Cañete et al.,
100 2010). The use of recombinant enzymes (Ikehara et al., 2010), some of them

101 with improved sensitivity (Zhang et al., 1994), has also been exploited. The
102 establishment of toxicity equivalent factors (TEFs) in alternative methods to
103 MBA for marine toxin detection is necessary to guarantee consumer protection
104 in monitoring programmes, as they allow a better estimation of the toxic
105 potential of a mixture of toxins with different potency (Botana et al., 2010). With
106 regards to this, the inhibitory potencies of different OA derivatives should be
107 investigated. Nevertheless, in this work OA has been taken as reference
108 compound of the diarrheic lipophilic toxins group.
109 The development of a PPIA using a recombinant PP2A catalytic subunit has
110 already been described in a previous work, where the genetically-engineered
111 enzyme was compared with a commercial one in terms of activity, stability and
112 inhibition by OA (Garibo et al., 2012). In the present work, an exhaustive
113 evaluation of the matrix charge effect on the PP2A activity has been performed
114 in order to apply the developed PPIA to the determination of OA and its acyl
115 derivatives in shellfish samples. Results have also been compared with those
116 obtained with a commercial PP2A. Mediterranean mussel, wedge clam, Pacific
117 oyster and flat oyster have been chosen as model shellfish species. Samples
118 determined as negative by the MBA for DSP toxins (toxin content lower than
119 160 μg OA eq/kg) and by LC-MS/MS analysis (diarrheic lipophilic toxin content
120 lower than the limit of quantification, LOQ = 30 μg OA/kg shellfish meat) have
121 been used for the study of the matrix effects. Once the matrix charge limits
122 established, the PPIA has been applied to the analysis of naturally-
123 contaminated Mediterranean mussels and results have been compared to those
124 obtained by LC-MS/MS analysis.
125

126 ## 2. Materials and Methods

127 ### *2.1 Reagents and materials*

128 Certified Reference Material of okadaic acid (CRM-OA) in methanol was
129 purchased from the Institute for Marine Biosciences of the National Research
130 Council (Halifax, Canada). The genetically-engineered PP2A catalytic subunit
131 was produced by Gene to Protein (GTP) Technology (Toulouse, France) and
132 contains a hexa-His tail at the C-terminus. A commercial protein phosphatase
133 2A (PP2A), isolated as the heterodimer of 60 kDa and 36 kDa subunits from
134 human red blood cells, was obtained from Upstate Biotechnology (New York,
135 USA). The activity of the stock solutions was between 766 and 1364 U/mL for
136 GTP Technology and 5,660 U/mL for Upstate Biotechnology, 1 U being defined
137 as the amount of enzyme required to hydrolyze of 1 nmol *p*-nitrophenyl
138 phosphate (*p*-NPP) in one min at room temperature. Components of buffers and
139 *p*-NPP were purchased from Sigma (Tres Cantos, España). For LC-MS/MS
140 analysis, gradient-grade methanol, formic acid and hyper-grade acetonitrile
141 were purchased from Merck (Darmstadt, Germany). Ammonium formate
142 (≥99.995%), sodium hydroxide pellets (≥99%) and hydrochloric acid 37% for
143 analysis were purchased from Sigma-Aldrich (St. Louis, MO, USA), Riedel-de
144 Haën (Seelze, Germany) and Panreac (Barcelona, Spain), respectively. All
145 solutions were prepared using Milli-Q grade water obtained from a Millipore
146 purification system (Bedford, USA).

147

148 ### *2.2 Shellfish samples*

149 Three negative Mediterranean mussel (*Mytilus galloprovincialis*) samples
150 according to LC-MS/MS analysis were obtained from Ebro Delta, Catalonia (NW

151 Mediterranean, Spain) in August, September and December 2008. Two
152 negative wedge clam (*Donax trunculus*) samples according to MBA for DSP
153 toxins and LC-MS/MS analysis were obtained from the shellfish monitoring
154 programme of Catalonia (Ebro Delta and Vilanova i la Geltrú) in October and
155 November 2009. Three negative Pacific oyster (*Crassostrea gigas*) samples
156 according to MBA for DSP toxins and LC-MS/MS analysis were obtained from
157 the shellfish monitoring programme of Catalonia (Ebro Delta) in January and
158 February 2010. Four negative flat oysters (*Ostrea edulis*) were used, two of
159 them provided by *Ostres de la Badia* (Santa Pola, Alicante, SW Mediterranean,
160 Spain) in January and June 2010, according to LC-MS/MS analysis, and the
161 other two obtained from the shellfish monitoring programme of Catalonia (Ebro
162 Delta) in December 2009 and January 2010, negative according to MBA for
163 DSP toxins and LC-MS/MS analysis.

164 Twelve positive Mediterranean mussel (*Mytilus galloprovincialis*) samples
165 according to MBA for DSP toxins (Yasumoto et al., 1978) and to LC-MS/MS
166 analysis were provided by *Amegrove* (O Grove, Spain) from Galicia (NE Atlantic
167 Ocean, Spain) in August 2010. These samples were obtained from different 4
168 rafts (C1, C2, C3, C4) at 1, 5 and 10 m depth during a DSP closure in Ría de
169 Arousa, inforced between July 2010 and November 2010 by INTECMAR
170 (INTECMAR, 2010). The samples were available because of the Amegrove's
171 own-checks on biotoxins. Integrated phytoplankton samples (0-15 m) were
172 collected from stations A0 (42º28'54''N, 08º57'48''W), at the entrance of the Ría
173 de Arousa, and A8 (42º29'48''N, 08º55'36''W), close to the rafts, using a PVC
174 hose and preserved with Lugol's solution. The Utermöhl method was used for
175 phytoplankton identification and quantification (Utermöhl, 1958): 25 mL of

176 sample were settled during 12 hours. The chamber was examined for
177 quantification of *Dinophysis* species and total phytoplankton.
178

179 ### *2.3 Lipophilic toxins extraction*

180 Crude extracts corresponded to shellfish samples (aliquot of 2 g from a flesh
181 homogenate of 100 g) blended with a hand-held homogeniser and extracted
182 with 9 mL of MeOH for 2 min at 17,500 rpm with an Ultra-Turrax® T25 Digital by
183 IKA® from Rose Scientific Ltd. (Alberta, Canada). Extracts were centrifuged at
184 3,000 rpm for 10 min in a Jouan centrifuge at room temperature. Supernatants
185 were removed. A second extraction was performed by the addition of 5 mL of
186 MeOH to the solid residue and 3-min agitation with a vortex. After centrifugation
187 under the same conditions, the two supernatants were joined and passed
188 through a 0.2-μm cut-off Whatman nylon membrane filter (Brentford, United
189 Kingdom). Samples were directly injected into the LC-MS/MS system. For
190 samples to be tested with the PPIA, extracts were evaporated in a Speed VAC
191 concentrator (Organomation Associates, Inc., Berlin, USA) under nitrogen at
192 room temperature, and the residues were resuspended in a buffer solution
193 containing 30 mM Tris-HCl, 20 mM $MgCl_2$, pH 8.4.
194

195 ### *2.4 Sample hydrolysis*

196 The protocol for the hydrolysis of lipophilic toxins in mussels was based on that
197 of Mountfort et al., 2001. Briefly, 125 μL of NaOH at 2.5 N were added to 1.25
198 mL of extract and homogenised for 10 seconds with a vortex. Extracts were
199 then incubated at 76 °C for 40 min in a Multi-Block®Heater from Lab-Line
200 Instruments, Inc. (Maharashtra, India). After cooling, 125 μL of HCl at 2.5 N

201 were added and hydrolysed extracts were passed through a 0.2-µm cut-off
202 Whatman nylon membrane. Samples were directly injected into the LC-MS/MS
203 system. For samples to be tested with the PPIA, extracts were evaporated in a
204 Speed VAC concentrator under nitrogen at room temperature, and the residues
205 were resuspended in a buffer solution containing 30 mM Tris-HCl, 20 mM
206 $MgCl_2$, pH 8.4.
207
208 ***2.5 Colorimetric PPIA***
209 The colorimetric PPIA was performed as follows: 50 µL of OA solutions (for the
210 calibration curves), shellfish extracts (for the evaluation of the matrix effects
211 from negative samples or the determination of toxins in positive samples) or
212 both (for the evaluation of a possible synergistic effect) at different
213 concentrations (starting at 100 µg/L for OA, 200 mg/mL for negative shellfish
214 samples and at the matrix charge limit for positive mussel samples, and diluting
215 to the half) were added in microtiter wells containing 100 µL of enzyme solution
216 at 1.25 U/mL). Then, 50 µL of 25 mM *p*-NPP solution were added and after 1-
217 hour incubation at room temperature in the dark, the absorbance at 405 nm was
218 measured with an automated multi-well scanning spectrophotometer (Biotek,
219 Synergy HT, Winooski, Vermont, USA). OA and shellfish extract solutions were
220 prepared in a buffer solution containing 30 mM Tris-HCl, 20 mM $MgCl_2$, pH 8.4.
221 Enzyme and substrate solutions were prepared in the same buffer, also
222 containing 2 mM DTT and 0.2 mg/mL BSA. Controls without PP2A, OA or
223 mussel extract were always used. Assays were performed in triplicate.
224 It is necessary to mention that the sensitivity of the assay is strongly affected by
225 the protein phosphatase activity, and small enzyme activity variations may

226 significantly change the inhibition percentages. Consequently, an OA calibration
227 curve was always performed in parallel to each positive mussel sample analysis
228 for the precise toxin quantification. The OA calibration curves obtained by PPIA
229 were analysed with SigmaPlot software package 10.0 (Systat Software, Inc.,
230 San José, California, USA) and fitted to sigmoidal logistic four-parameter
231 equations.

232

233 ***2.6 LC-MS/MS analysis***

234 Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis were
235 conducted on an Agilent 1200 LC (Agilent Technologies, Santa Clara, USA)
236 coupled with a 3200 QTRAP mass spectrometer through a TurboV^{TM}
237 electrospray ion source (Applied Biosystems, Foster City, USA).
238 Chromatographic separations were performed at 30 °C and 0.2 mL/min on a
239 Luna C8(2) column (50 mm × 1 mm, 3 μm) protected with a SupelcoGuard
240 C8(2) cartridge (4 mm × 2 mm, 3 μm), both from Phenomenex (Torrance, USA).
241 Other conditions followed the Harmonised Standard Operation Procedure
242 (SOP) for LC-MS/MS analysis of lipophilic toxins proposed by the European
243 Reference Laboratory (EURLMB, 2011), recently validated and recognised as
244 the reference method for the analysis of this group of toxins. Acidic
245 chromatographic elution was selected with mobile phases 100% water (A) and
246 95% acetonitrile (B), both containing 2 mM ammonium formate and 50 mM
247 formic acid. Other conditions related with MS/MS detection were optimised
248 directly through direct infusion of the CRM-OA standard. For DSP toxins,
249 multiple reaction monitoring (MRM) analysis was accomplished from the
250 precursor ions 803.5 and 817.5 *m/z* for OA/DTX-2 and DTX-1, respectively.

251 Product ions were common for all DSP toxins, with ions 255.2 *m/z* used for
252 quantification and 113.1 or 209.2 *m/z* used for confirmatory purposes. The LOD
253 and LOQ were at least 10 and 30 µg/kg OA in shellfish, respectively.
254

255 ***2.7 Statistical analyses***

256 To evaluate differences in the matrix charge effect on the PP2A activity for the
257 four shellfish species (non-hydrolysed and hydrolysed Mediterranean mussel,
258 wedge clam, Pacific oyster and flat oyster) and the two enzymes (GTP and
259 Upstate), the PP2A inhibition percentage was compared between both enzymes
260 for each species, and between species for each enzyme, using a paired *t*-test at
261 the different shellfish matrix charge levels. The paired *t*-test was also used to
262 evaluate differences in the PP2A inhibition when spiking buffer, non-hydrolysed
263 and hydrolysed Mediterranean mussel samples, for both enzymes. Differences
264 in the results were considered statistically significant at the 0.05 level. Prior to
265 analysis, data were tested for normality; Wilcoxon matched-pairs signed-ranks
266 test was used for non-normally distributed data sets instead of the paired *t*-test.
267 One-way analysis of variance was used to detect possible differences between
268 correction factors for each PP2A (GTP and Upstate) and Mediterranean mussel
269 sample (non-hydrolysed and hydrolysed) pair. The two-way ANOVA test was
270 also used to evaluate differences among sampling stations and depths in the
271 free and total DSP contents determined by LC-MS/MS analysis. The SigmaStat
272 software was used for the paired *t*-tests and the two-way ANOVA tests.
273 To describe how PP2A inhibition changes with DSP toxin content in shellfish
274 samples, and to predict the DSP toxin content (that would be determined by LC-
275 MS/MS) for a given value of PP2A inhibition, different non-linear regression

276 models were tested using the program Statgraphics Centurion XVI. The models
277 were adjusted to each of the 4 sets of results: free DSP and total DSP content
278 for each of the 2 enzymes *versus* the value determined by LC-MS/MS. Each set
279 of results contains 12 data points. The regression was considered statistically
280 significant at the 95% confidence level.

281

282 ## 3. Results

283 ### *3.1 Shellfish matrix charge effect on the PP2A activity*

284 Mediterranean mussel, wedge clam, Pacific oyster and flat oyster were chosen
285 as model shellfish species for the applicability study. As previously mentioned,
286 samples determined as negative by LC-MS/MS analysis (diarrheic lipophilic
287 toxin content lower than the LOQ = 30 μg OA and derivatives/kg) and in some
288 cases also by MBA for DSP toxins (toxin content lower than 160 μg OA eq/kg)
289 were chosen to evaluate the effect of the sample matrix on the enzyme activity.
290 Experiments were performed with a recombinant PP2A (from GTP) and a
291 commercial PP2A (from Upstate) in order to compare their robustness to matrix
292 effects. The PPIA was performed as usually, only changing the OA standard
293 solution by shellfish matrix with no toxin content. Figure 1 shows the PP2A
294 inhibition percentage at different shellfish matrix concentrations. A 10%
295 inhibition threshold was established, lower inhibition percentages being
296 considered as non-significant.

297 As expected, the same pattern was observed in all cases: the enzyme activity
298 was not affected at low matrix concentrations and started to increase
299 exponentially at high matrix concentrations. This matrix effect is not due to the
300 colour of the shellfish samples (controls without enzyme are performed in

301 parallel in order to subtract the corresponding absorbance value), but to the
302 presence of compounds that alter the natural environment of the enzyme and
303 thus its functionality.
304 There were statistically significant differences in the inhibition percentages
305 between enzymes for each species (Mediterranean mussel: $t = 2.6$, $p = 0.02$, df
306 = 10; wedge clam: $t = 2.9$, $p = 0.01$, df = 10; Pacific oyster: $t = 2.3$, $p = 0.04$, df =
307 10; flat oyster: $t = 5.6$, $p = 0.001$, df = 10), PP2A from GTP being able to
308 operate under higher shellfish matrix concentrations.
309 There were statistically significant differences in the inhibition percentages
310 between species for PP2A from GTP except for the wedge clam-Pacific oyster
311 pair (Mediterranean mussel-wedge clam: $t = -3.4$, $p = 0.01$, df = 10;
312 Mediterranean mussel-Pacific oyster: $W = 56$, $p = 0.01$; Mediterranean mussel-
313 flat oyster: $W=-64$, $p = 0.01$; wedge clam-Pacific oyster: $W = 24$, $p = 0.32$;
314 wedge clam-flat oyster: $t = 3.7$, $p = 0.01$, df = 10; Pacific oyster-flat oyster: t =
315 2.6, $p = 0.03$, df = 10). There were not statistically significant differences in the
316 inhibition percentages between species for PP2A from Upstate except for the
317 Mediterranean mussel-flat oyster pair (Mediterranean mussel-wedge clam: t =
318 1.3, $p = 0.21$, df = 10; Mediterranean mussel-Pacific oyster: $t = -0.7$, $p = 0.47$, df
319 = 10; Mediterranean mussel-flat oyster: $W = -18$, $p = 0.46$; wedge clam-Pacific
320 oyster: $t = -1.9$, $p = 0.08$, df = 10; wedge clam-flat oyster: $t = 1.5$, $p = 0.16$, df =
321 10; Pacific oyster- flat oyster: $t = 1.8$, $p = 0.10$, df = 10). The level of confidence
322 used to determine significance was 95% ($p < 0.05$). Taking into consideration
323 the inhibition percentages observed at the three highest matrix charges, it is
324 possible to list shellfish species from higher to lower matrix effect: "Pacific
325 oyster > wedge clam > Mediterranean mussel > flat oyster" for PP2A from GTP

326 and “Mediterranean mussel > Pacific oyster > wedge clam > flat oyster” for
327 PP2A from Upstate.
328 In the case of the Mediterranean mussel, the effect of hydrolysed extracts was
329 also evaluated in order to study the applicability of the developed PPIAs to the
330 determination of total DSP toxin content (including OA acyl derivatives) (Figure
331 1.A). There were statistically significant differences between hydrolysed and
332 non-hydrolysed samples for PP2A from Upstate ($W = 66$, $p < 0.001$) but not for
333 PP2A from GTP ($t = -1.1$, $p = 0.26$, $df = 9$). There were statistically significant
334 differences between enzymes for hydrolysed ($t = -4.6$, $p = 0.001$, $df = 9$) and
335 non-hydrolysed mussel samples ($t = 2.6$, $p = 0.02$, $df = 10$). Whereas the effect
336 of hydrolysed mussel samples was minor for the recombinant PP2A, the
337 commercial enzyme was drastically affected.
338 From results obtained in Figure 1, standardised matrix charge limits in view of
339 application in routine monitoring programmes were established depending on
340 the enzyme source and the shellfish species (Table 1).
341

342 ***3.2 OA calibration curves by PPIA***

343 As previously mentioned, a calibration curve was always performed in parallel
344 to each positive mussel sample analysis, due to possible slight differences in
345 the inhibition percentages from assay to assay. The OA calibration curves were
346 described by the sigmoidal logistic four-parameter equation (SigmaPlot software
347 package 10.0):

$$y = y_0 + \frac{a}{1+(x/x_0)^b}$$

348

349 where a and y_0 are the asymptotic maximum and minimum values, respectively,
350 x_0 is the x value at the inflection point and b is the slope at the inflection point.
351 Taking into account the 10% inhibition values of OA calibration curves and the
352 established charge limits, theoretical limits of detection (LODs) were calculated.
353 In Table 1 the LODs for both enzymes combined with the different shellfish
354 matrices, including hydrolysed mussel, are shown.

355

356 ***3.3 Analysis of positive mussel samples by PPIA and LC-MS/MS***

357 The PPIAs with recombinant and commercial PP2A were applied to the
358 determination of free and total DSP toxins in twelve non-hydrolysed and
359 hydrolysed Mediterranean mussel samples, respectively. In the determination of
360 OA equivalent contents in mussels, the IC_{50} values of the OA calibration curves
361 determined from the sigmoidal logistic four-parameter equations and the IC_{50}
362 values of the mussel extract dose-response curves determined from lineal
363 regressions were used. The IC_{50} values of the mussel extract curves were
364 determined from lineal regressions because some samples (those with low toxin
365 contents) did not attain the sigmoidal shape (*results not shown*). In those
366 samples where the highest mussel charge induced an inhibition percentage
367 lower than 50%, the determination of OA equivalent contents was performed
368 taking into account the inhibition percentage obtained at that highest mussel
369 charge (and the corresponding inhibition percentage of the OA calibration
370 curve). In the case of the analysis of hydrolysed mussel samples with PP2A
371 from Upstate, where all concentrations tested were higher than the matrix
372 charge limit, the determination of OA equivalent contents was performed taking
373 into account the inhibition percentage obtained at the lowest mussel charge

374 (and the corresponding inhibition percentage of the OA calibration curve). Table
375 2 shows the OA content estimates obtained by LC-MS/MS analysis and by the
376 PPIA with both enzymes. According to LC-MS/MS analysis, there were not
377 statistically significant differences among sampling rafts (free DSP: $p = 0.49$,
378 total DSP: $p = 0.23$), nor among sampling depths (free DSP: $p = 0.24$, total
379 DSP: $p = 0.07$). Inhibition enzyme assays slightly overestimated toxin content
380 compared to LC-MS/MS analysis, the overestimation being usually more
381 evident with the Upstate enzyme.

382 This overestimation was investigated in detail. Figure 2 shows the OA
383 calibration curves obtained in the PPIA with both enzymes in the absence and
384 presence of non-hydrolysed and hydrolysed Mediterranean mussel extracts.
385 The presence of mussel matrix, at a concentration below the matrix charge limit
386 (12.5 mg/mL for PP2A from GTP and 6.25 mg/mL for PP2A from Upstate),
387 increases the inhibition percentage due to the OA, shifting the calibration curves
388 towards lower OA concentrations. When using PP2A from GTP there were
389 statistically significant differences between the buffer and the non-hydrolysed
390 mussel samples ($W = 76$, $p < 0.001$), and between the buffer and the
391 hydrolysed mussel samples ($W = 78$, $p < 0.001$), but the differences were not
392 statistically significant between hydrolysed and non-hydrolysed mussel samples
393 ($t = 1.1$, $p = 0.26$, $df = 12$). In the same way, when using PP2A from Upstate
394 there were statistically significant differences between the buffer and the non-
395 hydrolysed mussel samples ($W = 76$, $p < 0.001$) and between the buffer and the
396 hydrolysed mussel samples ($t = -2.4$, $p = 0.03$, $df = 11$), but the differences
397 were not statistically significant between hydrolysed and non-hydrolysed mussel
398 samples ($t = 0.07$, $p = 0.93$, $df = 12$).

399 For the quantification of this synergistic inhibitory effect, the IC_{50} ratio between
400 the OA calibration curve in the presence of matrix and the OA calibration curve
401 in buffer was calculated. Since there were not statistically differences ($p = 0.61$)
402 in the IC_{50} ratio for each PP2A (GTP and Upstate) and mussel sample (non-
403 hydrolysed and hydrolysed) pair, the average IC_{50} ratio of 0.48 was applied as a
404 correction factor for all enzymes and mussel samples to improve the OA
405 quantifications (Table 2). The synergistic effect was also observed with non-
406 hydrolysed extracts of wedge clam, Pacific oyster and flat oyster (*results not*
407 *shown)*. For both enzymes, whereas Pacific oyster and flat oyster produce
408 approximately the same synergistic effect than mussel, the effect of wedge clam
409 is more important.
410 The corrected OA equivalent contents determined by the PPIA were correlated
411 to the values obtained in the LC-MS/MS analysis. The best models for the
412 regressions were "Double reciprocal" and "Square root-Y reciprocal-X" for the
413 analysis of free and total DSP, respectively. The percentage of the variability in
414 the predicted variable that has been explained by the models is between 75.62
415 and 95.01% for free DSP using the enzyme from Upstate and total DSP
416 measured using the enzyme from GTP, respectively. Results are shown in
417 Table 3.
418 With regards to phytoplankton, *Dinophysis acuminata* was present in the area
419 from April to October 2010 (Fig. 3). Other *Dinophysis* species present during the
420 event were *D. caudata* and *D. rotundata* in very low abundances. Other
421 dinoflagellate species known as potentially producers of DSP lipophilic toxins
422 were absent.
423

424 ## 4. Discussion

425 As expected, even if there is no OA in the shellfish sample, high concentrations
426 of shellfish matrix interfere with the PPIA. This is not only due to the matrix
427 coloration, effect that can be neglected by the use of appropriate controls, but
428 presumably to the effect of matrix compounds other than diarrheic lipophilic
429 toxins on the PP2A activity. Certainly, the presence of these compounds, when
430 used in high concentrations, modifies the enzyme environment and may alter its
431 functionality. Consequently, the establishment of matrix charge limits is
432 necessary to guarantee the precision of the OA contents calculated from the
433 PPIA.

434 Although the sensitivity of PPIAs may slightly vary from one day to another, in
435 relation to both the OA inhibition and the matrix charge effect, results clearly
436 show that the recombinant PP2A from GTP is able to operate under higher
437 shellfish matrix charges than the PP2A from Upstate. In the case of hydrolysed
438 mussel, the effect on the enzyme activity was minor when using the
439 recombinant enzyme but it was drastic for PP2A from Upstate. Since no acyl
440 derivatives of DSP toxins were detected in the LC-MS/MS analysis of the
441 samples used as negative, the stronger effect of hydrolysed matrix extracts
442 should be due to the hydrolysis step (reagents and/or extract heating). The
443 compounds used in the hydrolysis, even after evaporation of the solvents and
444 re-dissolution in the appropriate buffer, may be inhibiting the PP2A activity. Or
445 the heating step is somehow modifying the shellfish matrix, making it inhibitorier
446 to the enzyme. The LODs calculated taking into account the OA calibration
447 curves and the matrix charge limits indicate that, in principle, PP2A from
448 Upstate could not be used for the determination of OA contents near the

449 regulatory limit in Pacific oyster or hydrolysed Mediterranean mussel samples
450 (nevertheless, OA contents in hydrolysed mussel samples have been
451 determined with both enzymes in this work, as explained below and reported in
452 Table 2).
453 In the application of the developed PPIAs to the determination of OA and its
454 acyl derivatives in mussel samples, an overestimation in relation to LC-MS/MS
455 analysis was observed, as we also mentioned in a previous work (Cañete et al.,
456 2010). It seems that the overestimation has an origin on the shellfish matrix,
457 since its presence, even at concentrations that should not inhibit, produces a
458 synergistic effect to the OA inhibition. Nevertheless, it has not been possible to
459 identify the reason. The presence of some compounds in the shellfish matrix,
460 such as methanol-soluble lipids, has been hypothesised to exert an unspecific
461 inhibitory effect on the PP (Honkanen et al., 1996). However, we consider that
462 this hypothesis does not explain the synergistic effect, since the matrix
463 presence would have inhibited the PP even in the absence of OA (and it did not
464 at the matrix concentrations used in the assay). Neither the possible presence
465 of proteases, since they would have also been detected in the absence of OA
466 (in any case, the use of protease inhibitors in the assay did not induce any
467 significant change). The overestimation of PPIA in relation to LC-MS/MS has
468 been previously reported and attributed to the possible presence of an
469 unidentified interfering compound (Mountfort et al., 1999; González et al.,
470 2002). These authors reported higher OA contents from the PPIA than from
471 HPLC-FLD, which was attributed to the presence of some OA derivatives not
472 detectable by HPLC-FLD (since no hydrolysis was performed) or non-OA-
473 related inhibitors. Mountfort and co-workers (Mountfort et al., 2001) also

474 described the overestimation of PPIA in relation to HPLC-FLD analysis,
475 although these authors described it as an underestimation of the HPLC-FLD
476 analysis. In this case, they attributed the underestimation to possible losses in
477 yield at critical stages of sample preparation during the extraction protocol,
478 because they considered unlikely that the PPIA overestimated the OA contents
479 as yields in experiments with spiked shellfish closely matched theoretical yields.
480 Consequently, we consider appropriate to apply the correction factor
481 established from the ratio between the IC_{50} ratio between the OA calibration
482 curve in the presence of matrix and the OA calibration curve in buffer. In fact, it
483 would have been possible to neglect the correction factor if a matrix-matched
484 standard calibration had been performed.
485 Once the synergistic effect is overcome by the application of the correction
486 factor to the quantitative results, the performance of each one of the enzymes in
487 relation to the LC-MS/MS analysis estimation for non-hydrolysed and
488 hydrolysed samples can be compared. Results obtained with PP2A from GTP
489 correlate better with LC-MS/MS analysis than those obtained with PP2A from
490 Upstate. This may be related to the previous observation about the higher
491 robustness of PP2A from GTP compared to PP2A from Upstate. As expected,
492 correlations for the analysis of total DSP were better than those for free DSP.
493 This may be due to the fact that in the analysis of free DSP, LC-MS/MS is not
494 detecting the presence of acyl derivatives (which are detected by LC-MS/MS in
495 hydrolysed extracts), whereas they may be inhibiting the enzyme.
496 Consequently, the PPIA is interesting because it gives a measure of the total
497 inhibitory potency of a sample, including that from the OA acyl derivatives,
498 which are not detected by LC-MS/MS unless the hydrolysis step is performed.

499 In the application of the developed PPIA as screening tool, it is necessary to
500 prevent false negative results. Looking at Table 2, two samples (C1-1m and C1-
501 5m) were determined to have less than 160 μg OA eq/kg shellfish meat by the
502 PPIA with the recombinant enzyme (if the correction factor is considered). In
503 order to be safe, one can consider as appropriate the quantifications without the
504 correction factors or assess a “suspicious area” for samples with toxin contents
505 near the regulatory limit, which should be further analysed by LC-MS/MS. Only
506 one “negative” mussel sample but with toxin contents lower than 160 μg/kg was
507 analysed (not quantifiable free DSP toxins and 49 μg total DSP toxins/kg by LC-
508 MS/MS). The PPIAs resulted in 14 μg/kg and 40 μg/kg (corrected values) with
509 PP2A from GTP and Upstate, respectively. Although more “negative” mussel
510 samples with toxin contents close to the MPL should be analysed, one can
511 hypothesise that the “suspicious area” could be comprised between 80 and 160
512 μg/kg for PP2A from GTP and between 150 and 220 μg/kg for PP2A from
513 Upstate. Moreover, although the purpose of this work was to perform an
514 exhaustive study to better understand our system, in order to make the
515 screening faster, only one shellfish sample concentration (dose that induces
516 50% PP2A inhibition for samples with toxin content of 160 μg/kg) should be
517 analysed.
518 With regards to phytoplankton, on month before the collection of the shellfish
519 samples, a maximum of *D. acuminata* was recorded. In July, the abundance of
520 this species in the integrated samples of both stations was higher than 500
521 cells/L, recommended action limit for *D. acuminata*, which suggests closure or
522 intensified monitoring (Anderson et al., 2001). In August (at the moment of
523 sampling), however, the abundance of this species was lower than 500 cells/L.

524 Nevertheless, the abundance was around 120 cells/L, and this concentration
525 has already been associated to accumulation of lipophilic toxins in mollusks
526 above the legal limit and thus requiring the area closure (Pazos and Moroño,
527 2008). Consequently, the toxin profile of the positive Mediterranean mussel
528 samples, corresponding to OA and its acyl derivatives, was likely due to the
529 presence of *D. acuminata,* known as OA producer (Lee et al., 1989;
530 FAC/IOC/WHO, 2004), and which moreover has been the main problem in the
531 Spanish Rías (Reguera et al., 2012).
532

533 ## 5. Conclusions

534 The applicability of PPIA assays to the determination of OA and its acyl
535 derivatives, as reference compounds of the lipophilic toxins group, in shellfish
536 samples has been investigated. Although in terms of sensitivity no differences
537 are observed between enzymes, our recombinant PP2A catalytic subunit can
538 operate under higher shellfish matrix charges than the commercial enzyme,
539 especially when analysing hydrolysed mussel samples. Shellfish matrix charge
540 limits have been established in order to guarantee the accuracy in the
541 determination of OA equivalent contents. A synergistic effect between shellfish
542 matrix and OA has been observed and overcome by the establishment of a
543 correction factor. Results obtained by the PPIAs have been compared to those
544 obtained by LC-MS/MS. Good agreements have been observed, especially for
545 PP2A from GTP and hydrolysed samples, where total DSP toxin contents were
546 determined.
547 As a result, a useful analysis tool for the determination of OA equivalent
548 contents in shellfish has been produced. This tool allows the analysis of a large

549 number of samples simultaneously and with short analysis times. The low cost
550 per sample and ease of the procedure make this tool useful in monitoring
551 programmes to control shellfish toxicity, in parallel to LC-MS/MS analysis. For
552 example, PPIA could be used to analyse hydrolysed shellfish samples
553 (necessary to determine acyl ester derivatives of OA, DTX-1 or DTX-2, globally
554 know as DTX-3), increasing the sample throughput, decreasing the demand of
555 expensive instrumental equipment in control laboratories, with benefit for large
556 monitoring programmes, and improving the efficiency of these monitoring
557 programmes and public health protection due to the reduction of the response
558 time. Additionally, the analysis of a higher number of shellfish samples
559 (because of higher sampling rates, larger geographic areas and/or different
560 sampling depths) could be performed, favouring representativeness in
561 surveillance systems. This may be of especial relevancy where oceanographic
562 conditions (stratification, currents) or phytoplankton population dynamics
563 enhance the risk of contamination.
564 Although the use of sample purification protocols after toxin extraction can be
565 used to diminish the effect of the matrix on the enzyme activity, this was not our
566 purpose. The work is aimed at evaluating the applicability of the developed
567 PPIAs assays as fast analysis tools with minimum sample treatment.
568 Nevertheless, such purification protocols may be envisaged in order to
569 decrease the LODs and apply the developed PPIA to the analysis of low/trace
570 toxin contents in phytoplankton or shellfish with research purposes.
571
572 **Acknowledgements**

573 This work has been funded by INIA and FEDER through the RTA2008-00084-
574 00-00 and RTA2009-00127-00-00 projects, by INTERREG SUDOE IVB and
575 FEDER through the SOE1/P1/E129 ALARMTOX project, and by INKOA
576 Sistemas and Université de Perpignan-Via Domitia through the BIOKA
577 EUROTRANSBIO project. Dr. Campàs acknowledges financial support from the
578 *Ministerio de Ciencia e Innovación* and the European Social Fund through the
579 *Ramón y Cajal* program. Diana Garibo acknowledges scholarships from the
580 ALARMTOX project and the *Comissionat per a Universitats i Recerca* of the
581 *Departament d'Innovació, Universitats i Empresa* of the *Generalitat de*
582 *Catalunya*. The authors acknowledge *Amegrove, Ostres de la Badia,*
583 JACUMAR project ("*Cultivo de mitílidos: expansión y sostenibilidad"*) and the
584 shellfish monitoring programme of Catalonia (*Departament d'Agricultura,*
585 *Ramaderia, Pesca, Alimentació i Medi Natural*, *Generalitat de Catalunya*) for
586 kindly providing shellfish samples, and GTP Technology for providing the
587 recombinant PP2A.

588

589 **References**

590 - Albano, C., Ronzitti, G., Rossini, A.M., Callegari, F., Rossini, G.P. 2009. The
591 total activity of a mixture of okadaic acid-group compounds can be calculated
592 by those of individual analogues in a phosphoprotein phosphatase 2A assay.
593 Toxicon 53(6), 631-637.
594 - Anderson, D.M., Andersen, P., Bricelj, V.M., Cullen, J.J., Rensel, J.E.J. 2001.
595 Monitoring and management strategies for harmful algal blooms in coastal
596 waters, APEC #201-MR-01.1. Singapore: Asia Pacific Economic Program;

597 and Paris: Intergovernmental Oceanographic Commission Technical Series
598 No. 59.
599 - Bialojan, C., Takai, A. 1988. Inhibitory effect of a marine sponge toxin, okadaic
600 acid, on protein phosphatases. Biochemical Journal 256(1), 283-290.
601 - Botana, L.M., Vilarino, N., Alfonso, A. 2010. The problem of toxicity equivalent
602 factors in developing alternative methods to animal bioassays for marine-
603 toxin detection. TRAC-Trends in Analytical Chemistry 29(11), 1316-1325.
604 - Caillaud, A., de la Iglesia, P., Campàs, M., Elandaloussi, L., Fernández, M.,
605 Mohammad-Noor, N., Andree, K., Diogène, J. 2010. Evidence of okadaic
606 acid production in a cultured strain of the marine dinoflagellate *Prorocentrum*
607 *rhathymum* from Malaysia. Toxicon 55(2-3), 633-637.
608 - Campàs, M., Marty, J.-L. 2007. Enzyme sensor for the electrochemical
609 detection of the marine toxin okadaic acid. Analytica Chimica Acta 605(1),
610 87-93.
611 - Cañete, E., Campàs, M., de la Iglesia, P., Diogène, J. 2010. NG108-15 cell-
612 based and protein phosphatase inhibition assays as alternative
613 semiquantitative tools for the screening of lipophilic toxins in mussels.
614 Okadaic acid detection. Toxicology in Vitro 24(2), 611-619.
615 - Commission Regulation (EC) No 853/2004 of the European Parliament and of
616 the Council of 29 April 2004 laying down specific hygiene rules for food of
617 animal origin. Official Journal of the European Union, L226, 22-82.
618 - Commission Regulation (EC) No 15/2011 of the European Parliament and of
619 the Council of 10 January 2011 amending Regulation (EC) No 2074/2005 as
620 regards recognised testing methods for detecting marine biotoxins in live
621 bivalve molluscs. Official Journal of the European Union, L6, 3-6.

622 - Della Loggia, R., Sosa, S., Tubaro, A. 1999. Methodological improvement of
623 the protein phosphatase inhibition assay for the detection of okadaic acid in
624 mussels. Natural Toxins 7(6), 387-391.
625 - European Union Reference Laboratory for Marine Biotoxins (EURLMB) 2011.
626 EU-Harmonised Standard Operating Procedure for determination of lipophilic
627 marine biotoxins in molluscs by LC-MS/MS, version 4, July 2011.
628 (http://www.aesan.msps.es/CRLMB/docs/docs/metodos_analiticos_de_desar
629 rollo/EU-Harmonised-SOP-LIPO-LCMSMS_Version4.pdf; accessed by April
630 4th, 2012).
631 - FAO/IOC/WHO (2004). Report of the Joint FAO/IOC/WHO ad hoc Expert
632 Consultation on Biotoxins in Bivalve Molluscs.
633 (ftp://ftp.fao.org/es/esn/food/biotoxin_report_en.pdf; accessed by April 4th,
634 2012).
635 - Garibo, D., Devic, E., Marty, J.-L., Diogène, J., Unzueta, I., Blázquez, M.,
636 Campàs, M. 2012. Conjugation of genetically-engineered protein
637 phosphatases to magnetic particles for okadaic acid detection. Journal of
638 Biotechnology 157(1), 89-95.
639 - González, J.C., Leira, F., Fontal, O.I., Vieytes, M.R., Arévalo, F.F., Vieites,
640 J.M., Bermúdez-Puente, M., Muñiz, S., Salgado, C., Yasumoto, T., Botana,
641 L.M. 2002. Inter-laboratory validation of the fluorescent protein phosphatase
642 inhibition assay to determine diarrhetic shellfish toxins: intercomparison with
643 liquid chromatography and mouse bioassay. Analytica Chimica Acta 466(2),
644 233-246.

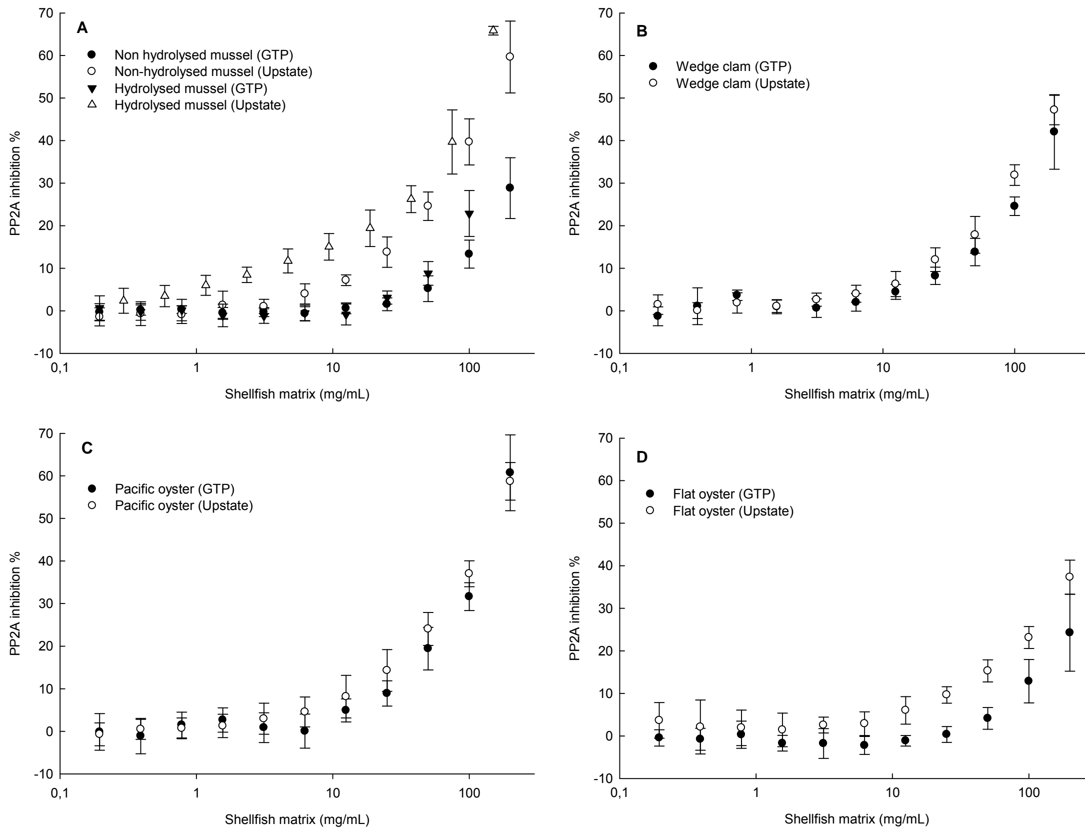
645 - Honkanen, R.E., Mowdy, D.E., Dickey, R.W. 1996. Detection of DSP-toxins,
646 okadaic acid, and dinophysis toxin-1 in shellfish by serine/threonine protein
647 phosphatase assay. Journal of AOAC International 79(6), 1336-1343.
648 - INTECMAR (2010). Notificaciones de resoluciones de levantamento de peche,
649 de prohibición de extracción y de autorización de extracción.
650 (http://www.intecmar.org/PDFs/Historicos/Biotoxinas/Resolucions/Resolucion
651 s_2010.pdf; accessed by April 4th, 2012).
652 - Ikehara, T., Imamura, S., Yoshino, A., Yasumoto, T. 2010. PP2A inhibition
653 assay using recombinant enzyme for rapid detection of okadaic acid and its
654 analogs in shellfish. Toxins 2(1), 195-204.
655 - Lee, J.S., Igarashi, T., Fraga, S., Dahl, E., Hovgaard, P., Yasumoto T. 1989.
656 Determination of diarrhetic shellfish toxins in various dinoflagellate species.
657 Journal of Applied Phycology 1, 147-152.
658 - Leira, F., Vieites, J.M., Vieytes, M.R., Botana, L.M. 2000. Characterization of
659 9H-(1,3-dichlor-9,9-dimethylacridin-2-ona-7-yl)-phosphate (DDAO) as
660 substrate of PP-2A in a fluorimetric microplate assay for diarrhetic shellfish
661 toxins (DSP). Toxicon 38(12), 1833-1844.
662 - Mountfort, D.O., Kennedy, G., Garthwaite, I., Quilliam, M., Truman, P.,
663 Hannah, D.J. 1999. Evaluation of the fluorimetric protein phosphatase
664 inhibition assay in the determination of okadaic acid in mussels. Toxicon
665 37(6), 909-922.
666 - Mountfort, D.O., Suzuki, T., Truman, P. 2001. Protein phosphatase inhibition
667 assay adapted for determination of total DSP in contaminated mussels.
668 Toxicon 39(2-3), 383-390.

669 - Pazos, Y., Moroño, A. 2008. Microplancton tóxico y nocivo en las Rías
670 Gallegas en los años 2003 a 2006. In Gilabert, J. (Ed.), Avances y
671 tendencias en fitoplancton tóxico y biotoxinas (pp.13-28). Cartagena:
672 Universidad Politécnica de Cartagena.
673 - Reguera, B., Velo-Suárez, L., Raine, R., Park, M.G. 2012. Harmful *Dinophysis*
674 species: A review. Harmful Algae 14, 87-116.
675 - Steidinger, K.A. 1993. Some taxonomic and biological aspects of toxic
676 dinoflagellates. In I.R. Falconer (Ed.), Algal Toxins in Seafood and Drinking
677 Water (pp. 1-28). London: Academic Press.
678 - Tsuchiya, T., Ikeda, N., Obara, K., Hartshorne, D.J. 1997. A type 2A protein
679 phosphatase from clam smooth muscle. Use of 4-methylumbelliferyl
680 phosphate as substrate. Comparative Physiology and Biochemistry 118(1),
681 17-21.
682 - Tubaro, A., Florio, C., Luxich, E., Sosa, S., Della Loggia, R., Yasumoto, T.
683 1996. A protein phosphatase 2A inhibition assay for a fast and sensitive
684 assessment of okadaic acid contamination in mussels. Toxicon 34(7), 743-
685 752.
686 - Utermöhl, H. 1958. Zur Vervollkommnung der quantitativen Phytoplankton-
687 Methodik. Mitteilungen der Internationalen Vereinigung für Theoretische und
688 Amgewandte Limnologie 9, 1-38.
689 - Vieytes, M.R., Fontal, O.I., Leira, F., Baptista de Sousa, J.M.V., Botana, L.M.
690 1997. A fluorescent microplate assay for diarrheic shellfish toxins. Analytical
691 Biochemistry 248(2), 258-264.
692 - Yasumoto, T., Murata, M. 1993. Marine toxins. Chemical Reviews 93(5), 1897-
693 1909.

694 - Yasumoto, T., Oshima, Y., Yamaguchi, M. 1978. Occurrence of a new type of
695 shellfish poisoning in the Tohoku district. Bulletin of the Japanese Society of
696 Scientific Fisheries 44(11), 1249-1255.
697 - Zhang, Z., Zhao, S., Long, F., Zhang, L., Bai, G., Shima, H., Nagao, M., Lee,
698 E.Y.C. 1994. A mutant of protein phosphatase-1 that exhibits altered toxin
699 sensitivity. The Journal of Biological Chemistry 269(25), 16997-17000.
700

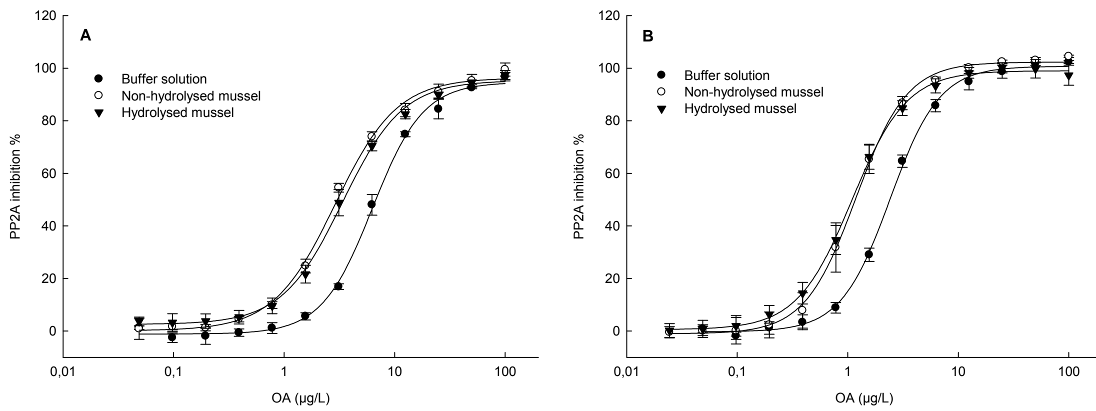
701 **Figure 1.** PP2A inhibition percentage at different matrix concentrations of non-
702 hydrolysed and hydrolysed Mediterranean mussel (A), non-hydrolysed wedge
703 clam (B), non-hydrolysed Pacific oyster (C) and non-hydrolysed flat oyster (D)
704 with PP2A from GTP and Upstate.

705



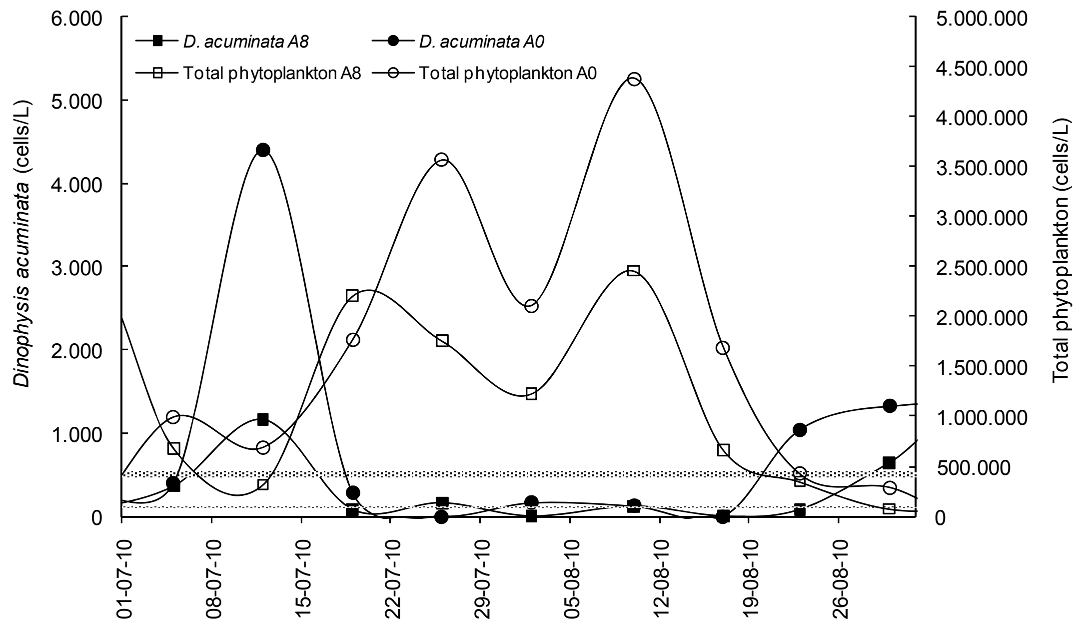
706
707

708 **Figure 2.** PP2A inhibition percentage at different OA concentrations in buffer
709 solution, non-hydrolysed and hydrolysed mussel with PP2A from GTP (A) and
710 Upstate (B).



711
712

713 **Figure 3.** Phytoplankton abundance (*Dinophysis acuminata* and total
714 phytoplankton) in A0 and A8 stations near the rafts were shellfish samples were
715 collected. Thick gray line at 500 cells/L indicates the alert value for *Dinophysis*
716 *acuminata*. Thin gray line at 120 cells/L indicates the *Dinophysis acuminata*
717 minimal abundance associated to DSP events in the Galician Rías.



718

719

720 **Table 1.** Charge limits and LOD_{10} for OA in different shellfish matrices.

721

Shellfish sample	***Enzyme***	***Charge limit (mg/mL)***	***LOD_{10} (µg/kg)***
Mussel	GTP	50.0	22
	Upstate	12.5	111
Hydrolysed mussel	GTP	25.0	44
	Upstate	2.3	604
Wedge clam	GTP	12.5	88
	Upstate	12.5	111
Pacific oyster	GTP	12.5	88
	Upstate	6.3	222
Flat oyster	GTP	50.0	22
	Upstate	12.5	111

722 723 724 725 726 727

728

729 **Table 2.** Free and total DSP toxin contents (µg/kg) in Mediterranean mussel
730 samples determined by LC-MS/MS and PPIA with PP2A from GTP and Upstate
731 (values non-corrected and corrected with the 0.48 factor). Relative standard
732 deviation values were not shown for clarity of the results and were always below
733 10%.

		Free DSP				***Total DSP***			
		C1	C2	C3	C4	C1	C2	C3	C4
LC-MS/MS	1m	165	302	319	340	193	400	331	416
	5m	204	401	666	504	219	657	681	679
	10m	462	512	305	449	631	660	436	655
Non-corrected									
GTP	1m	235	511	410	425	307	416	498	490
	5m	214	928	972	710	313	1029	975	1094
	10m	1014	1146	405	602	1383	1367	544	1256
Upstate	1m	426	661	574	706	435	466	542	1027
	5m	356	1213	944	1358	413	1303	1002	2095
	10m	891	987	479	1210	1390	1417	688	3119
Corrected									
GTP	1m	113	245	197	204	147	200	239	235
	5m	103	445	467	341	150	494	468	525
	10m	487	550	194	289	664	656	261	603
Upstate	1m	205	317	276	339	209	224	264	493
	5m	171	582	453	652	198	638	481	1006
	10m	428	474	230	581	667	680	330	1497

734
735

736 **Table 3.** Regression equations, correlations and percentages of variability for
737 the correlations between the corrected OA equivalent contents determined by
738 the PPIA with GTP and Upstate enzymes and the values obtained in the LC-
739 MS/MS analysis of Mediterranean mussel samples, in relation to free and total
740 DSP toxin contents.

	Enzyme	Equation	Correlation	R^2
Free DSP	GTP	$y = 1/(0.000923724 + 0.474349/x)$	0.9354	87.50%
	Upstate	$y = 1/(0.000486659 + 0.826633/x)$	0.8696	75.62%
Total DSP	GTP	$y = (29.6028 - 2238.44/x)^2$	−0.9747	95.01%
	Upstate	$y = (28.8389 - 2664.06/x)^2$	−0.9163	83.97%

741