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1 **Generation of a panel of high affinity antibodies and development of a biosensor-based**
2 **immunoassay for the detection of okadaic acid in shellfish**

3

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9

10 **Abbreviated title: Biosensor-based assay for okadaic acid**

11

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15

16 **Abstract**

17 Okadaic acid (OA) and its derivatives, DTX-1 and DTX-2, are marine biotoxins associated with
18 diarrhetic shellfish poisoning. Routine monitoring of these toxins relies on the mouse bioassay.
19 However, due to the technical unreliability and animal usage of this bioassay, there is a need for
20 convenient and reliable alternative assay methods. A panel of monoclonal antibodies against OA
21 was generated and the most suitable was selected for surface plasmon resonance (SPR)-based
22 assay development. SPR-based biosensors have been shown to be highly reproducible immuno-
23 analytical tools, with potential for large scale screening applications. The cross reactivity of the
24 selected antibody with DTX-1 was found to be 73%, confirming its suitability for assay
25 development. The OA and derivative assay was designed as an inhibition assay covering the
26 concentrations 1 to 75 ng/ml, with a sensitivity of 22.4 ng/ml. The regulatory limit for OA and
27 DTXs is 160 ng/g, equivalent to 12.8 ng/ml in crude extract, and this concentration was within
28 the linear range of the assay which was 11.2 to 38.8 ng/ml. The assay was highly reproducible
29 with a coefficient of variability (%CV) of <7.3 %. Preliminary validation showed no matrix
30 interference from mussel extracts and good recovery of added standard in mussel extracts, with
31 %CV of <9.35%. This assay could provide a useful and convenient screening tool for OA and
32 its derivatives with a comprehensive extraction protocol for shellfish monitoring programmes.

33

34

35 **Keywords**

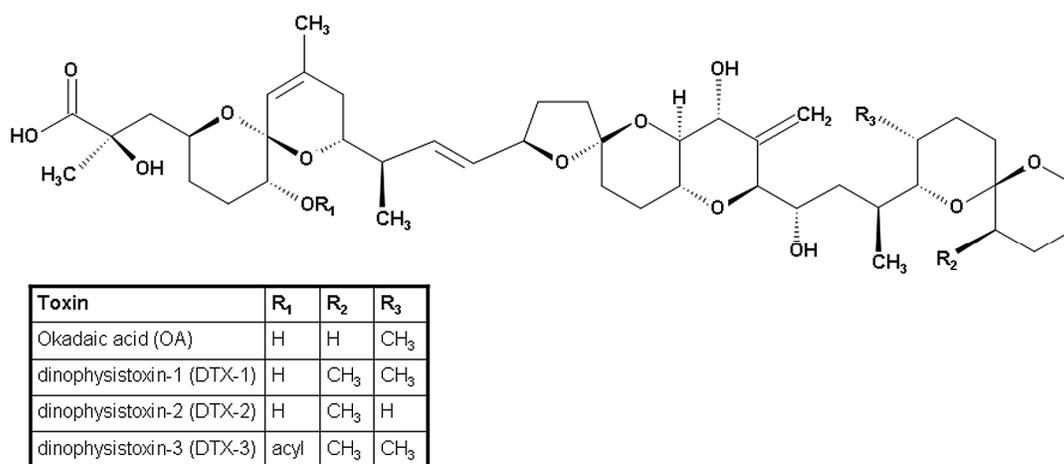
36 biosensor, okadaic acid, DSP, monoclonal antibody, shellfish, immunoassay

37

38 **1. Introduction**

39 Okadaic acid (OA) and its derivatives, the dinophysis toxins DTX-1 and DTX-2, are structurally
 40 related, lipophilic, toxic, polyether compounds produced by dinoflagellates of the genera
 41 Prorocentrum and Dinophysis (Lee *et al.*, 1989) (Figure 1). These biotoxins are associated with
 42 diarrhetic shellfish poisoning (DSP). OA was first isolated from the sponge *Halichondria*
 43 *okadaii* (Tachibana *et al.*, 1981) and later, OA and its derivatives, including a third derivative
 44 DTX-3, were purified from contaminated shellfish (Yasumoto *et al.*, 1984). DTX-3, the 7-O-acyl
 45 derivative, was found to be a metabolic by-product of the parent toxins in the shellfish and not a
 46 *de novo* product synthesised by phytoplankton (Suzuki *et al.*, 1999).

47



48

49 **Figure 1.** Chemical structure of okadaic acid and the dinophysis toxins, DTX-1, -2 and -3.

50

51 Filter-feeding marine species which are consumed by humans, such as mussels (*Mytilus edulis*),
 52 clams (*Siliqua patula*) and scallops (*Pecten maximus*), accumulate these toxins in their digestive
 53 tissues, facilitating their entry into the human food chain and causing DSP. Although no fatalities
 54 have been reported, the worldwide occurrence of DSP has made it a serious threat for the

55 shellfish industry and public health. National shellfish monitoring programmes have been
56 implemented to protect consumers, as well as the shellfish industry, and to promote international
57 harmonisation of biotoxin monitoring. In Europe, the level of OA must not exceed 160 ng/g of
58 shellfish (European Communities decision, 2002/225/EC).

59 Shellfish monitoring for the presence of DSP toxins relies on the mouse bioassay (MBA)
60 (Yasumoto *et al.*, 1984) and rat bioassay (Kat, 1983), in which three mice or rats are fed with
61 shellfish extract as stipulated by EU regulations (EU Commission regulation No. 15/2011,
62 amending EC regulation No. 2074/2005). A sample is considered positive if two out of three
63 mice die and if a diarrhetic response is observed in any of the three rats. However, the MBA test
64 lacks specificity and is recognised as having poor reproducibility and high variability (Jellett,
65 1993; Campbell *et al.*, 2011). The assay is also prone to interference from free fatty acid, leading
66 to false positive (Suzuki *et al.*, 1996). Alternative methods of detection have to be used for
67 routine monitoring of shellfish as of the end of 2014 due to technical and ethical problems
68 associated with the MBA to fulfill requirements set by the European Union Reference
69 Laboratory (EU-RL) (EU Commission regulation No. 15/2011, amending EC regulation No.
70 2074/2005). A number of alternative methods have already been proposed for the detection of
71 OA and derivatives including liquid chromatography-mass spectrometry (LC-MS)-based
72 analysis, which inter-laboratory validation study demonstrated its suitability as alternative
73 detection method (Van den Top *et al.*, 2011), and a colorimetric protein phosphatase 2A (PP2A)
74 assay was also demonstrated suitable as alternative method (Smienk *et al.*, 2012, 2013).
75 Immunoassays have also been developed for the detection of OA, such as ELISA-based assay
76 developed by Kreuzer *et al.* (1999), which relied on commercial antibodies. Automated Surface
77 plasmon resonance (SPR) - based biosensors are attractive alternative to these assays which can

78 be time consuming. The technology initially developed for research, such as screening of
79 biological samples for binding partners and kinetic analysis, is a very useful and reliable
80 quantitative tool for detection of contaminants in biological fluids. When compared to other
81 analytical quantitative methods, such as high performance LC (HPLC), LC-MS and plate-based
82 ELISAs, SPR-based biosensors offer significant advantages in reproducibility, speed,
83 automation, simplicity and the possibility for high throughput analysis with minimal sample
84 preparation.

85 This work describes the generation of a panel of high-affinity monoclonal anti-OA antibodies,
86 and the development and optimisation of an inhibition biosensor-based immunoassay using SPR.

87

88 **2. Materials and methods**

89 **2.1. Instrumentation**

90 A BIACORE 2000™ biosensor instrument and CM5 sensor chips (research grade) were used
91 (Biacore Life Science, GE Healthcare, UK). The BIACORE 2000™ was controlled by
92 BIACORE control software version 3.2 running under Windows XP. The instrument running
93 temperature was 25 °C.

94

95 **2.2. Reagents**

96 HEPES buffered saline supplemented with EDTA and surfactant (HBS-EP; 10 mM HEPES, 0.15
97 M NaCl, 3 mM EDTA, 0.005% surfactant P20, pH 7.4), and the amine coupling kit (containing
98 *N*-hydroxysuccinimide (NHS), *N*-ethyl-*N*'(3-ethylaminopropyl) carbodiimide (EDC), and
99 ethanolamine hydrochloride) were obtained from Biacore Life Science. OA was purchased from
100 LC Laboratories (U.S.A.) and DTX-1 from Wako Laboratories (Japan). Sodium hydroxide

101 (pellet, NaOH) was purchased from BDH Chemical Ltd. (UK) and acetonitrile analytical grade
102 from Romil (Lennox, Ireland). Protein G Sepharose™ was a product of Amersham Biosciences
103 (Sweden). Bicinchoninic acid (BCA) protein assay kit was obtained from Thermo Scientific
104 (Ireland). Bovine serum albumin (BSA), ovalbumin (OVA), EDC, NHS, *N,N*-
105 dimethylformamide (DMF) and all other reagents were purchased from Sigma and were of the
106 highest grade available.

107

108 **2.3. Generation of mouse monoclonal antibodies against OA**

109 OA was conjugated to bovine serum albumin (BSA) for immunisation using EDC and NHS
110 coupling, according to the method of Kreuzer *et al.* (1999). In brief, EDC and NHS were added
111 to OA in DMSO, at 20 and 3.3 molar excess over OA, respectively. Following 30 min activation
112 at 37 °C, BSA was added at a 50:1 BSA to OA ratio and the conjugation mixture was incubated
113 for 24 h at 37 °C. The OA-BSA conjugate was purified by dialysis against phosphate buffered
114 saline (PBS), pH 7.2 overnight at 4 °C. OA was coupled to OVA for use in screening assays
115 following the same procedure.

116 Six to eight weeks old Balb/C mice were injected three times at four week intervals with OA-
117 BSA at a concentration of 50 µg in 150 µl of PBS and emulsified by addition of an equal volume
118 of Freund's complete adjuvant. A week after the last intraperitoneal boost, the tail vein was bled
119 and the serum tested for the presence of anti-OA antibody using antibody capture and indirect
120 competitive ELISA (see section 2.4 below). The mouse with the highest affinity of the antiserum
121 for its antigen, as determined by effective dose 50 (ED-50; the concentration of free OA required
122 to inhibit the binding of the antiserum or antibody to the immobilised antigen by 50%), was
123 selected for the generation of monoclonal antibodies by cellular fusion. Myeloma SP2/mIL-6

124 cells (Harris *et al*, 1992) were fused with spleen cells of the selected animal in the presence of
125 polyethylene glycol (Köhler and Milstein, 1975). Individual clones were isolated by limiting
126 dilution and antibody-producing clones were selected by their ability to bind and displace free
127 OA on a competitive indirect immunoassay. A panel of eight clones were then cultured for bulk
128 antibody production as previously described (Ker-hwa Ou and Patterson, 1997).

129 Anti-OA antibodies were purified from tissue culture supernatant by affinity chromatography on
130 a 5 mL Protein G Sepharose 4 Fast Flow column. The tissue culture supernatant was bound to
131 the matrix and washed with 0.02 M sodium phosphate buffer, pH 7.4. IgG was eluted with 0.1 M
132 glycine-HCl, pH 2.5 until absorbance at 280 nm reached 0.05 and fractions were immediately
133 neutralised with 1 M Tris, pH 9. The fractions containing antibody were determined by
134 absorbance reading at 280 nm and dialysed against PBS at 4 °C. The yield per flask (150 ml) was
135 approximately 20 mg, as determined by BCA assay.

136

137 **2.4. Screening immunoassays**

138 An antibody capture immunoassay was used to screen the anti-sera response and tissue culture
139 supernatant. OA-OVA conjugate, prepared in the same manner as OA-BSA above, was coated at
140 2 µg/ml in 0.05 M carbonate buffer pH 9.6 onto microtitre plate wells (Nunc Maxiporp) and
141 incubated for 90 min at 37 °C. The plates were blocked with 3% non-fat milk powder for 1 h at
142 37 °C. Serial dilutions of serum or tissue culture supernatant in PBS with 0.05% BSA (PBS-B)
143 or neat tissue culture supernatant were added (100 µl/well) and the plate was incubated for 1.5 hr
144 at 37 °C. Bound antibody was detected with 100 µl of horse radish peroxidase- (HRP) labelled
145 rabbit anti-mouse IgG diluted at 1:2000 in PBS-B. Between each step the plate was washed four
146 times with 300 µl PBS/ 0.05% tween 20. The diluted serum or tissue culture supernatant or

147 antibody was added to the wells with free OA (0.1 to 10 ng/ml) standards for the indirect
148 competitive immunoassay to test the specificity of the anti-sera.

149

150 **2.5 Immobilisation of OA on CM5 sensor chip**

151 Simultaneous immobilisation of OA onto all flow cells of the CM5 sensor chip was performed
152 following modifications of a previously described method (Gillis *et al.*, 2002). For covalent
153 immobilisation, carboxyl groups of the sensor surface were activated by derivatisation with NHS
154 mediated by EDC. EDC and NHS were mixed (1:1) as per kit and 50 μ l of the mixture was
155 deposited on the surface for 20 min activation. This step was repeated once. The amine
156 functionalised surface was then prepared by adding 50 μ l of 1 M ethylene diamine, pH 8.5, to the
157 activated surface for 1 h. Any remaining activated groups were deactivated with 1 M
158 ethanolamine, pH 8.5, for 20 min. OA (1 mg) was dissolved in 250 μ l of DMF and mixed with
159 225 μ l of 10 mM sodium acetate, pH 4.5, containing 5 mg of EDC and 2 mg of NHS. OA was
160 then immobilised on the surface by placing 50 μ l of this solution in static contact with the amine-
161 functionalised surface for 2 h. The surface was then conditioned with repeated injection of 25 μ l
162 of 100 mM NaOH, to remove any non-covalently bound material.

163

164 **2.6. Antibody selection**

165 To examine the binding kinetics of the eight antibodies selected, each antibody was injected over
166 the OA immobilised on the sensor chip surface and conditions to remove the bound antibody
167 were investigated. Preliminary binding data were collected for each antibody by injecting a
168 known concentration of antibody over the chip (0.7 μ g/ml of antibody for 12 min at 20 μ l/min)
169 and then allowing it to dissociate in HBS-EP buffer for 15 min. The interaction curve for each

170 antibody on the chip was then compared to select the most suitable candidate for concentration
171 assay development.

172 OA standards of 1 – 75 ng/ml were prepared in HBS-EP buffer. Standard curves were obtained
173 by mixing the antibody in HBS-EP buffer with OA standards to a 200 µl final volume. The
174 mixture was injected for 1 min over the chip at 25 µl/min, and regenerated by 1 min injection of
175 20 % acetonitrile in 100 mM NaOH. All curves were fitted using a four-parameter equation with
176 BIAevaluation software and ED-50s determined to select the antibody that gave the most
177 sensitive standard curve for assay development. Cross-reactivity of the selected antibody to
178 DTX-1 was then evaluated by assaying the antibody with DTX-1 standards on the biosensor-
179 based assay. DTX-1 standards ranging from 1 to 75 ng/ml in HBS-EP were prepared from a 100
180 µg/ml stock. The percentage cross-reactivity was defined as the ED-50 of the standard curve
181 divided by the ED-50 of the cross reactant curve and multiplied by 100 (O’Fegan, 2000).

182

183 **2.7. Concentration assay**

184 Non-contaminated mussels were purchased from a local outlet (Oyster Creek Seafood Ltd.,
185 Ireland) and contaminated mussels were obtained from the Marine Institute (Galway, Ireland) as
186 part of their routine screening programme. Hepatopancreas were excised for extraction and crude
187 methanolic extracts were prepared as follows: 2 g of hepatopancreas was homogenised in 12.5
188 ml 100 % methanol using a vortex. The mixture was centrifuged and methanolic extracts were
189 collected. Methanol extraction was repeated once more on pellets and extracts were pooled with
190 a final volume of 25 ml and filtered through a 0.2 µm membrane (Hess *et al.*, 2004). OA
191 standards were also prepared in different dilutions of methanol (50 %, 80 % and 100 %) to

192 examine the effect of the solvent on the assay. The curves were then compared against the curve
193 in HBS-EP buffer

194 All sample analysis optimisation was aimed to achieve the required sensitivity with binding
195 between 200 and 500 resonance units (RU) on the sensor surface. The optimised assay
196 conditions were as follows: the antibody was diluted at 1:750 in HBS-EP buffer and then mixed
197 9:1 with the OA standard or sample, injected for 2 min over the chip at a flow rate of 25 μ l/min
198 and regenerated with 1 min injection of 20% acetonitrile in 100 mM NaOH. Preliminary
199 validation of the assay was carried out following guidelines from Wong *et al.* (1997) and
200 O’Fegan (2000). The desired characteristics of the standard curves were defined as follows: the
201 sensitivity (ED-50), lower limit of detection (LLOD; standard concentration corresponding to B0
202 minus three times its standard deviation, with B0 being the antibody binding with no antigen),
203 working range (ED-20 to ED-80) and the reproducibility (%CV for each standard). Repeated
204 assay of three quality control standards (11, 20 and 62 ng/ml, n = 5) in one run determined the
205 intra-assay variation. Inter-assay variation was determined over four consecutive assays, using
206 the same set of quality control standards. Recovery of added standards was carried out by spiking
207 crude methanolic extracts with OA concentrations ranging from 10 to 200 ng/ml and
208 extrapolating the concentration of the spiked extract from the standard curve. A linearity study
209 was carried out by diluting OA-positive sample extracts with known OA concentration in HBS-
210 EP buffer to determine the ability of the assay to obtain results directly proportional to the
211 concentration of the analyte in the sample.

212

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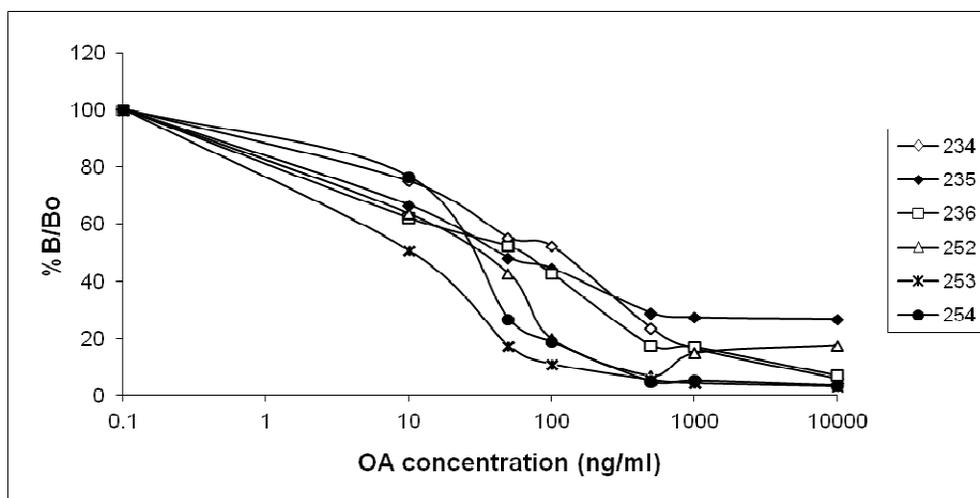
214

215 **3. Results and discussion**

216 **3.1. Generation of OA-specific monoclonal antibodies**

217 Five mice were immunised with the OA-BSA conjugate. One week after the last boost, the sera
218 were screened for the presence of anti-OA antibody in an antibody capture immunoassay using
219 plates coated with OA-OVA conjugate. Titres, defined by dilution of sera giving an absorbance
220 reading of 1, ranging from 1:32,000 to 1:64,000 were obtained. To compare responses between
221 mice, dose-response curves were constructed with each of the sera using OA standards, ranging
222 from 10 to 1000 ng/ml (Figure 2). All mice gave a positive response, but serum from an
223 individual mouse, designated 253 with a titre of 1:64,000 gave the most sensitive dose-response
224 curve (Figure 2). Mouse 253 was therefore selected for fusion with myeloma cell line to generate
225 monoclonal antibodies. 176 clones generated from mouse 253 were initially tested positive in the
226 screening assay and 28 showing displacement at 5 ng/ml were selected for further evaluation.
227 The 8 most sensitive clones (labelled Ab 1 to 8) were kept for further displacement studies and
228 bulk antibody production. Displacement at 0.5 ng/ml ranged from 40 to 75%, which was
229 considered adequate sensitivity for analysis of OA. The binding of the antibodies to OA was then
230 evaluated on the biosensor.

231



232

233 **Figure 2.** Dose-response curves obtained with serum from OA-BSA immunised mice.

234

235 3.2. Preliminary evaluation on the SPR platform

236 As a preliminary to the development of an SPR-based assay for OA, an evaluation of binding

237 kinetics of the eight monoclonal antibodies was undertaken on sensor surface immobilised OA.

238 The chip was initially conditioned with repeated injection of 100 mM NaOH to remove any

239 remaining non-covalently bound material. A typical analysis cycle is presented in Figure 3. The

240 optimal regeneration condition for each antibody was initially determined where the regeneration

241 step removes any bound material without affecting the ligand activity. Each antibody was

242 injected over the chip and regeneration conditions were optimised. The surface was fully

243 regenerated at 10% acetonitrile in 100 mM NaOH for the antibodies Ab 2, 3, 4, 5, 7, 8 and at

244 20% acetonitrile in 100 mM NaOH for Ab 1 and Ab 6. The difference between regeneration

245 solutions suggested a slight difference between antibody affinities to the immobilised OA: the

246 stronger the binding of the antibody to the antigen, the higher its affinity to the antigen and

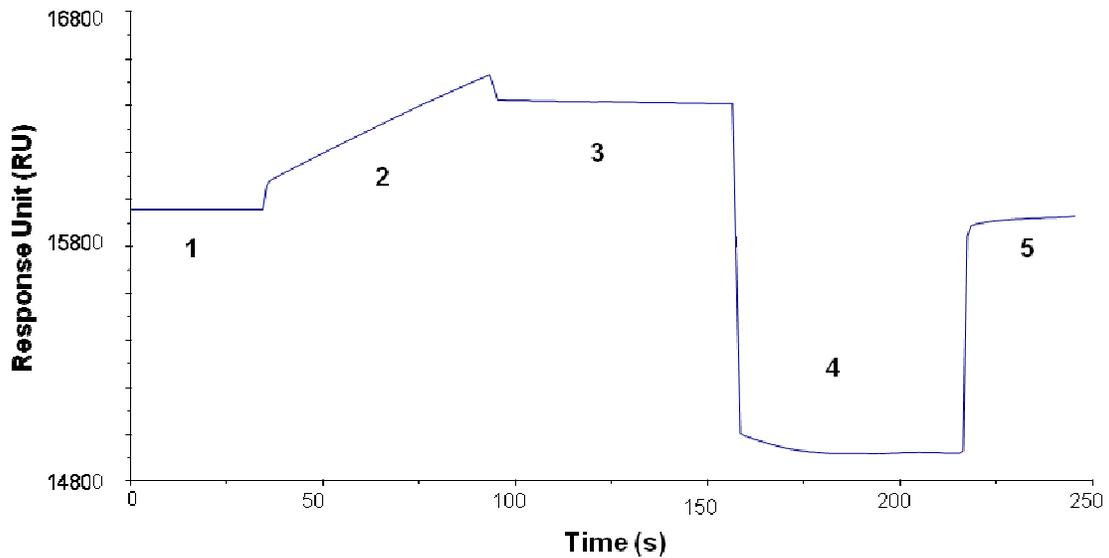
247 therefore, the more concentrated the regeneration solution required to remove the bound

248 antibody. As Ab1 and Ab 6 needed a slightly more concentrated regeneration solution in

249 acetonitrile for removal from the surface (20% versus 10% for the other six antibodies),
250 suggesting that Ab 1 and 6 represent the best antibody choice to further develop a robust assay
251 (Dillon *et al.*, 2003).

252

253

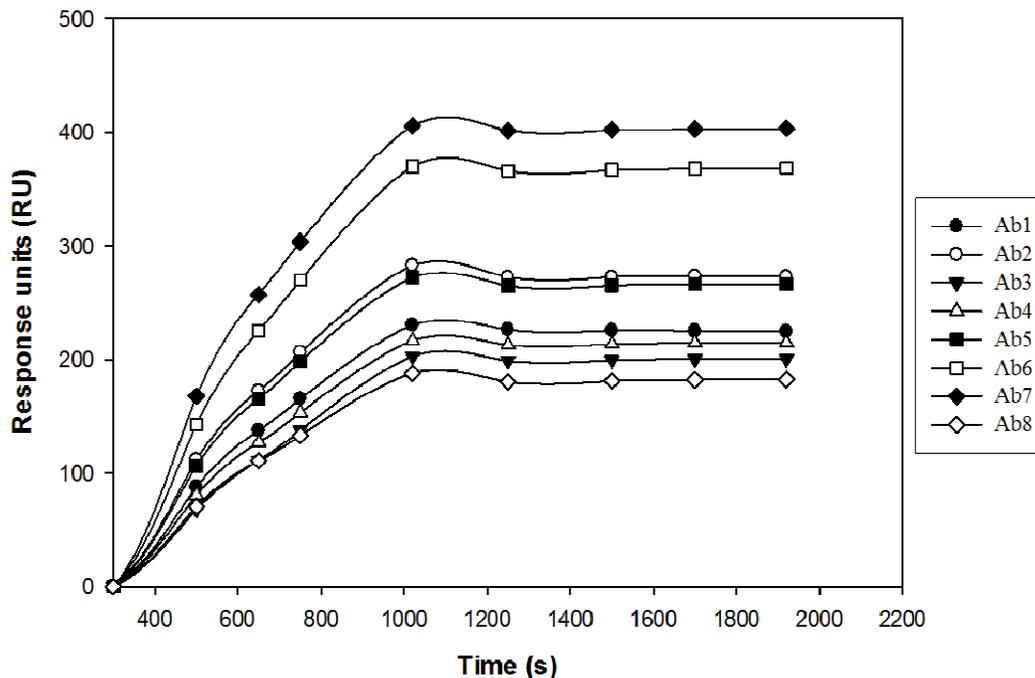


254

255 **Figure 3.** Typical analysis cycle using SPR-based biosensor. 1) Flow of buffer over the
256 immobilised OA on the sensor surface (baseline). 2) Injection of the antibody: sample/standard
257 mixture, the response increases as the antibody binds onto the immobilised OA (association
258 phase). 3) The injection is finished and the buffer flows over the bound antibody (dissociation
259 phase). 4) The surface is regenerated; all the non-covalently bound material is removed. The dip
260 in the response measured is due to the difference between the refractive index of the regeneration
261 buffer and the refractive index of the regular HBS-EP buffer. 5) The baseline returns to its
262 normal level as the buffer flows over the cleaned surface.

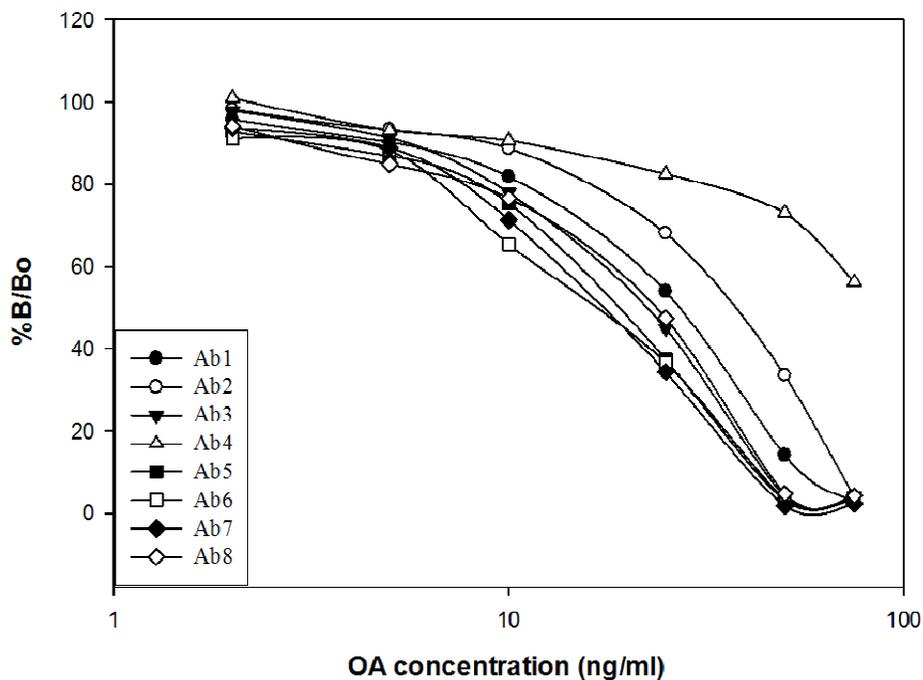
263

264 Qualitative kinetic data were obtained by comparing the dissociation part of the interaction
265 curves, after injecting one concentration of antibody onto the OA surface. Although this is not
266 the recommended procedure for acquiring binding data, qualitative comparison of interaction
267 curves can be a useful tool in selecting the most suitable antibody for assay development
268 (Karlsson *et al.*, 1991). Figure 4 shows the binding profiles of the eight antibodies. The
269 dissociation part of the curve is reported to be the most critical value as a lower dissociation rate
270 reflects a more stable binding (Karlsson *et al.*, 1991). A visual comparison of the dissociation
271 part of the curve showed no discernible difference between the eight antibody binding stabilities,
272 which suggested that all eight antibodies had equally strong binding affinities for OA.
273



274
275 **Figure 4.** Comparison of the interaction curves of the eight antibodies after injection of 0.7
276 $\mu\text{g/ml}$ of antibody over immobilised OA for 12 min at 20 $\mu\text{l/min}$ and 15 min dissociation in
277 HBS-EP buffer. The dissociation part of the curve is reflective of the antibody stability.

278 Separate standard curves were generated for each antibody and ED-50s for each curve were
279 compared (Figure 5). Ab 6 was found to produce the most sensitive curve, as determined by the
280 lowest ED-50 and was thus selected for further assay development. The cross-reactivity of Ab 6
281 towards the commercially-available DTX-1 was 73%, which indicated that the antibody selected
282 could bind other similar structures. Ab 6 was therefore suitable for assay development for the
283 detection of OA and its co-occurring derivative DTX-1, said to have a relative toxicity of 1 when
284 compared to OA (Aune *et al.*, 2006) and higher toxicity *in vitro* than the other DTXs (Fernández
285 *et al.*, 2014)

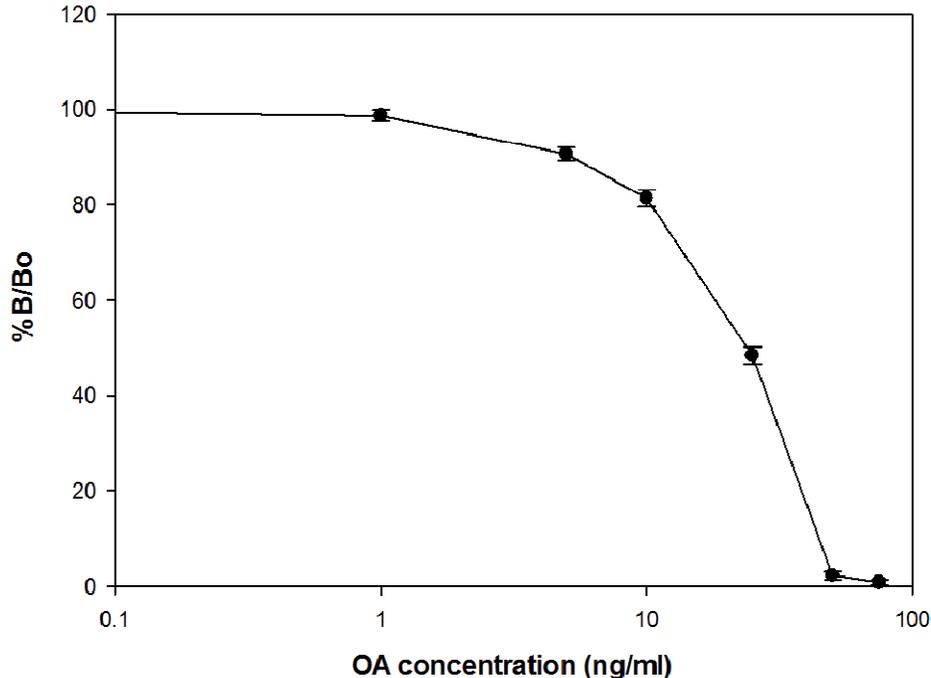


286
287 **Figure 5.** Comparison of standard curves from eight monoclonal anti-OA antibodies on a SPR-
288 based immunoassay.

289
290
291

292 **3.3. Concentration assay**

293 The OA and DTX assay was designed as an inhibition assay with the selected antibody, Ab 6,
294 mixed with the sample or standard at a ratio of 9:1 and to a final volume of 200 μ l. Figure 6
295 represents the mean of 10 curves obtained separately with duplicate concentration of standards.
296 The curve was highly reproducible, with %CV between the 10 curves less than 5%. The assay
297 covered the concentration range 1 to 75 ng/ml, had a linear range between 11.2 and 38.8 ng/ml
298 and ED-50 was 22.4 ng/ml. The regulatory limit of OA and DTXs, set at 160 ng/g, is equivalent
299 to 12.8 ng/ml in crude extract (regulatory cut-off point) which was within the linear range of
300 the assay. Repeated assays in one run (n=4) of three quality control samples resulted in the
301 following concentration-dependent intra-assay %CVs: at 11 ng/ml, 2.8%; at 20 ng/ml, 1.4% and
302 at 62 ng/ml, 0.9%. Inter-assay variation (n=5) was determined over 5 consecutive assays using
303 the same set of control standards and was 7.3%, 1.9 % and 2.1% at 11, 20 and 62 ng/ml
304 respectively. The assay shows good precision with %CV less than 7.3%, considered acceptable
305 in assay validation (DeSilva *et al.*, 2003)



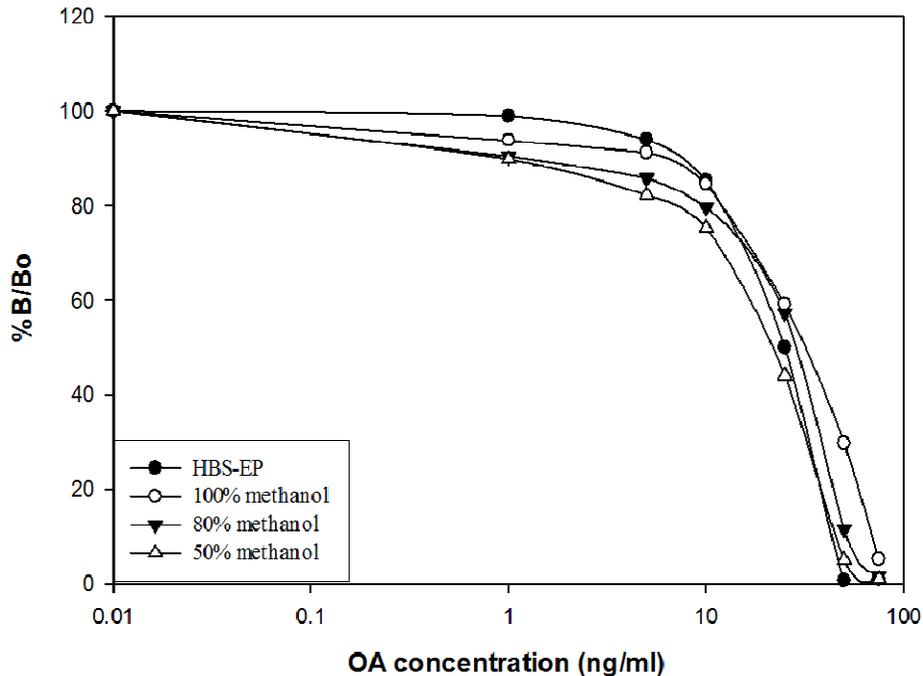
306

307 **Figure 6.** Composite standard curves for analysis of OA in HBS-EP buffer Ab 6. The standard
 308 curve is derived from the mean results for 10 sets of standards analysed in duplicate. The error
 309 bars indicate the standard deviations for the 10 sets of standards.

310

311 **3.4. Application of the assay to marine sample analysis**

312 Potential interference from the methanolic extraction buffer and from shellfish extracts was
 313 evaluated. The optimised protocol was adjusted to minimise any interference noted. Crude
 314 mussel extracts were prepared in 100% methanol and the assessment of the interference of
 315 methanol on the assay was carried out by preparing sets of OA standards in different
 316 concentrations of methanol (50, 80 and 100%) to compare with the standard curve in HBS-EP
 317 buffer (Figure 7). Minimal interference from methanol was noted, especially when standards
 318 were prepared in 50% methanol and 50% buffer. Hence, a 1:2 of the sample extracts in HBS-EP
 319 buffer following extraction was used for sample analysis in the assay.



320

321 **Figure 7.** Influence of the extraction buffer on the characteristics of the standard curve:
 322 comparison of standard curves obtained with OA standards prepared in different concentration of
 323 methanol (50%, 80% and 100%) and OA standards prepared in HBS-EP buffer.

324

325 A batch of OA- and DTX-negative mussels was purchased and pooled hepatopancreas extracts
 326 were prepared to examine the effect of mussel extract matrices on the assay performance. OA
 327 was added to negative mussel extract at concentrations ranging from 10 to 200 ng/ml. Spiked
 328 extracts were quantified on SPR-based assay and recoveries of 90.3 – 97.9% were obtained
 329 (Table 1). Accurate recovery of added standard indicated minimum matrix interference.

330

331

332

333

334 **Table 1.** Recovery of added standard in negative mussel extracts.

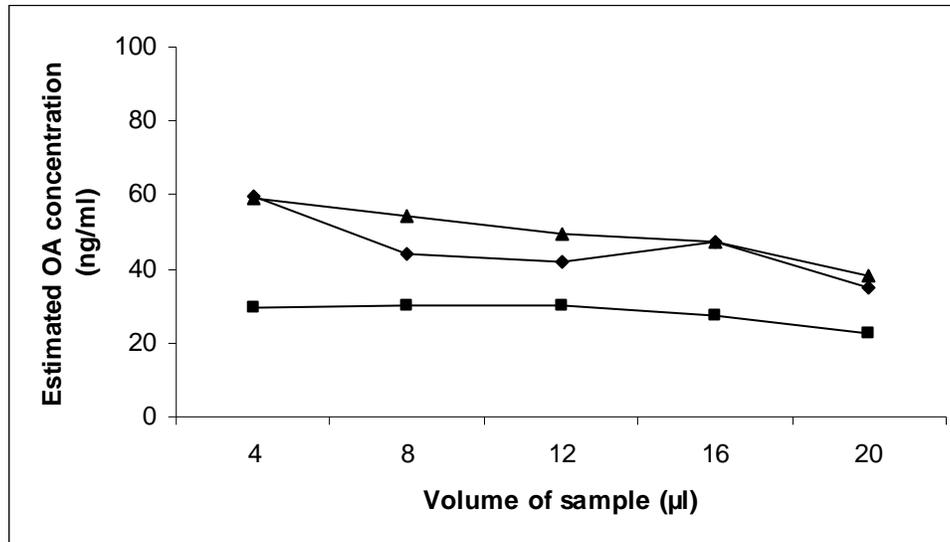
Spiked OA standards (ng/ml)	Mean conc. determined (n=4, ng/ml)	% CV	% Recovery
10	9.1	9.3	91
20	19.6	3.6	98
40	37.7	1.6	94.2
80	77.6	4.4	97
160	146.5	3.1	91.6
200	180.6	6.8	90.3

335

336

337 Three OA- and DTX-positive mussel homogenates were provided by the Marine Institute as part
 338 of their routine monitoring programme. Crude extracts were prepared and a range of different
 339 dilutions were made to a final volume of 20 µl in buffer before assay. The concentration of OA
 340 measured was directly related to the effective volume assayed over the range examined, with R²
 341 values greater than 0.85 confirming the linearity of the assay (Figure 8). These data also
 342 confirmed that there was minimal matrix interference arising from the sample extract in the assay
 343 and the suitability of the assay for OA detection in mussel samples. Experiments carried out to
 344 evaluate matrix interference during this study, such as effect of methanol on standard curve,
 345 linearity and recovery experiments all supported the absence of matrix effects in our assay.

346



347
 348 **Figure 8.** Relationship between the effective volume of sample extract assayed and the
 349 concentration of OA measured for three positive mussels extracts. The volume of diluted sample
 350 assayed in each case was 20 µl. The final OA concentration in the sample was estimated each
 351 time from the measured concentration multiplied by the dilution factor.

352
 353 Although the developed SPR-based assay described is not as sensitive as ELISA-based assays
 354 commercially available and previously described in the literature, such as DSP ELISA Kit
 355 (L35000420-096, Biosense Laboratories AS, Norway) and the indirect competitive ELISA
 356 developed by Lu *et al.* (2011), it has proven to be a robust (% CV<7.3%) and highly
 357 reproducible assay, detecting OA at nanogram concentrations and around the mandated cut-off
 358 point. No extensive sample clean-up procedure is required, by comparison to HPLC and MS-
 359 based methods, which makes the assay more convenient with a comprehensive extraction
 360 protocol. The instrument is fully automated and results are available within minutes after
 361 injection, as no incubation time is needed and a high throughput option is feasible.

362 The ability of the same antibody (Ab 6) to detect OA on a different analytical platform was also
363 previously evaluated. The antibody was applied to an electrochemical biosensor and its ability to
364 detect OA produced in this study in shellfish extract was demonstrated. Although the assay had
365 lower sensitivity, the automation of the SPR-based assay in this study allowed for reduced assay
366 time (minutes as opposed to hours) (Campas *et al.*, 2008). Various biosensor applications have
367 been previously developed for the detection of OA. Kreuzer *et al.* (2002) presented a screen-
368 printed electrode system for the measurement of a variety of phytotoxins including OA. The
369 assay had a sensitivity of 32 ng/ml, was simple, cost-effective and rapid but was low throughput.
370 Similarly, a quartz crystal microbalance immunosensor was developed but the assay sensitivity
371 was not good enough to fulfill EU requirements (Tang *et al.*, 2002). By contrast, the SPR-based
372 assay presented here is suitable for high throughput analysis with a level of sensitivity in
373 accordance with the EU legislation. Antibodies against OA were also produced by Stewart *et al.*
374 (2009a) and the single laboratory validation for routine monitoring of OA using SPR biosensor
375 (Biacore Q) was also presented; however the sample preparation required evaporation to dryness
376 prior to re-suspension in compatible analysis buffer (Stewart *et al.*, 2009b). In the assay
377 presented, minimal interference from the extraction buffer and shellfish matrix with simple
378 sample preparation was demonstrated.. This study presents a convenient, time-effective and
379 confirms the usefulness of SPR biosensing for detection and monitoring of environmental
380 contaminants.

381

382 **4. Conclusion**

383 A panel of monoclonal antibodies against OA was produced and one was selected to develop a
384 fully automated SPR-based immunoassay. The antibody showed the desired ability to recognise

385 structurally related biotoxins (DTX-1), and good sensitivity, allowing the detection of OA in the
386 nanomolar range. The optimised assay was highly reproducible and was successfully applied to
387 crude mussel extracts and is thus suitable for application to high throughput analysis of OA and
388 DTX-1 in shellfish. As no extensive clean-up is required, the assay is time-effective (5 min per
389 sample). This assay could provide a useful and convenient screening tool with a comprehensive
390 extraction protocol for shellfish monitoring programmes.

391

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399 **6. References**

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