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Development of ELISAs for detecting domoic acid, okadaic acid, and saxitoxin and their applicability for the detection of marine toxins in samples collected in Belgium

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3 **Development of ELISAs for detecting domoic acid, okadaic acid,**
4 **and saxitoxin and their applicability for the detection of marine**
5 **toxins in samples collected in Belgium**
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Abstract

Okadaic acid (OA), a diarrhetic shellfish poison (DSP), domoic acid (DA), an amnesic shellfish poison (ASP) and saxitoxin (SAX), a paralytic shellfish poison (PSP), are three of the best-known marine biotoxins. The mouse bioassay is the method most widely used to detect many of these toxins in shellfish samples, but animal welfare concerns have prompted researchers to seek alternative methods of detection. In this study, three direct competitive enzyme-linked immunosorbent assays (ELISAs), each based on antibodies raised in rabbits against a conjugate of the analyte of interest, were developed for marine biotoxin detection in mussel, oyster, and scallop. One assay was for okadaic acid, one for saxitoxin, and one for domoic acid (DA) usually detected and quantified by HPLC-UV. All three compounds and a number of related toxins were extracted quickly and simply from the shellfish matrices with a 9:1 mixture of ethanol and water prior to analysis. The detection capabilities ($CC\beta$ values) of the developed ELISAs were 150 ug kg^{-1} for okadaic acid, 50 ug kg^{-1} for domoic acid, and 5 ug kg^{-1} or less for saxitoxin. The assays proved satisfactory when used over a 4-month period for the analysis of 110 real samples collected in Belgium.

Keywords: enzyme immunoassay; domoic acid; okadaic acid; saxitoxin; validation.

Introduction

Over the past two decades, the frequency, intensity, and geographic distribution of harmful algal blooms has increased, along with the number of toxic compounds found in the marine food chain. Different explanations for this trend have been given, such as increased scientific awareness of toxic algal species, increased utilisation of coastal waters for aquaculture, transfer of shellfish stocks from one area to another, cultural eutrophication due to domestic, industrial, and agricultural wastes, increased mobility of humic substances and trace metals from soil due to deforestation and/or acid rain, and unusual climate conditions (Hallegraeff et al. 1995).

Phycotoxins produced by algae pose a significant potential threat to human health. A variety of symptoms are reported to arise after consumption of contaminated shellfish, including stomach cramps, abdominal pain, diarrhoea, headaches, memory loss, paralysis, and in some extreme cases even death. In addition to the human health risk, the accumulation of these toxins in shellfish can also have severe consequences for the shellfish industry.

Monitoring the various toxin groups is therefore essential. Several families are responsible for poisonings, the symptoms and severity of which depend on the toxin family. Paralytic shellfish poisoning (PSP) is associated with a wide number of derivatives of saxitoxin (SAX). Diarrhetic shellfish poisoning (DSP) is caused by a group of polyether toxins including okadaic acid (OA), the dinophysistoxins (DTX-1, -2, -3...), pectenotoxin, and yessotoxin. Neurotoxic

1
2
3 shellfish poisoning (NSP) is the result of exposure to a group of polyethers
4
5 called brevetoxins, and amnesic shellfish poisoning (ASP) is caused by domoic
6
7 acid (DA).
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11 Because of the threat to human health, many countries (including European
12
13 Union Member States) have enacted regulations on the maximum permitted
14
15 levels of various phycotoxins. In the European Union, Regulation (EC) No
16
17 853/2004 lays down specific hygiene rules for food of animal origin. For live
18
19 bivalve molluscs, this regulation stipulates that, in addition to meeting
20
21 microbiological criteria and having organoleptic characteristics associated with
22
23 freshness and viability, live bivalve molluscs must not contain marine biotoxins
24
25 in total quantity measured in the whole body (or any edible part separately)
26
27 exceeding the following limits: 800 $\mu\text{g kg}^{-1}$ for the PSP toxins, 20 mg kg^{-1}
28
29 domoic acid equivalents for the ASP toxins, 160 $\mu\text{g kg}^{-1}$ okadaic acid
30
31 equivalents for okadaic acid, dinophysistoxins, and pectenotoxins together. For
32
33 yessotoxins and azaspiracids, the maximum permissible level is 1 mg kg^{-1}
34
35 yessotoxin equivalents and 160 $\mu\text{g kg}^{-1}$ azaspiracid equivalents.
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50 The method officially recognised by regulatory authorities for the detection of
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52 the aforementioned toxins is the mouse bioassay. Alternative methods can be
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54 used, provided they are no less effective and ensure the same level of public
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56 health protection. A series of instrumental methods (Hess et al. 2005; Diener et
57
58 al. 2006; AOAC 2005), immunoassays (Botre et al. 2000; Garthwaite et al.
59
60

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3 1998, 2001; Kawatsu et al. 2000, 2002; Morton et al. 1996; Osada et al. 1995,
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5
6 Smith et al. 1994, 1995; Vale et al. 1999; Renz et al. 1988; Usleber et al. 1991;
7
8
9 Traynor et al. 2006; Llamas et al. 2007; Fonfria et al 2007), and functional
10
11 assays have been developed for the determination of phycotoxins in shellfish.
12

13
14 This paper describes the development of rapid screening immunoassays for
15
16 detecting domoic acid, okadaic acid, and saxitoxin. Polyclonal antibodies were
17
18 raised against each of these compounds and used to develop the corresponding
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20 ELISAs, which were then used over a period exceeding 4 months for the
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22 analysis of 110 real samples collected in Belgium.
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31 **Materials and methods**

32 *Reagents, chemicals, and apparatus.*

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34 Domoic acid, okadaic acid, saxitoxin, and other phycotoxin standards and
35
36 certified reference materials (CRM-ASP-Mus-C, CRM-DSP-Mus-b) were
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38 purchased from the National Research Council of Canada's Institute for Marine
39
40 Biosciences (Halifax, Canada).
41
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46
47 Human serum albumin (HSA), bovine serum albumin (BSA), 1-ethyl-3-(3-
48
49 dimethylaminopropyl) carbodiimide, N-hydroxysuccinimide, and complete
50
51 Freund's adjuvant were obtained from Sigma (St Louis, MO, USA). New
52
53 Zealand female rabbits were used for immunisation. Nunc Maxisorp surface 96-
54
55 well polystyrene microtitre plates were purchased from Nunc (Roskilde,
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57
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1
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3 Denmark). All solvents were of LC or analytical reagent grade. Mussel, oyster,
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5 and scallop samples were obtained from local supermarkets.
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9 A Multiskan EX Thermo Fisher Scientific microplate reader (Zellik, Belgium)
10
11 was used to read the absorbance in the ELISA-plate wells at 450 nm.
12
13

14 *Preparation of immunogens.*

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16 Domoic acid and okadaic acid were conjugated with HSA by means of
17
18 carbodiimide and N-hydroxysuccinimide. To 50 μL dimethyl sulfoxide (DMSO)
19
20 containing 1 mg domoic acid or okadaic acid, 20 μL 1-ethyl-3-(3-
21
22 dimethylaminopropyl) carbodiimide hydrochloride solution (25 mg mL^{-1} in
23
24 DMSO) and 10 μL N-hydroxysuccinimide solution (30 mg mL^{-1} in DMSO) were
25
26 added. After stirring for 1.5 h at 25°C, 0.25 mL of HSA solution (20 mg mL^{-1} in
27
28 0.085 M borate buffer containing 0.06 M NaCl, pH 8.2) was added to the
29
30 mixture. After incubation for 1.5 h at 25°C, the reaction mixture was loaded
31
32 onto a PD-10 column (Pharmacia Biotech, Uppsala, Sweden) equilibrated with
33
34 phosphate buffered saline (PBS). After elution, the protein-containing portion
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36 (3.5 mL) was collected. The toxin-protein conjugates were then stored at -30°C
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38 until used.
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53 The saxitoxin immunogen was prepared according to Renz V and Terplan G
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55 (1988). Typically, BSA (2 mg) in sodium acetate buffer (0.1 mol L^{-1} , pH 4.4, 0.5
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57 mL) was mixed with saxitoxin diacetate (150 μg) in the presence of
58
59 formaldehyde (37% W/W, 40 μL). The reaction was left to proceed at room
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3 temperature for 72 h, then at 4°C for another 12 h. The reaction mixture was
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5
6 dialysed against 1 mmol L⁻¹ acetic acid solution for 6 h, then against deionised
7
8
9 water for 18 h. After dialysis the SAX-BSA was precipitated overnight at 4°C
10
11
12 with aluminium potassium sulfate (10%, 800 uL) and NaOH (1 mol L⁻¹, 500
13
14 uL). The resulting precipitate was washed six times with cold PBS, pH 7.2.

15 16 17 *Preparation of enzyme-labelled drugs.*

18
19
20 The two phycotoxins (domoic acid and okadaic acid) were conjugated to
21
22
23 horseradish peroxidase (HRP) enzyme label by the same method as for the
24
25
26 immunogens.

27
28 Saxitoxin was coupled to HRP by the periodate reaction [16]; HRP (4 mg) was
29
30
31 added to a sodium periodate solution (0.1 mol L⁻¹, 0.2 mL) and the solution was
32
33
34 gently mixed for 20 min at room temperature. The mixture was dialysed
35
36
37 overnight at 4°C against sodium acetate buffer, pH 4.4, and the pH of the final
38
39
40 solution was adjusted with carbonate buffer (0.2 mol L⁻¹, pH 9.6, 20 µL). The
41
42
43 activated enzyme was added to saxitoxin as an acetate salt (80 ug) in acetic acid
44
45
46 (0.1 mol L⁻¹, 250 uL) and the pH was adjusted to 7.5 with sodium carbonate
47
48
49 buffer (0.5 mol L⁻¹, pH 9.6).

50
51 After 45 min at room temperature, NaBH₄ (4 mg mL⁻¹, 0.1 mL) was added. The
52
53
54 mixture was incubated for 15 min at 4°C and finally dialysed against PBS (0.15
55
56
57 mol L⁻¹). The saxitoxin conjugate was stored at -20°C.

58 59 60 *Immunisation of rabbits.*

1
2
3 Immunogen emulsions were injected subcutaneously into four sites on the
4
5 animal. Rabbits were immunised every 28 days with 200 ug immunogen, and
6
7 blood samples were taken from the marginal vein of the ear 10 days after each
8
9 immunisation (from the third immunisation onward). Test bleeds were
10
11 monitored every 28 days for the presence of antibodies. When titres stabilised,
12
13 the rabbits were exsanguinated and the resulting antisera stored at -20°C.
14
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19
20 *Assessment of antibodies and enzyme conjugates.*
21

22 All antibodies and enzyme conjugates were initially assessed by means of
23
24 ELISA chequerboards to determine optimum working dilutions.
25
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28 *Specificity of antibodies.*
29

30 Specificity means the ability of a method to distinguish between the analyte
31
32 being measured and other substances. Sixty blank shellfish samples (20 mussel,
33
34 20 oyster, and 20 scallop samples) were analysed in blind duplicate.
35
36
37

38 The specificities of the three antibodies were assessed by determining the extent
39
40 to which each one cross-reacted, in the ELISA, with analytes having a chemical
41
42 structure similar to that used to raise the antibody. The degree of antibody
43
44 specificity within the amnesic, diarrhetic, and paralytic poison families was
45
46 estimated by means of the competitive ELISAs described in section 2.7. Cross-
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48 reactivity was calculated by means of the formula:
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ID 50 of compound used to raise antibody X 100

ID 50 of the competing compound

Competitive ELISA procedure.

Ninety-six-well Nunc Maxisorp microtitre plates (Roskilde, Denmark) were coated beforehand with purified sheep antibody directed against rabbit IgG (CER, Marloie, Belgium). The wells were filled with 50 μ L working standard prepared in assay buffer (the standard was chosen according to the residue to be detected), 50 μ L diluted sample, or 50 μ L assay buffer (Bo). The ELISA buffer composition was as follows: 0.15 M NaCl, 0.056 M Na₂HPO₄.2 H₂O, 0.009 M NaH₂PO₄.2 H₂O, 0.2% gelatin, 0.05% Tween 20, 0.01% 8-anilino-1-naphthalenesulfonic acid ammonium salt, and 0.0028 M ascorbic acid. Two additional wells were filled with 150 μ L assay buffer and were dedicated to nonspecific binding (NSB). The peroxidase conjugate and the antiserum (diluted to its optimal concentration in assay buffer), were then dispensed into the wells (the conjugate and antiserum were chosen according to the residue to be detected). The diluted antibody was added to each well except for the two wells corresponding to NSB. The microtitre plate was incubated at 4°C for 2 hours (OA and DA ELISAs) or overnight (SAX ELISA).

The wells were then emptied and washed three times with washing buffer (0.15 M NaCl, 0.05% Tween 20). A 150- μ L aliquot of chromogen mixture [(3, 3', 5, 5'-tetramethylbenzidine) (TMB) and enzyme substrate H₂O₂; v/v)] was added to each well. The enzyme reaction was stopped by addition of 6 M sulfuric acid

1
2
3 (50 μ L). The absorbance of the solution at 450 nm was read within 30 min. The
4
5
6 average optical density (OD_{450}) of the Bo wells, containing all constituents
7
8
9 except the phycotoxin competitor, was taken as 100% activity. Percent
10
11
12 inhibition was calculated as:

$$\frac{(OD_{450 \text{ test}} - OD_{450 \text{ NSB}})}{(OD_{450 \text{ Bo}} - OD_{450 \text{ NSB}})} \times 100$$

22 *Sample extraction.*

23
24
25 Tissues were homogenised in a blender (3 min), which was cleaned and rinsed
26
27
28 with methanol and distilled water after each sample. One gram of tissue
29
30
31 homogenate was vortexed for 1 min in the presence of 5 ml ethanol-water (9/1).
32
33
34 After centrifugation (10 min at 3000 g), the supernatant was decanted and the
35
36
37 tissues extracted again with a new aliquot (3 mL) of the same ethanol-water
38
39
40 mixture. The final volume of the extract was made up to 10.0 mL and filtered
41
42
43 through a 25-mm filter with a 0.45- μ m-pore-size nylon filter membrane
44
45
46 (Whatman). The extract was stored at 4°C and diluted in ELISA buffer prior to
47
48
49 ELISA-based detection and quantification.
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52

53 *Determination of detection capability.*

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55
56 The detection capability of an analytical procedure is defined in Decision
57
58
59 2002/657/EC as the smallest amount of a substance that can be detected,
60
identified, and/or quantified in a sample with an error probability of β .

1
2
3 Twenty blanks of known non-contaminated mussels and twenty blank mussel
4 samples fortified at 150 ug kg⁻¹ with domoic acid, 50 ug kg⁻¹ with okadaic acid,
5
6 or 2 ug kg⁻¹ with saxitoxin, were prepared "blind to the analyst" and analysed in
7
8 duplicate. The ELISA plates were then processed as previously described.
9
10

11
12 The detection capability CC_β (cut-off) was calculated as the concentration at
13
14 which less than 5% of false-compliant results remained. If 19 samples among 20
15
16 fortified samples were declared positive, then CC_β = level of fortification. If 18
17
18 or less of the fortified samples were declared positive, then CC_β > level of
19
20 fortification. If all the fortified samples were declared positive then CC_β < level
21
22 of fortification. The method was repeated by gradually increasing the level of
23
24 fortification until CC_β was determined. This validation procedure was repeated
25
26 for the other two matrices of interest (oyster and scallop).
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40 **Results and Discussion**

41
42 ELISA assays for the various toxin classes were developed. Emphasis was
43
44 placed on keeping the methods and all reagents as similar as possible in the
45
46 three assay procedures.
47
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51
52 The performance characteristics sensitivity, specificity, and detection capability
53
54 (CC_β) of the three ELISAs in screening for domoic acid, okadaic acid, and
55
56 saxitoxin in shellfish were determined in order to validate the ELISAs and to
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1
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3 test the possibility of using them to screen shellfish for low levels of
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6 phycotoxins. The three methods were validated according to the criteria of
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8
9 European Decision 2002/657/EC (Commission 2002) for a screening method.
10

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14 *Antibody sensitivity.*

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17 All three of the shellfish toxin immunogens resulted in the production of
18
19
20 antibodies. Sera exhibiting antibody titres were assessed for their sensitivity and
21
22
23 specificity. Each rabbit serum was tested with the corresponding peroxidase
24
25
26 conjugate.

27
28 The assay sensitivity was calculated as 50% inhibition of deviation (ID 50), the
29
30
31 concentration of shellfish toxin necessary to cause 50% antibody binding. The
32
33
34 competitive ELISA format described in previously section was used to
35
36
37 determine the assay sensitivity. A set of shellfish toxin standards (in the range of
38
39
40 0.005-10 ng mL⁻¹) was used. The average optical density of zero standard wells
41
42
43 (Bo) represents 100% activity and the remaining standards were normalised
44
45
46 with respect to the optical density of Bo.

47
48 Several parameters were tested to determine the optimal conditions for each
49
50
51 ELISA, notably incubation temperature and incubation time. These parameters
52
53
54 were evaluated by comparing the antibody sensitivity (ID 50) while taking into
55
56
57 account the practical requirements of potential users. First, the results obtained
58
59
60 at two incubation temperatures, 4 and 37°C were compared. The sensitivity was
substantially better (no data presented) when the incubation temperature was

1
2
3 4°C. Second, the results obtained at two incubation times, overnight or 2 h, were
4
5 compared. The best incubation times were 2 hours for domoic acid and okadaic
6
7 acid and overnight for saxitoxin. The best incubation temperature was 4°C for
8
9 the three ELISAs. Under these conditions, the respective mid-point of the DA,
10
11 OA, and SAX calibration curve (ID 50) obtained were 1.8 , 1.3 and 0.04 ng ml⁻¹.
12
13 Representative standard curves for domoic acid, okadaic acid and saxitoxin,
14
15 obtained with the optimised ELISA are shown respectively in Figures 1. It
16
17 appears that inhibition of binding of domoic acid was linear in the range 0.1 –
18
19 10 ng mL⁻¹. It was also reproducible in this range, its standard deviation being
20
21 no more than ± 5%.
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33 *Antibody specificity.*

34
35 The cross-reactivities in assay buffer of the anti-OA, anti-DA, and anti-SAX
36
37 antibodies are given in Table 1. The ELISAs were performed with increasing
38
39 amounts of competitor. When no ID 50 could be determined within the tested
40
41 concentration range because the binding calculated in percent was superior to
42
43 50% for the highest concentration, the ID used in the cross-reactivity formula
44
45 was the ID observed at that concentration and the cross-reactivity was recorded
46
47 as inferior to the result of the calculation.
48
49
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53
54 The anti-DA antibody showed 100% cross-reactivity with domoic acid and no
55
56 significant cross-reactivity with any other shellfish toxin. As the total domoic
57
58 acid concentration in the certified reference material (CRM) MUS-1 was
59
60

1
2
3 accurately determined (Table 2), it would appear that the antiserum cross-reacts
4 well with all major isomers of domoic acid and that this ELISA provides a
5
6 measurement of total domoic acid (the summed concentrations of the various
7
8 domoic acid isomers).
9

10
11
12
13
14 The anti-OA antibody was tested for cross-reactivity towards two OA analogues
15
16 of the same toxin family: the dinophysistoxins DTX-1 and DTX-2. It showed
17
18 78% cross-reactivity towards DTX-1 and 2.6% cross-reactivity towards DTX-2,
19
20 as compared to OA. An ELISA developed for DSP detection should detect
21
22 okadaic acid and the dinophysistoxins equally, but no such ELISA is available.
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The anti-OA antibody was tested for cross-reactivity towards two OA analogues of the same toxin family: the dinophysistoxins DTX-1 and DTX-2. It showed 78% cross-reactivity towards DTX-1 and 2.6% cross-reactivity towards DTX-2, as compared to OA. An ELISA developed for DSP detection should detect okadaic acid and the dinophysistoxins equally, but no such ELISA is available. Detection of DTX-3 requires an extra base hydrolysis step in the extract preparation procedure to remove the acyl group and convert the toxin to DTX-1. Commercially available ELISAs for this group include the DSP-Check[®] ELISA test kit from UBE Industries, Tokyo, Japan and the Rougier Bio-Tech[®] ELISA test kit. The former is used worldwide to screen for OA and DTX-1 at a claimed detection limit of 20 ng g⁻¹. The latter exploits a monoclonal anti-OA antibody and an anti-idiotypic antibody that competes with OA for binding sites on the anti-OA antibody. This antibody exhibits a much higher sensitivity (10-20 fold) for OA than for either DTX-1 or DTX-2, whereas DTX-3 does not cross-react at all.

The anti-SAX antiserum showed 100% cross-reactivity with saxitoxin, 26.2% with GTX 5 (gonyautoxin-5), 19.2% with decarbamoyl saxitoxin, and 5.6% with GTX 2/3 (gonyautoxin-2 and gonyautoxin-3). It showed very low cross-

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3 reactivity with neo-saxitoxin (1.4%). It is a well-recognised problem that all PSP
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5 antibodies fail to recognise all members of the toxin family and thereby
6
7 underestimate the total toxin content. This notably concerns the PSPs, as anti-
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9 SAX antibodies show poor cross-reactivity with neo-saxitoxin and vice versa
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11 (Usleber et al. 1994; Huang et al. 1996). The commercial ELISA test kits have
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13 only been developed for SAX. However, they are not totally specific for SAX
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15 and some reactions are induced to decarbamoyl-SAX (dcSAX) and neoSAX.
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17 Usleber et al. (1997) have concluded that ELISA results correlated well with
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19 mouse bioassay results when analysing scallops. However, Kasuga et al. (1996)
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21 have concluded that the mouse assay can not be replaced with ELISA for the
22
23 purpose of screening inshore shellfish samples, as unpredictable cross-reactions
24
25 have occurred, as well as underestimations of toxicity of some naturally
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27 contaminated shellfish samples, harvested in the sea near Japan. The
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29 predominance of GTX's and the lack of SAX in shellfish samples resulted in the
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31 underestimation of toxin content by the ELISA kit in comparison with the PSP
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33 mouse bioassay. The major advantages of ELISA are simplicity, and rapidity of
34
35 the test procedure, and a higher sensitivity compared with other methods.
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37 However, quantitative agreement between ELISA and mouse bioassay depends
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39 on the antibody specificity and on the toxin profile of the shellfish. Thus both
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41 over- and under-estimation of total toxicity may occur.
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58 In order to avoid the poor cross-reactivity of saxitoxin antibodies with neo-
59 saxitoxin and vice versa, one could use two or more ELISAs based on antibody
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3 recognition of different members of the same toxin family. Chu et al. (1996) has
4
5 demonstrated the feasibility of this approach, finding a very high correlation
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7 between the results mouse PSP bioassays and those obtained by summing the
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9 results of SAX and neo-SAX ELISAs. The ease and speed of ELISAs makes it
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11 relatively simple to conduct several ELISAs in parallel. The use of two ELISAs
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13 to cover a particular toxin group requires only a little additional work.
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19 Another approach [9] is to develop a competitive enzyme immunoassay based
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21 on a gonyautoxin 2/3 (GNTX2/3)-specific monoclonal antibody and a saxitoxin-
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23 horse radish peroxidase conjugate. GNTX2/3, dc-GNTX2/3, C1/2, GNTX1/4,
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25 STX and neoSTX were detectable at concentrations lower than the regulatory
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27 limit of 80 ug/100 g of shellfish tissue.
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33 As far as screening purposes are concerned, ELISAs offer major advantages
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35 compared with the mouse bioassay, which is criticized in Europe because of
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37 animal welfare. Considering the presence of cross-reactions with lower binding
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39 specificity and the potential lack of response to other toxins than saxitoxin
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41 within the PSP group, the practical application of these assays will probably
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43 remain limited, unless acceptable performance characteristics can be
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45 demonstrated in formal collaborative studies according to AOAC International
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47 or ISO accepted procedures.
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58 *Detection capability.*
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3 The smallest content of the substance that may be detected by each ELISA was
4 assessed by determining the detection capability ($CC\beta$) of the assay for the
5 shellfish toxin to be detected. To evaluate the performance of our ELISAs, three
6 matrices were chosen: mussel, oyster, and scallop. For each matrix, known
7 negative samples were unfortified (blank) and fortified with phycotoxins at
8 various concentrations, extracted, and analysed by means of the optimised
9 ELISAs. The detection capability was determined for each toxin in each matrix.
10
11 In the mussel, oyster and scallop matrix, the respective $CC\beta$ values for DA, OA,
12 were 150 and 50 $\mu\text{g kg}^{-1}$. For the saxitoxin, the respective $CC\beta$ values for
13 mussel, oyster and scallop matrix were 1, 2 and 5 $\mu\text{g kg}^{-1}$.

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The results presented in Figures 2, 3, 4 demonstrate that the blank and spiked
oyster samples met the $CC\beta$ criterion: all blank oyster samples gave a response
below the $CC\beta$, while at least 19 out of 20 spiked samples gave a signal above
the $CC\beta$ and were thus classified as suspect ($\beta=5\%$).

In practice, the means of the absorbance values obtained for each sample were
divided by the absorbance of the zero standard (B_0) and multiplied by 100. For
each group of 20 samples, the mean ratio B/B_0 (%) ± 2 standard deviations was
calculated (Figures 2, 3, 4). These data show that there was no B/B_0 (%) range
overlap between the blank and spiked samples.

For example, in the case of domoic acid in oyster (Figure 2), a 91% threshold is
the lower limit of the blank range and 89% is the upper limit of the spiked

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3 sample range (cut-off). In the routine application of such a qualitative method a
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6 sample was considered suspect if the binding percentage was below or equal the
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9 cut-off. Thus the assays developed could be used in highly reliable manner to
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12 differentiate between samples free from domoic acid or okadaic acid or
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14 saxitoxin from those with these phycotoxins above the $CC\beta$ values.
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20 *Extraction, recovery.*
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25 The present method has the advantage that a single solvent extraction system
26
27 adequately extracts the ASP, DSP, and PSP toxins. Ethanol is a preferred
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29 solvent because of its low toxicity and volatility. In most assays it causes lesser
30
31 matrix effects than methanol. The extraction efficiency was evaluated by
32
33 comparing the calibration curves constructed before and after extraction of blank
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35 matrices spiked with a known amount of phycotoxin. An extraction efficiency
36
37 above 90% was obtained for domoic acid, okadaic acid, and saxitoxin,
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39 indicating that the proposed extraction is suitable for use in screening for both
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41 aqueous and lipophilic toxins. Matrix effects of alcohol extracts in the ELISA
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43 were eliminated by dilution of the shellfish extract with PBS until the standard
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45 curves obtained for toxins dissolved in ELISA-buffer-diluted shellfish extract
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47 coincided with those obtained for toxins dissolved in ELISA buffer.
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52 Toxin recovery was determined by spiking shellfish samples with the target
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54 toxin and also by analysing the following CRMs: CRM-ASP-Mus-C and CRM-
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3 DSP-Mus-b. CRM-ASP-Mus-C is a mussel tissue homogenate containing 41 ± 2
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6 mg kg^{-1} domoic acid, as well as several related compounds (isomers). The sum
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9 of the concentration of domoic acid and C5'-epidomoic acid is certified at 44 ± 3
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12 mg kg^{-1} . In CRM-DSP-Mus-b, a CRM for okadaic acid and DTX-1, the toxin
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15 levels are 10.1 mg kg^{-1} for okadaic acid and 1.3 mg kg^{-1} for DTX-1.

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17 The results for recovery of DA, OA and saxitoxin added to the extracts of
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20 mussels, oysters and scallop are shown in table 2 . DA was detected in CRM-
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23 ASP-Mus-C with a mean recovery of 105% and coefficient of variation of 9%.

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26 OA was detected in CRM-DSP-Mus-b with a mean recovery of 109% and
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29 coefficient of variation of 11%. Saxitoxin added to the mussel was detected with
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32 a mean recovery of 112% and coefficient of variation of 8%.

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37 *Analysis of real samples from the Belgian market.*

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42 One hundred and ten samples (42 mussels, 61 scallops and 7 oysters) were
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45 collected in Belgium between October 2007 and January 2008. One hundred and
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48 three of these samples were collected by the Belgian Veterinary Inspectors for
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51 Public Health and seven by scientific personnel from different shops located in
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54 the south of Belgium (Walloon region). Samples were frozen and kept at
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57 approximately -20°C until analysis. The 110 real samples were analysed with
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60 the three validated ELISAs developed for screening shellfish for domoic acid,
okadaic acid, and saxitoxin. The analyses were performed on 11 different days

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3 over a period exceeding four months. Over this period, the methods showed
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5 good robustness. QC samples were analysed to check the performance of the
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7 assay. The results for negative QC samples and for positive QC samples spiked
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9 at the CC β concentration were very satisfactory. Reagent blanks were always
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11 found compliant and the spiked reagent blanks were always determined as
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13 suspect. This confirms that the detection capability set during the initial
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15 validation was an acceptable threshold value.
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22 Table 3 gives an overview of the contaminated samples. It was concluded that
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24 saxitoxin residues were present in one sample (mussel) at the concentration of 2
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26 $\mu\text{g kg}^{-1}$ and okadaic acid in one sample (mussel) at the CC β concentration.
27
28 Domoic acid was detected and quantified in 9 samples (concentration above the
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30 CC β). The incidence of domoic-acid-contaminated samples was 0% for mussel,
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32 0% for oyster, and 15% for scallop. The domoic acid content was above the 20
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34 mg kg^{-1} limit (the EU-adopted maximum level) in 5 samples (8%).
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42 Since the present validated ELISAs for domoic acid, okadaic acid, and saxitoxin
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44 in shellfish are screening methods, suspect samples need to be confirmed by
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46 mass spectrometry. The contaminated samples were analysed by LC-MS/MS to
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48 confirm their contaminated status and to avoid overestimation due to
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50 interference. Analysis was done on a Quattro Premier (Waters) mass
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52 spectrometer in the ESI mode, using traces 312>266 and 312>161 for
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54 quantification and confirmation of domoic acid (Holland et al. 2003) and traces
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56 804>255 and 804>113 for quantification and confirmation of okadaic acid (Fux
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3 et al. 2007). These analyses confirmed the contaminant identities and
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5 concentrations determined by the ELISAs.
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9 In addition, the ELISAs applied to mussel, oyster, and scallop samples
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11 successfully detected domoic acid and okadaic acid when they were shown to be
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13 present by LC-MS/MS. Hence, the occurrence of false negatives in the ELISAs
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15 for domoic acid and okadaic acid appears unlikely.
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22 **Conclusions**

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25 This work has led to the development of three quick, sensitive ELISAs for the
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27 detection of domoic acid, okadaic acid, and saxitoxin respectively in shellfish.
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31 The proposed technique requires little expensive apparatus or equipment. A
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33 major advantage of ELISAs over other methods is the ability to process
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35 relatively large numbers of samples over a short lapse of time. A single
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37 technician can prepare and extract up to 20 samples in approximately 4 hours.
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41 More rapid analyses of commercial shellfish samples would reduce the time the
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43 product must be held in storage and allow the prompt issue of the official
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45 documentation on shellfish toxin content required by some export markets. The
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47 reported methods have been validated according to the criteria laid down by the
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49 EU in Commission Decision 2002/657/EC and were used for more than 4
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51 months for the analysis of the 110 real samples collected in Belgium. The
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53 domoic acid content was above the 20 mg kg⁻¹ limit (EU-adopted maximum
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55 level) in 5 scallop samples (8%).
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For Peer Review Only

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21 Portuguese bivalves. *Toxicon* 37(11): 1565-77.
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Table 1: Cross-reactivities of polyclonal antibodies raised against domoic acid (DA), okadaic acid (OA), and saxitoxin (SAX) towards various marine toxins.

Marine toxin	Cross-reactivity (%)		
	DA	OA	SAX
Domoic acid	100	< 0.1	< 0.1
Okadaic acid	< 0.1	100	< 0.1
Dinophysistoxin DTX-1	< 0.1	78	< 0.1
Dinophysistoxin DTX-2	< 0.1	2.6	< 0.1
Saxitoxin	< 0.1	< 0.1	100
Decarbamoyl saxitoxin	< 0.1	< 0.1	19.2
Gonyautoxins GTX 2/3	< 0.1	< 0.1	5.6
GTX 5	< 0.1	< 0.1	26.2
Decarbamoyl GTX 2/3	< 0.1	< 0.1	0.2
Neosaxitoxin	< 0.1	< 0.1	1.4
Decarbamoyl neosaxitoxin	< 0.1	< 0.1	0.5
Gonyautoxins GTX 1/4	< 0.1	< 0.1	<0.1
C1 and C2	< 0.1	< 0.1	0.2
B ₁ and B ₂	< 0.1	< 0.1	<0.1

Table 2: Recovery or trueness, coefficient of variation (CV) obtained in analyses of domoic acid (DA), okadaic acid (OA), and saxitoxin (SAX) in mussel, oyster, and scallop.

Spiked sample or CRM	Mussel Recovery or <u>Trueness</u> (CV)	Oyster Recovery or <u>Trueness</u> (CV)	Scallop Recovery or <u>Trueness</u> (CV)
DA spiked sample (5 mg kg ⁻¹)	95% (11)	108% (13)	96% (10)
OA spiked sample (0.16 mg kg ⁻¹)	86% (7)	85% (6)	101% (9)
SAX spiked sample (0.005 mg kg ⁻¹)	112% (8)	116% (10)	106% (11)
CRM-ASP-Mus-C (44 mg kg ⁻¹)	<u>105%</u> (9)	-	-
CRM-DSP-Mus-b (11.4 mg kg ⁻¹)	<u>109%</u> (11)	-	-

Table 3: Domoic acid (DA), okadaic acid (OA), and saxitoxin (SAX) contamination in real samples of mussel, oyster, and scallop collected in Belgium.

	Domoic acid ELISA	Okadaic acid ELISA	Saxitoxin ELISA
Number of samples (mussel, oyster, scallop) analysed by each ELISA	110	110	110
Number of mussel samples analysed	42	42	42
Number of mussel (concentration > CC β)	0	1	1
Number of mussel (concentration > MRL)	0	0	0
Number of oyster samples analysed	7	7	7
Number of oyster (concentration > CC β)	0	0	0
Number of scallop samples analysed	61	61	61
Number of scallop (concentration > CC β)	9	0	0
Number of scallop (concentration > MRL)	5	0	0
Proportion of scallop (concentration > MRL)	8%	0%	0%

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3 **Figure 1:** ELISA standard curves for domoic acid (DA), okadaic acid (OA) and
4 saxitoxin (SAX) in assay buffer spiked respectively with DA, OA and SAX
5 standards. Calibration curve parameters: anti-DA antibody dilution, 1/16,000;
6 DA conjugate dilution, 1/4,000; incubation time, 2 hours; incubation
7 temperature, 4°C; anti-OA antibody dilution, 1/32,000; OA conjugate dilution,
8 1/32,000; incubation time, 2 hours; incubation temperature, 4°C; anti-SAX
9 antibody dilution, 1/32,000; SAX conjugate dilution, 1/1000; incubation time,
10 overnight; incubation temperature, 4°C. Under these conditions, the respective
11 mid-point of the DA, OA, and SAX calibration curve (ID 50) obtained were 1.8
12 , 1.3 and 0.04 ng ml⁻¹.
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34 **Figure 2:** Detection capability (CC_β): graphic representation of 20 blanks
35 (mean +/- 2 standard deviation) and 20 oyster samples spiked at 150 ug kg⁻¹
36 domoic acid (mean +/- 2 standard deviation).
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45 **Figure 3:** Detection capability (CC_β): graphic representation of 20 blanks
46 (mean +/- 2 standard deviation) and 20 oyster samples spiked at 50 ug kg⁻¹
47 okadaic acid (mean +/- 2 standard deviation).
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56 **Figure 4:** Detection capability (CC_β): graphic representation of 20 blanks
57 (mean +/- 2 standard deviation) and 20 oyster samples spiked at 2 ug kg⁻¹
58 saxitoxin (mean +/- 2 standard deviation).
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Figure 1:

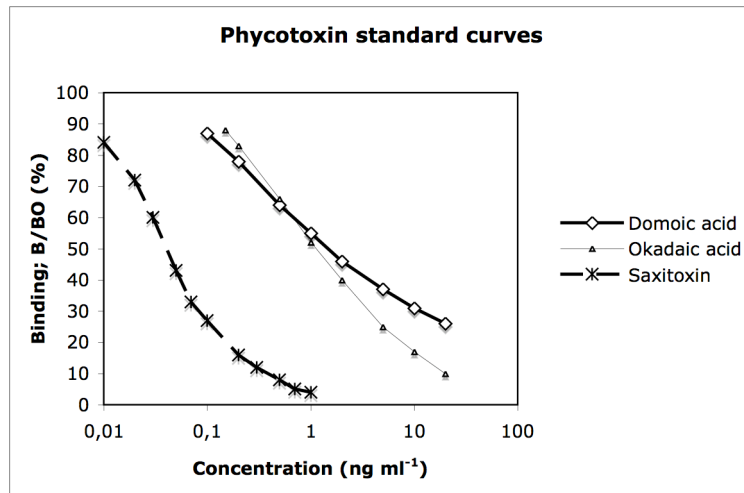
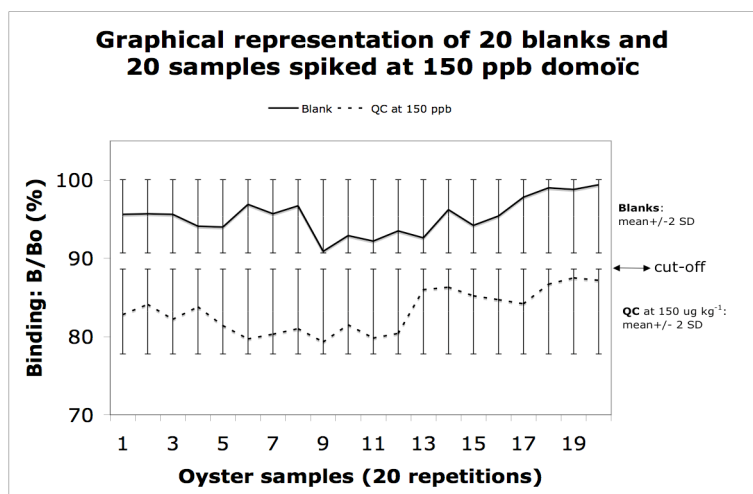
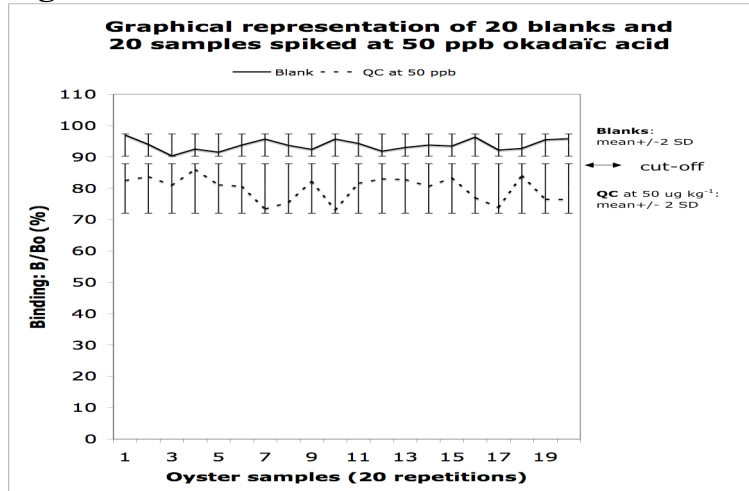


Figure 2:



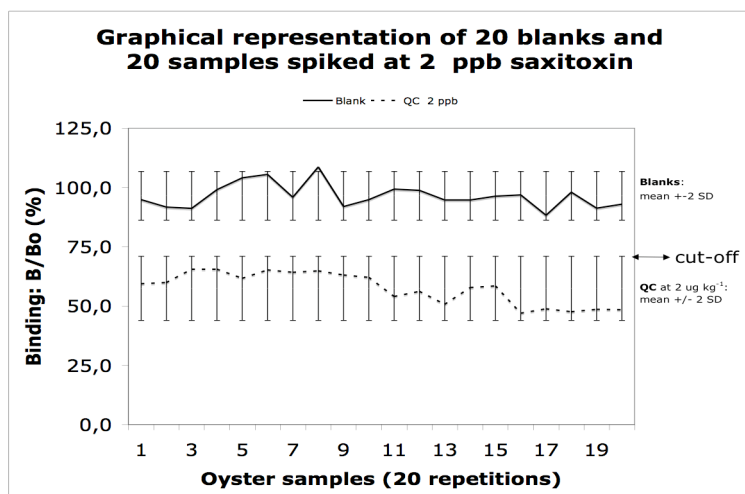
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Figure 3:



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Figure 4:



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