*CONffIDENCE: Contaminants in food and feed: Inexpensive detection for control of exposure*

# Progress towards the optoelectronic mouse for multi-shellfish toxin analysis

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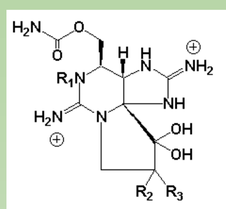
## Introduction

**Despite ethical and technical concerns the biological method, or more commonly referred mouse bioassay, remains as the reference method for phycotoxin analysis in shellfish. This is particularly the case for the global monitoring of diarrheic (DSP) and paralytic shellfish poisoning (PSP) and emerging toxins, whereas the detection of the amnesic shellfish poisoning (ASP) toxin domoic acid is performed by HPLC analysis. HPLC-FLD and LC-MS methods have been developed for PSP and DSP toxin analysis respectively but due to difficulties with both these procedures these techniques have not been fully implemented as a replacement to the mouse bioassay. In recent years, surface plasmon resonance (SPR) technology has been displayed as a highly promising immunoassay based tool since it offers rapid real time detection requiring minimal reagents and standards due to the microfluidic system. The latter being essential because of the limited availability and supply of toxin standards. Importantly, this technology has the capability of achieving the requirements set out by European legislation for an alternative method in terms of validation and transferability. Although, alternative methods of detection to the biological methods have been described, to date each analytical method is specific for individual toxins and their immediate analogues, with each toxin group requiring individual tests. The ideal scenario for the monitoring of marine biotoxins would be to incorporate as many of the toxins onto a single assay format - one test for all.**

## Aim

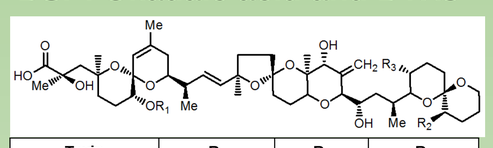
**The aim of this research was to develop a multiplex SPR biosensor assay capable of detecting a combination of PSP/DSP/ASP and emerging toxins. The major toxin targets to be detected in shellfish tissue for each group are:**

**PSP: Saxitoxin and analogues**



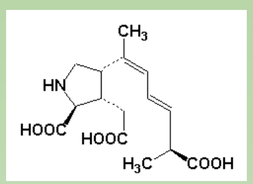
| STX | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| STX | H | H | H |
| GTX-II | H | H | $OSO_3^-$ |
| GTX-III | H | $OSO_3^-$ | H |
| NeoSTX | OH | H | H |
| GTX-I | OH | H | $OSO_3^-$ |
| GTX-IV | OH | $OSO_3^-$ | H |

**DSP: Okadaic acid and DTXs**

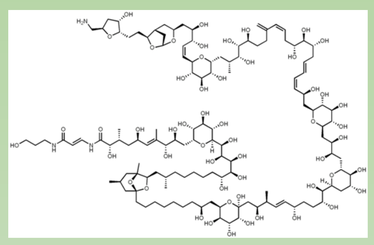


| Toxin | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| Okadaic Acid | H | H | H |
| DTX-1 | H | $CH_3$ | $CH_3$ |
| DTX-2 | H | $CH_3$ | $CH_3$ |

**ASP: Domoic acid (DA)**



**Emerging toxin: Palytoxin**



## Methods

### Multiplex Biosensor Analysis

**Optical surface plasmon resonance (SPR) biosensors are a fast and label-free alternative to classical binding assays such as ELISA, RIA and RRA. Their scope is limited by the cross-reactivity of the bio-recognition element. However, multiplexing can be achieved by the development of multi-flow cell SPR and/or by the development of multi-spots within a single SPR flow cell for different bio-recognitions. A prototype 16-plex (4 spots x 4 flow cells) SPR instrument was developed (Figure 1). This device is a molecular interaction array system that can perform concentration analysis of multiple analytes. Within CONffIDENCE this technology was applied to multiplex marine biotoxin analysis. A toxin assay was incorporated into each of the four flow cells of a single biosensor chip thus allowing samples to be analyzed under different cycle conditions. These toxin assays are performed using an indirect inhibition assay format, in which specific antibodies are mixed with standards / shellfish samples and run over a chip with immobilised target toxins. No toxin present response is high, toxin present response is low (Figure 2).**

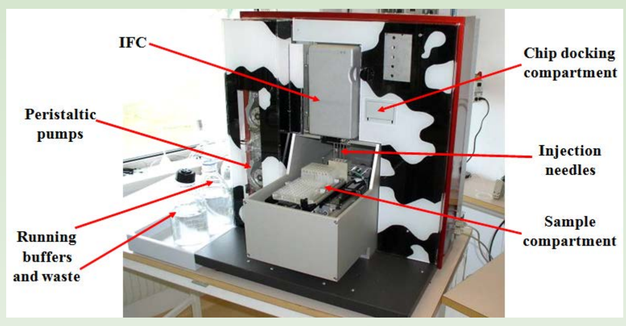



Figure 1: Biacore prototype multiplex biosensor

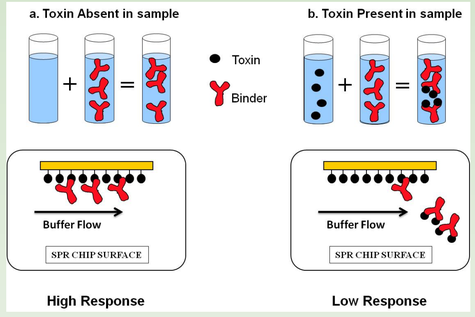



Figure 2: Schematic of SPR optical biosensor inhibition assay format

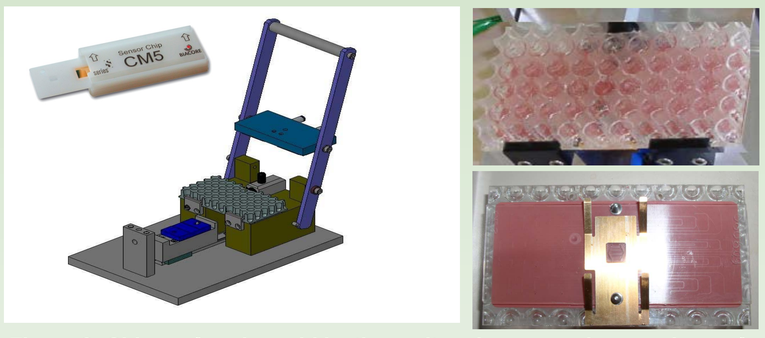



Figure 3: Chip surface immobilisation unit and upper and lower views of loading wells (x16 left and right) for immobilisation of components

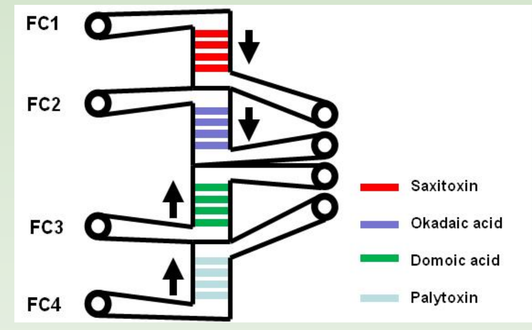



Figure 4: Schematic of chip surface with toxins immobilised as per flow cell of the instrument

### Chip Surface Immobilisation

**The chip surface immobilization unit (Figure 3) was designed with 16 isolated microfluidic channels for the immobilization of up to 16 different ligands on a chip surface. When docked on the prototype instrument, the sensor then forms 4 microfluidic flow cells with 4 immobilised spots within each flow cell (Figure 4). For the immobilisation of each toxin an amine surface was prepared in each flow cell. Domoic acid and okadaic acid were conjugated to the amine surface using carbodiimide chemistry whereas saxitoxin was conjugated using a modification of the Mannich reaction.**

### Assay Development

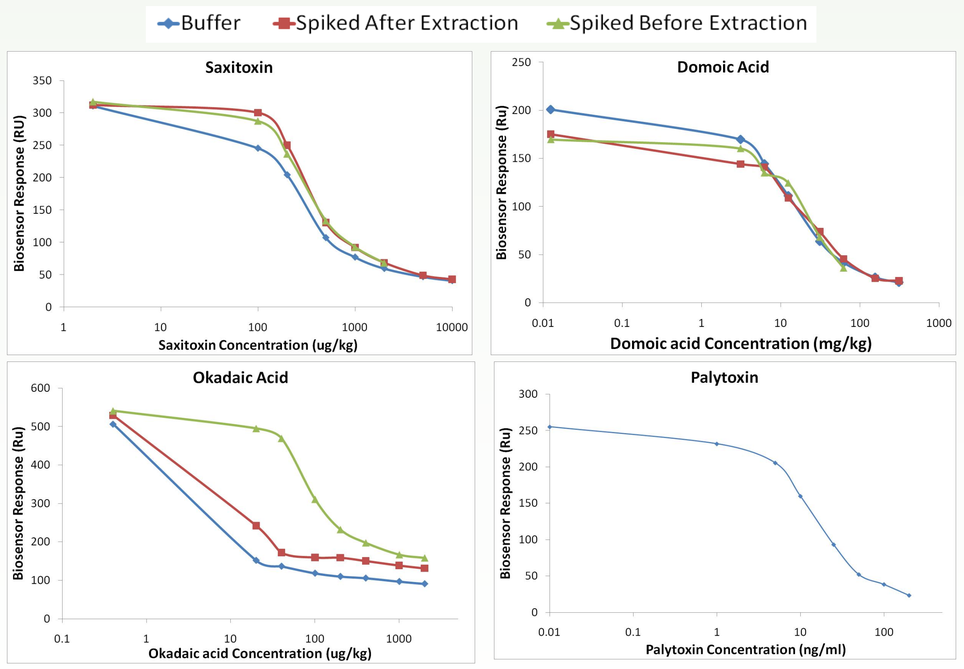
**Each flow cell with toxin immobilised was assessed using antibodies to each of the toxin targets. The antibody dilutions and regeneration solutions were optimised for each assay. Each antibody was mixed in a 1 to 1 ratio with toxin standards, injected over the chip surface, the binding response measured and the chip regenerated for the next analysis. Sample injection and regeneration is performed automatically.**

**Mussels were homogenised and extracted using a simple rapid alcohol extraction procedure followed by dilution in buffer. The matrix dilution applied was to achieve the sensitivity required for the European regulatory levels. Homogenised negative mussels were spiked at varying levels before and after extraction to determine the matrix effects on the assay and the extraction efficiency.**

## Results

**Calibration curves were produced on each flow cell for the specified toxin in buffer and mussel tissue spiked before and after extraction. Limited matrix effects were observed for saxitoxin and domoic acid but significant matrix effects and low extraction efficiency were observed for okadaic acid. The sensitivity ($IC_{50}$) achieved in the mussel extracted curves is suitable for the detection of these toxins at and below the regulatory level with buffer calibration curves being suitable for saxitoxin and domoic acid.**

| Toxin | Regulatory limit | Antibody | Matrix Dilution | $IC_{50}$ Extracted Curve | Dynamic Range ($IC_{20}$ to $IC_{80}$) Extracted Curve |
|---|---|---|---|---|---|
| Saxitoxin | 800µg STXeq/kg | QUB 7 | 1 in 40 | 348 µg/kg | 144 - 874 µg/kg |
| Domoic | 20mg/kg | 12D21D12F5 | 1 in 250 | 16 mg/kg | 4 - 53 mg/kg |
| Okadaic | 160µg/kg | R739 | 1 in 8 | 83 µg/kg | 36 - 192 µg/kg |
| Palytoxin | 30 µg/kg (EFSA) | PLTX MAB | N/A | 12 ng/ml | 4 – 33 ng/ml |




## Conclusions

**A single multiplex chip was produced for the simultaneous detection of PSP/DSP/ASP toxins and the emerging toxin in European waters, palytoxin.**

**A multiplex prototype biosensor assay was developed for the key European regulated toxins at the regulatory limits and calibration curves produced.**

**Final assay development for palytoxin is underway.**

**Reference:** ***Campbell et al.*****, 2011. Use of a novel micro-fluidic device to create arrays for multiplex analysis of large and small molecular weight compounds by surface plasmon resonance. Biosensors and Bioelectronics, 26 (6) 3029-3036.**

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