Project no. 513967

Project acronym: BIOTOXmarin

Project title:

DEVELOPMENT OF NOVEL ANALYTIC TOOLS FOR THE DETECTION OF MARINE BIOTOXINS

Instrument: STREP

Thematic Priority: PRIORITY 5, FOOD QUALITY AND SAFETY

Final Activity Report

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Project coordinator:
Prof. Dr.Dr. Heinz C. Schröder

Version 1

Project coordinator organisation:
Johannes Gutenberg Universität Mainz

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Part I: Project Execution

Introduction

The contamination of seafood with algal toxins can cause severe neuronal and gastrointestinal disorders but also allergies in human. Sporadic outbreaks of poisoning by ingestion of shellfish which have accumulated marine biotoxins have become a world-wide problem. The economic consequences caused by the production of marine biotoxins during algal blooms in the coastal regions are enormous. In this project, fast, simple and cost-effective detection methods for marine biotoxins in seafood as well as patient sera were developed, based on the application of high-affinity capture antibodies and novel artificial receptor mimics against the toxins. The new tools for the detection (and quantification) of marine biotoxins developed in the proposed project are based on the application of the new Polymer Instruction technology and the highly sensitive Integrated Optical Grating Coupler (IOGC) biosensor technology, and the use of high-affinity antibodies for sensitive ELISA und Western blotting techniques. User-friendly chip / dip-stick assay methods as well as new bioassays based on interaction of okadaic acid with phosphoprotein phosphatase 2A (microtitre-plate based PP2A inhibition assay) or the activation/phosphorylation of MAP kinase p38 were developed. The developed technologies were compared with existing techniques for evidence of improved efficiency and accuracy. Prototype kits were manufactured by the companies.

Contractors involved

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The state of the art

There are four forms of poisoning caused by consumption of contaminated seafood, which markedly differ in course and symptoms: paralytic shellfish poisoning (PSP), neurotoxic shellfish poisoning (NSP), amnesic shellfish poisoning (ASP) and diarrhetic shellfish poisoning (DSP). In addition, ciguatera fish poisoning (CFP) is a disorder associated with dinoflagellate toxins that accumulate in tropical fish meat. ASP can be a life-threatening syndrome. It is characterized by gastrointestinal symptoms such as nausea, vomiting, abdominal cramps and diarrhea as well as neurological symptoms. DSP causes gastrointestinal symptoms only. This disease is not fatal and characterized by severe diarrhea, nausea, vomiting, abdominal cramps and shivers. In contrast, NSP causes an intoxication syndrome with both neurological and gastrointestinal symptoms. NSP toxins can also cause respiratory asthma-like symptoms by formation of toxic aerosols through wave movements. PSP is a life-threatening syndrome like ASP. The symptoms are purely neurological and the onset is fast. The main PSP toxins are saxitoxins and gonyautoxins, NSP toxins are brevetoxins, ASP toxins are domoic acid and DSP toxins are okadaic acid, dinophysistoxins, pectenotoxins, azaspiracids and yessotoxins. The toxic dinoflagellates producing them can be isolated from both natural and anthropogenic algal blooms.

Work performed and results achieved

The main achievements and breakthroughs can be summarized as follows.

- **Isolation and characterisation of two new analogues of yessotoxin (YTX): 1-desulfo carboxyhomoYTX and 1-desulfo carboxyhomoYTX (Breakthrough: discovery of new biotoxins).**

- **Isolation and characterisation of novel analogues of oxazinin-1: oxazinin-4, -5, -6, and -7 (Breakthrough: discovery of new biotoxins).**

- **Method for larger-scale production and preparation of highly pure samples of yessotoxin (Commercial application: production of standards).**

- **Identification of novel spirolides: 27-hydroxy-13,19-didesmethylC and others (Breakthrough: discovery of new biotoxins).**

- **Development of a new LC-MS method for sensitive, specific and direct determination of palytoxin (P-PTX) (Breakthrough: discovery of new biotoxins).**

- **Development of a new method (HILIC/MS) for determination of domoic acid (DA) (Breakthrough: novel sensitive, specific and direct method for detection of marine biotoxins).**

- **Demonstration of apoptotic effect (increase in $[Ca^{2+}]_i$ level and caspase activities) of isolated marine biotoxins in neuronal cells (Yessotoxin, okadaic acid and others) in neuronal cells (Breakthrough: Elucidation of new bioactive effects of marine biotoxins).**

- **Preparation of high affinity monoclonal and polyclonal antibodies against marine biotoxins (yessotoxin, okadaic acid, domoic acid and others) (Commercial application: component of novel assays for detection of marine biotoxins).**

- **Development of a new competitive ELISA based on these antibodies (Commercial application: new assays for detection of marine biotoxins).**

- **Development of instructed polymer receptor phases with "molecular memory" for the isolation, identification and binding of marine biotoxins (Breakthrough: application of a novel principle for the detection and isolation of marine biotoxins).**

- **Development of cheap, rapid and user-friendly lateral flow assays for marine biotoxins (dip-stick / chip assay) for detection of biotoxins (Breakthrough: application of a novel principle for the detection of marine biotoxins).**

- **Development of a bioassay for detection of activation / phosphorylation of p38 MAP kinase (Breakthrough: assay based on a general marker for biotoxin exposure).**

- **Development of a highly sensitive immunosensor for marine biotoxins (detection range between 0.001-10 ng/ml) (Commercial application: assay for detection of marine biotoxins based on novel principle).**

- **Development of an optical waveguide sensor based on immobilized receptor phases and OWLS technology (Commercial application: novel principle for detection of marine biotoxins).**

- **Improvement of the sensitivity of the microtitre-plate based PP2A inhibition assay (Commercial application: routine assay for detection of marine biotoxins).**

This project which involved 4 companies had a strong focus on exploitation of the results: 5 patents have been approved and more than 10 patent applications have been submitted.

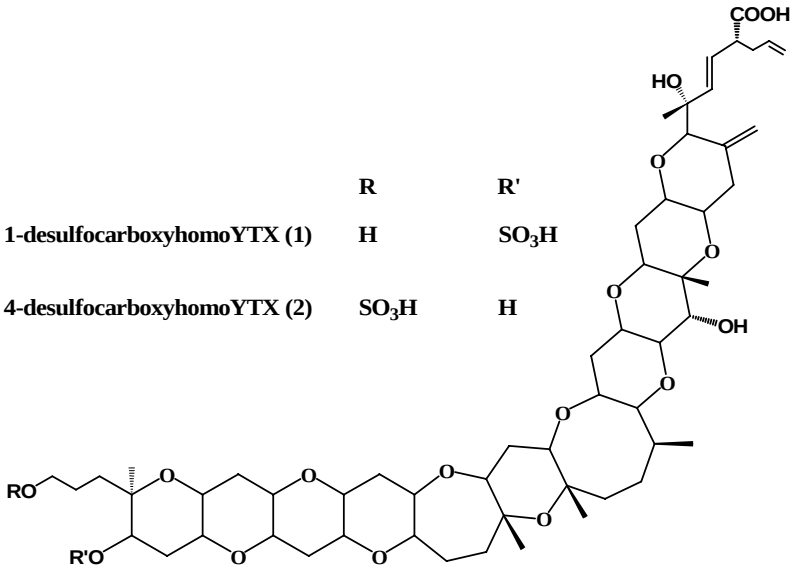
Moreover, the results of this project have been summarized in more than 15 publications in scientific journals and presented at scientific meetings / conferences.

1. Isolation and chemical characterization of marine biotoxins

1.1. Isolation and chemical characterization of marine biotoxins

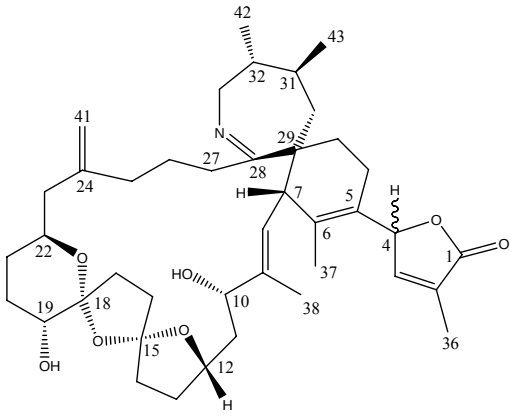
This project has provided further insights into the complex toxin profile, in particular in the Mediterranean Sea. Due to their potential noxious impact on aquaculture and human health, it is particularly worthy to emphasize the isolation and structural determination of a) desulfocarboxyhomoyessotoxins and b) spirolides in the Northern Adriatic Sea as well as c) the detection of palytoxins along almost the whole Italian coastline.

Desulfocarboxyhomoyessotoxins. Two new analogues of yessotoxin were isolated and characterized: 1-desulfo carboxyhomoYTX and 4-desulfo carboxyhomoYTX. The structure of the new toxins has been established in 1D- and 2D-NMR experiments.



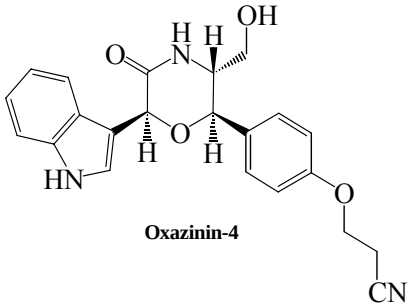
In spite of their small structural innovation in comparison to other yessotoxins, the occurrence of desulfocarboxyhomoyessotoxins in Adriatic mussels seriously affects some very recent EU law provisions. In fact, these new desulfoyessotoxins - instead of concentrating into the typical hydromethanolic layer - are unexpectedly recovered in the lipophilic layer (where DSP toxins are usually extracted), thus failing the purpose of a recent EU protocol set up with the aim of separating yessotoxins from DSP-toxins. Hence a revision of the EU control procedure is urgently needed.

Spirolides. *Alexandrium ostenfeldii*, a dinoflagellate that has bloomed in the Adriatic Sea since November 2003, nowadays represents the major source of biotoxin contamination in that sea. Analysis of the toxin profile of Adriatic *A. ostenfeldii* cultures as well as that of mussels grown in areas massively infested by this dinoflagellate led us to detect, among others, a novel spirolide (27-OH-13,19-didesmethyl spirolide C). During the algal bloom, *A. ostenfeldii* was producing fairly high amounts of spirolide 13-desmethyl C together with some unknown spirolide isomers. The spirolides (SPXs) are a group of macrocyclic imines first identified in extracts of mussels and scallops from Nova Scotia, Canada.

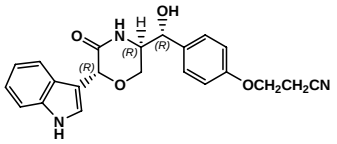


13-Desmethylspirolide C

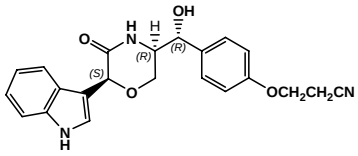
The analysis of toxic mussels collected along the coast of the Northern Adriatic Sea also allowed the isolation and full characterisation of novel analogues of oxazinin-1, namely oxazinin-4, -5, -6, and -7. Their stereostructures have been established on the basis of MS and NMR data. The elucidation of the absolute stereochemistry of these new oxazinin analogues was achieved by the perfect overlapping of their CD spectra with those of synthetic models.



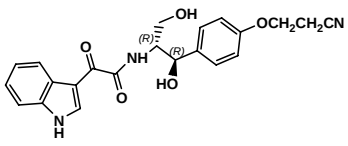
Oxazinin-4



oxazinin-5



oxazinin-6



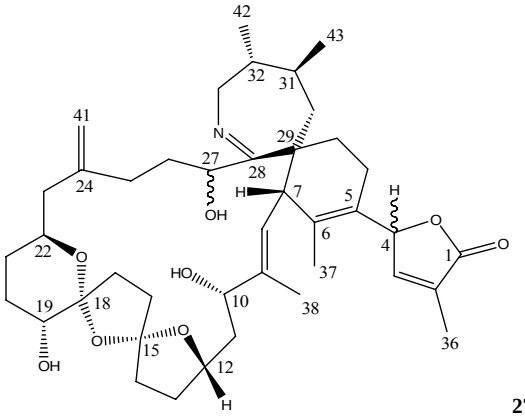
preoxazinin-7

Palytoxins. A new threat to human health is currently impending over the Mediterranean Sea: *Ostropsis ovata* that has caused alarming toxic outbreaks since summer 2005. Relying on a new LC-MS method set up in frame of this project, we were able to identify palytoxin - a marine toxin ranking among the most potent natural molecules so far known – and a new much more abundant palytoxin-like molecule (ovatoxin-a) as the causative agents of all these toxic events.

1.2. Isolation of the main phycotoxins from cultured toxic producer organisms

Cultures of *Protoceratium reticulatum* were extracted in order to provide pure samples of yessotoxin (YTX) suitable for bioactivity studies. In order to obtain sufficient amounts of yessotoxin and its analogues for biological studies, the optimal growth conditions for this organism were investigated, in terms of different salinity and nutrient (N/P) conditions. Yessotoxin was ozonized and coupled to ovalbumin for raising antibodies. Quantification of the compound was performed by HPLC/MS.

Analysis of large scale batch culture of *A. ostenfeldii* by LC/MS techniques allowed the identification of several spirolides (most of them unknown). The major compound was 27-hydroxy-13,19-didesmethylC. The planar structure of this new spirolide, never isolated before, was elucidated through extensive NMR investigation and MS/MS analyses.



27-Hydroxy-13,19-didesmethylspirolide C

Gonyaulax spinifera cells were isolated from the Adriatic seawater and successfully cultured. LC/MS analysis led us to identify *Gonyaulax spinifera* as another producer of yessotoxin. Its large scale culture could be used as a further source of YTX.

1.3. Development of LC-MS monitoring methods for determination of marine biotoxins in mollusks and cells

In 2005, serious respiratory distress was observed in humans exposed to marine aerosols on the beach of Genova (Italy). The toxic outbreak co-occurred with a massive proliferation of the tropical microalga *O. ovata* in seawater and disappeared when the *O. ovata* population decreased. We developed a new method for sensitive, specific and direct determination of palytoxin (P-PTX) based on combination of reversed phase liquid chromatography with mass spectrometry (LC-MS). Spiking experiments before and after extraction allowed the assessment of limits of detection and quantitation for palytoxin, accuracy, intra-day and inter-day reproducibility of the method. In 2006 a monitoring program was carried out; the collected samples were analyzed for the presence of palytoxin by the newly developed method.

LC-MS analyses indicated the occurrence of palytoxin along with a much more abundant palytoxin-like compound never reported so far, which we named ovatoxin-a. On the basis of molecular formula, fragmentation pattern, and chromatographic behavior, the structure of ovatoxin-a appeared to be strictly related to that of palytoxin. We also run analyses of cultured *O. ovata* to unequivocally demonstrate that putative palytoxin and ovatoxin-a contained in field samples were actually produced by *O. ovata* itself.

In addition, a new method for the sensitive, specific and direct determination of domoic acid (DA), the causative toxin of ASP syndrome, was developed. This method is

based on a combination of hydrophilic interaction liquid chromatography with mass spectrometry (HILIC/MS).

1.4. Determination of the bioactivity of the isolated marine biotoxins

P1 determined the effect of toxic supernatants from dinoflagellates and isolated marine biotoxins (pure compounds) on viability of primary neuronal cells and PC12 cells (MTT assay) and on intracellular free calcium concentration $[Ca^{2+}]_i$ (fura-2 method) in these cells. In addition, the effect of the toxic supernatants and the isolated toxins on caspase activities involved in apoptotic pathway was determined using various fluorogenic substrates (neuronal cells). Yessotoxin as well as various dinoflagellate supernantants (Fig. 1) caused an increase in $[Ca^{2+}]_i$ level both in neuronal cells and in PC12 cells. Okadaic acid and yessotoxin caused a significant increase in caspase activities (apoptotic effect) in both PC12 cells and neuronal cells. Okadaic acid and okadaic acid-containing culture supernanants from various dinoflagellates induced an increase in specific activities of caspase-1, caspase-3 and caspase-6 in primary neuronal cells and at a lower extent, also in PC12 cells.

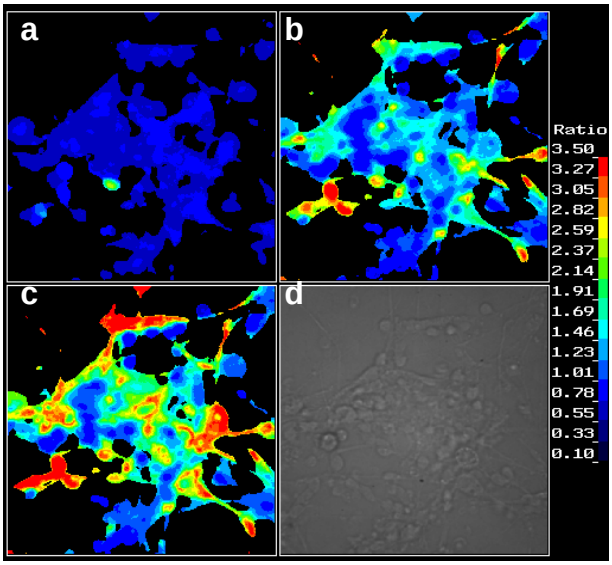


Fig. 1. Changes of intracellular free calcium level ($[Ca^{2+}]_i$) in primary neuronal cells after addition of dinoflagellate supernatants. The 340/380 nm ratios are shown. (**a**) Primary neuronal cells before addition of dinoflagellate supernatant; (**b**) 1 min and 30 s after addition of dinoflagellate supernatant; (**d**) and at the end of the measurement (10 min after addition of dinoflagellate supernatant). After incubation one light photo of was made (**d**). Magnification x 400.

2. Preparation of antibodies against marine biotoxins and development of sensitive immunoassays

We prepared monoclonal and polyclonal antibodies against a series of marine biotoxins: okadaic acid, yessotoxin, domoic acid and brevetoxin. The antigens (biotoxins) were either isolated within the consortium from dinoflagellate supernatants (yessotoxin) or purchased from commercial sources (okadaic acid, domoic acid, brevetoxin and palytoxin). The antibody conjugates used for the immunization of mice and rabbits were prepared by coupling to the peptide FID33 (33 amino acids; okadaic acid and domoic acid via their COOH group, yessotoxin after bromination), or to ovalbumin (domoic acid, yessotoxin after treatment with ozone), or to KLH (brevetoxin). The toxin conjugates were injected into both Balb/c mice and

rabbit. The preparation of monoclonal antibodies was performed following established techniques.

Instead of the FID33 conjugate of yessotoxin (brominated yessotoxin), a yessotoxin-ovalbumin conjugate was also successfully used for immunization of mice and and rabbits, and preparation of monoclonal antibodies. Yessotoxin was conjugated to ovalbumin after treatment with ozone. Experiments to raise antibodies against palytoxin were not successful because of the extremely high toxicity of this biotoxin.

Anti-domoic acid antibodies (monoclonal and polyclonal) were obtained by using both FID33 conjugate of domoic acid or a domoic acid-ovalbumin conjugate.

The antibodies were used in competitive ELISA. The principle of the competitive ELISA is shown in Fig. 2. The biotoxin is covalently linked to an ELISA plate (A). The plate is then incubated with the purified (monoclonal or polyclonal) antibody against the biotoxin (A,B). If the extract to be tested contains the biotoxin, competition for the bound antibody and thus dissociation of the bound antibody from the plate will occur (C,D). In the next step a specific peroxidase-conjugated secondary antibody is bound to the remaining antibody on the plate (E). This complex formation is visualized using the peroxidase substrate TMB (F).

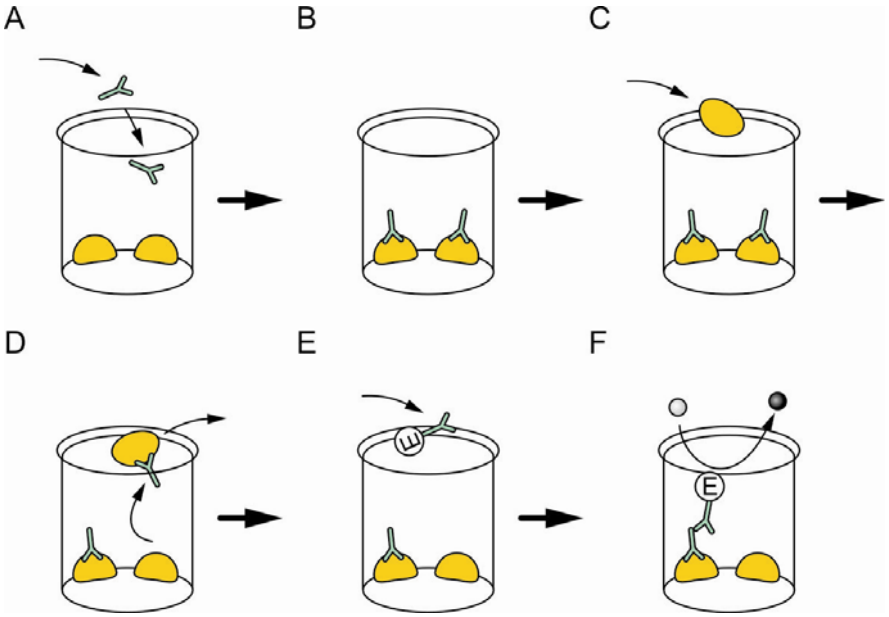


Fig. 2. Principle of the competitive ELISA test.

The detection limit using the monoclonal anti-okadaic acid antibody was 5 ng/ml. The detection limit using the purified polyclonal anti-okadaic acid serum (rabbit) in competitive ELISA was approximately 1 ng/ml.

Validation of the competitive ELISAs was performed using hepatopancreas extracts of contaminated mussels (okadaic acid) ands controls. The extracts were prepared in 80% methanol (80/20 MeOH/water v/v).

The anti-yessotoxin, anti-domoic acid and anti-okadaic acid antibodies were also used in Western blotting and immunofluorescence microscopy experiments. Hepatopancreas extracts from polluted and non-polluted mussels *Mytilus galloprovincialis* were tested. Extracts from polluted samples showed a strongly positive reaction, whereas extracts from non-polluted mussels only weakly reacted with the antibodies. The immunoblotting experiments revealed that okadaic acid and domoic acid are bound to specific proteins.

In a further set of experiments the antibodies were used for detection of okadaic acid in bacteria containing sponge tissue. By applying the polyclonal antibodies against okadaic

acid, the bacteria become brightly stained in immunohistochemistry. The technique of electron immunogold labeling was applied to identify the structures which reacted with the antibodies.

3. Development of Instruction receptor phases for the isolation, identification and binding of marine biotoxins

The PolymerInstruction technology is based on three steps: in the first step linear polymers with specific receptor groups, with "docking positions", are produced (Fig. 3, left). In a second step, the resulting functionalized polymers are connected, defining the flexibility and porosity of the polymer network (Fig. 3, right). The mesh of this net is then tailored to the size and functionality of the substance which has to be recognized (induced fit); Fig. 4. The instructed polymer has a "molecular memory"; it is able to recognize the substance/template or molecules with a structure similar to the template even in complex mixtures.

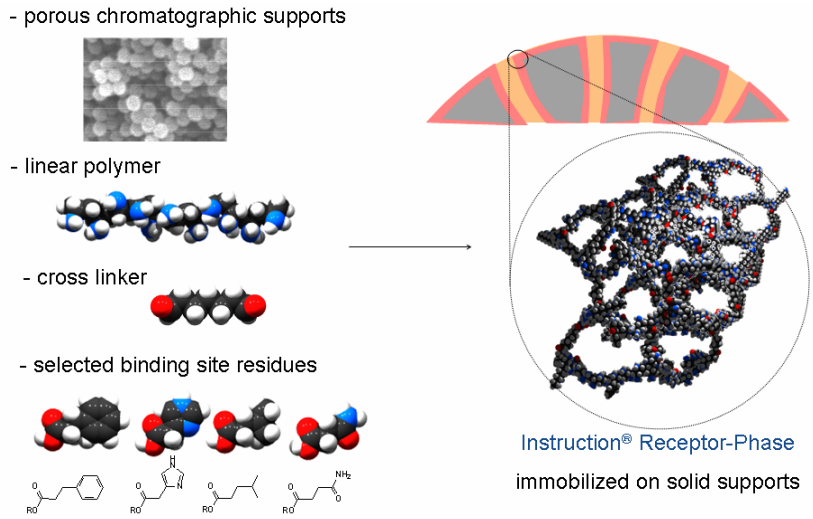


Fig. 3. Instruction receptor-phases. Overview of the phase design.

A large number of 140 Instruction® Receptor-Phases have been designed, synthesized and characterized. Most promising candidates were designed using silica as inorganic chromatographic support. Polyvinylamine turned out as most suitable basis polymer. Binding experiments were performed together with the project partners. To obtain additional Receptor-Phases with improved design and potential affinity to okadaic acid and other marine biotoxins, such as azaspiracides, ciguatoxin, dinophysistoxin, domoic acid, pectenotoxin, saxitoxin, tetradotoxin and yessotoxin, different combinations of binding site residues were incorporated to the polymeric network. Chemical composition, degree of substitution, degree of cross-linking and binding capacity of the developed Receptor-Phases were determined by suspension-NMR spectroscopy, inverse SEC, CHN-analysis and titration. We succeeded to identify affinity binding site residues with high retention for okadaic acid. The specificity and binding strengths were adjusted, designed and improved by "training" substances with functional groups similar to the biotoxins (okadaic acid).

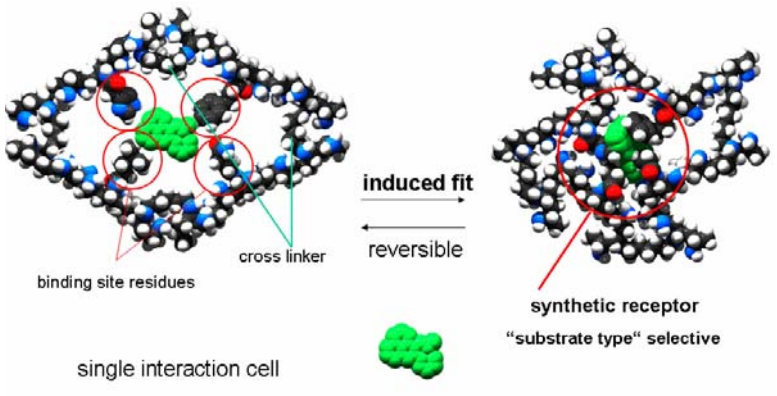


Fig. 4. Substrate/ligand interaction. Reversible induced fit.

The improved receptor phases were immobilized to appropriate sensor surfaces. A new immobilization method was developed to coat Receptor-Phases onto planar non-porous silica surfaces. Optical waveguide sensors were coated with layers of the Receptor-Phases which had been proved to show a potential specificity to bind okadaic acid. These coated chips were examined for their applicability as a substitute for antibodies to detect biotoxins using the Integrated Optical Grating Coupler (IOGC) sensor technology. Surface characterization by RIM showed the success of the developed immobilization method. The Receptor-Phase layer was even, stable and smooth and therefore well applicable to the IOGC technology. The experiments indicated that optical waveguide sensors coated with Instruction® Receptor-Phases may be applicable for a sensitive detection of marine biotoxins using IOGC sensor technology. Receptor-Phases are chemically designed polymers with a unique stability and high cost advantage compared to monoclonal IgGs for application in mass screenings.

4. Development of chips for detection and quantification of marine biotoxins

We developed a cheap, rapid and user-friendly stick assay for detection of biotoxins. The development of this assay is based on the antibodies prepared in this project. Diverse principles of the target immunodetection methods have been established. The immunochromatographic assay is based on the transport of tag-labelled antibody (or antigen) to its binding partner-specific antigen (antibody) immobilized on the surface of a membrane. Methods for stable localization of okadaic acid and domoic acid on nitrocellulose membranes have been developed.

This principle of the assay is as follows (Fig. 5A). The biotoxin-specific antibody is labeled with polystyrene microspheres. They are applied on the glass fibre release pad. The transfer is induced by the capillary power of the aqueous buffer through membrane pores of a defined size. While following the flow path, the sample first comes in contact with the tag-labelled primary antibody, which is bound to the biotoxin. According to principle of competitive displacement, the released tag-labelled antibody migrates to a capture zone of membrane-immobilized biotoxin and membrane-immobilized secondary antibody. Result: Applying samples without biotoxin, there is a strong signal on the test line (immobilized biotoxin) and a weaker one on the control line (immobilized secondary antibody). Applying the same system with biotoxin, the signal on the test line (immobilized biotoxin) got weaker and the one on the control line stronger regarding the negative control (Fig. 5B). A prototype of the chip / dip-stick assay has been developed.

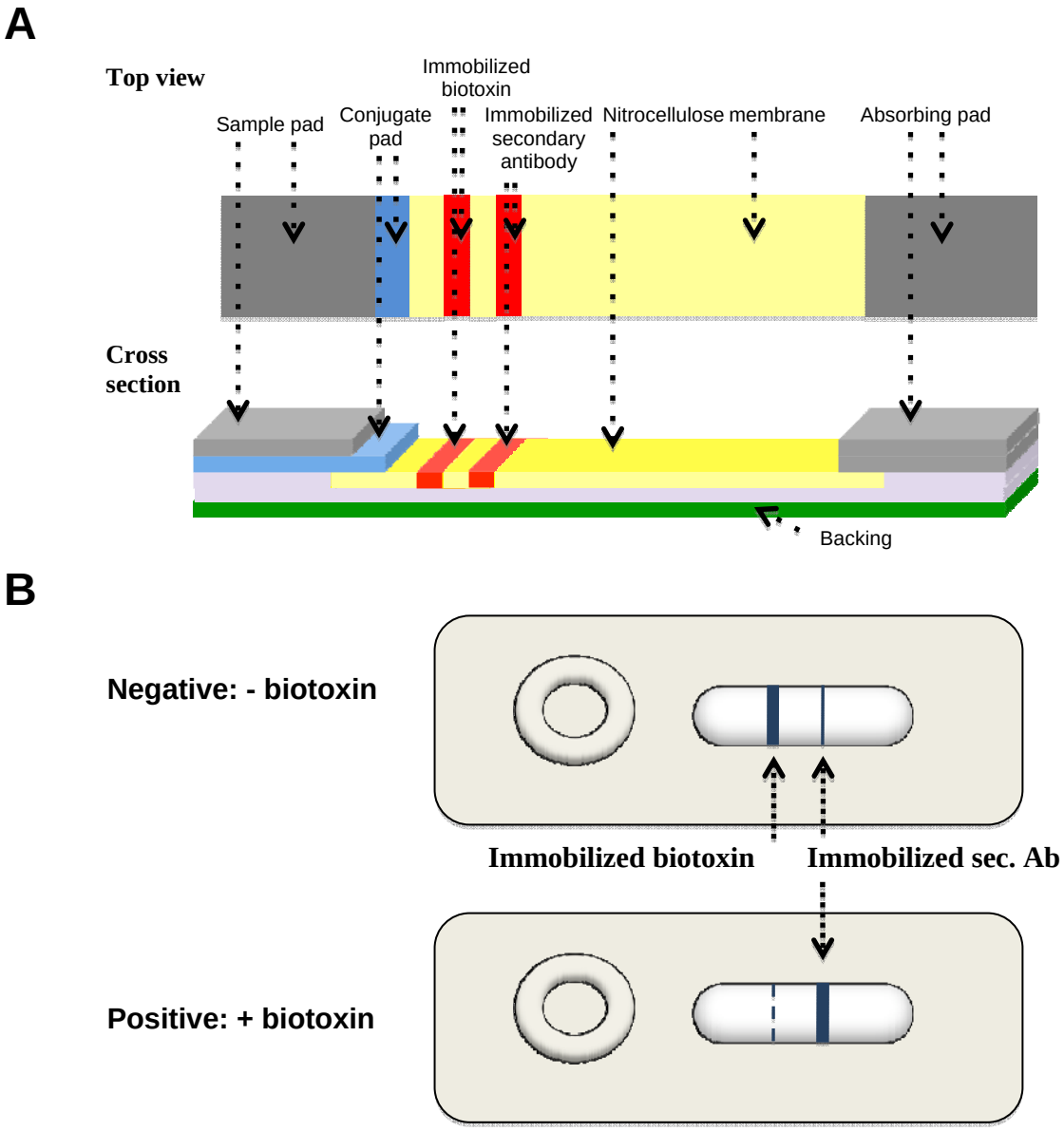


Fig. 5. (A) Principle of the lateral flow assay (chip / dip-stick assay). **(B)** Results obtained with negative (minus biotoxin and positive (plus biotoxin) sample.

In addition, we developed an enzyme-linked immunosorbent assay (ELISA) for detection of the phosphorylation level of the p38 MAP kinase p38 (a general marker for biotoxin exposure). This ELISA assay is based on the ability of the anti-pp38 antibody to bind with high affinity to a peptide containing the phosphorylated TGY recognition motif. A pair of antibodies directed against the phosphorylated MAPK and the non-phosphorylated MAPK is used. These antibodies were prepared against a phosphorylated MAPK peptide and a non-phosphorylated MAPK peptide which were coupled to ovalbumin. This assay has been used to detect the increase in phosphorylated p38 in mussels contaminated with okadaic acid.

5. Development of biosensors based on the OWLS technology

We developed an immunosensor for marine biotoxins based on the OWLS technology. Epoxy functionalized waveguide surfaces were used for covalent immobilization of the biomolecules. Real-time measurements revealed that the epoxi functionalized sensor surface is stable, gives reproducible association curves. The sensor chip can be regenerated several

times without loosing activity after the antibody has been bound to the immobilized antigen. We optimized the regeneration solution so that it gives enough dissociation strength to break the antibody-antigen binding but not the covalent binding of the conjugated antibody to the sensor surface.

In experiments to detect okadaic acid in a direct manner anti-okadaic acid antibodies were immobilized on the amino modified sensor surface; sensor responses were obtained at a concentration of 1 ng/ml. In the next step, a competitive immunoassay was developed to obtain a higher sensitivity of the assay. In the competitive assay, standard solutions containing okadaic acid were mixed with monoclonal antibodies. The mixture was incubated and then injected into the OWLS measuring system (Fig. 6). The amino silanized sensors were activated with glutaraldehyde to immobilize the okadaic acid conjugate (okadaic acid-ovalbumin) to the sensor surface. The optimal dilution of the monoclonal anti OA antibody for the competitive assay was determined. Using optimal parameters, the detection range of the competitive detection method was between 0.001-10 ng/ml (Fig. 7).

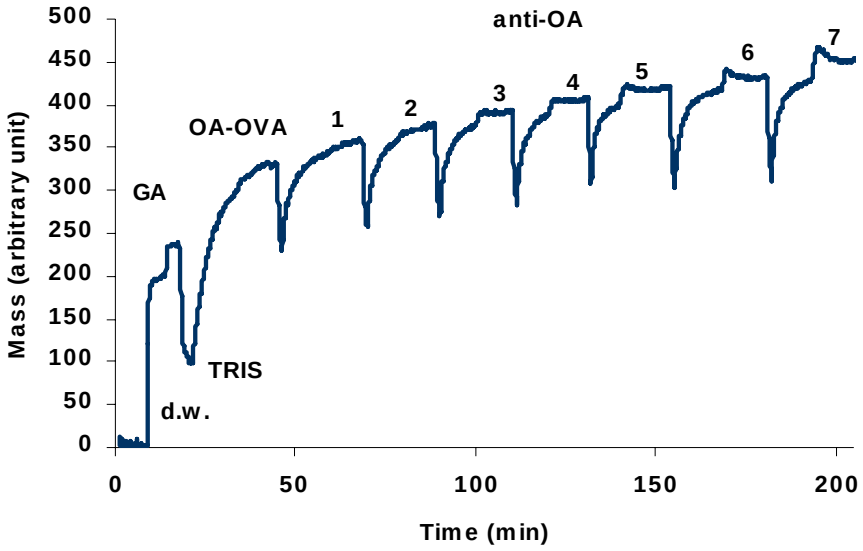


Fig. 6. Typical sensor response showing immobilization of glutaraldehyde (GA), okadaic acid-ovalbumin (OA-OVA), and different concentration of antibody solutions. Antibodies bound to the OA-conjugate were washed off with 50 mM HCl after each cycle. (Dilution of antibody; 1: 1000-fold; 2: 500-fold; 3: 250-fold; 4: 167-fold; 5: 125-fold; 6: 100-fold; 7: 50-fold).

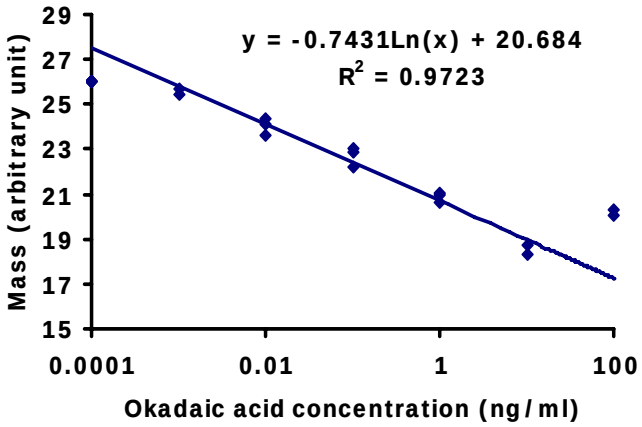


Fig. 7. Calibration curve for indirect measurement of okadaic acid.

6. Development of a microtitre-plate based PP2A inhibition assay

Experiments to optimize the assay conditions of the protein phosphatase inhibition assay revealed that *p*-nitrophenyl phosphate can be used as a substrate of the PP2A purified from *Buccinum undatum*. We were successful to develop a procedure for purification of PP2A from *B. undatum*. The purified enzyme cross-reacts with anti-human PP2A antibodies. The purification procedure can also be applied to other species. In the reporting period, we also succeeded to clone a phosphoprotein phosphatase (PP1) from a sponge, which is active also at low temperature. The sensitivity of the PP2A assay was improved. Alternative solid phase extraction cartridges were investigated and tested with different shellfish tissue matrices. Comparitive analysis of these samples and study of recovery was carried out using LC-MS/MS. DSP positive material was prepared from negative mussel homogenate and certified material. This was distributed for analysis by all partners within the project. Data obtained for the material was collected for comparison.

7. Development of a bioassay based on activation of MAP kinases

We developed an immunoassay for detection of total and activated/phosphorylated p38 MAP kinase (Western blot and/or ELISA) for monitoring the health state of mollusks and possible exposure to marine biotoxins.

The tissue distribution of p38 MAP kinase was investigated in oyster *Crassostrea gigas* and scallop *Pecten maximus.* The experiments revealed a significant expression of p38 MAP kinase in the heart but not in the digestive gland. These results show that heart cells are of interest as an *in vitro* model for toxicological studies. In addition, we demonstrated by immunocytochemistry that okadaic acid is accumulated in cultured oyster heart cells.

The expression and activation of p38 after exposure to okadaic acid and/or lipopolysaccharide (LPS) was studied using a sandwich ELISA that measures the concentration of phosphorylated p38 MAP kinase. Cell lysates of okadaic acid or LPS-treated heart showed an increase in pp38 MAP kinase compared with the untreated control (ELISA; Fig. 8A). These results are in agreement with the results of the Western blot (Fig. 8B).

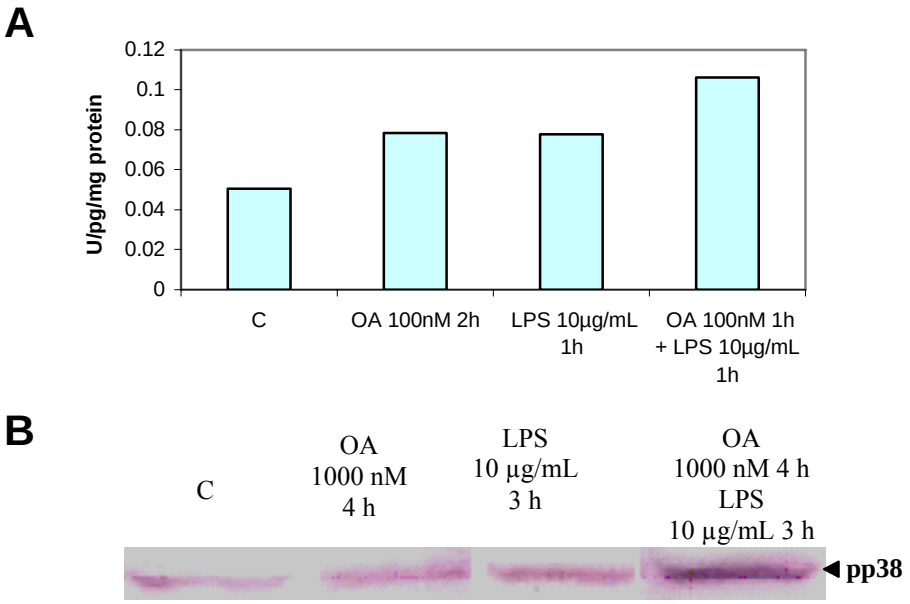


Fig. 8. *In vivo* p38 activation/phosphorylation. Cell lysates of *C.gigas* heart were collected after incubation with OA and/or LPS for the indicated times, and evaluated for pp38 MAP kinase concentration using the ELISA kit (A) or were subjected to Western immunoblotting using the appropriate antibody against phosphorylated p38 MAP kinase (B).

OA was found to exert a positive inotropic effect in cultured cells isolated from the atrium of oyster heart. The experiments showed that OA increased the beating rate (Fig. 9). Therefore, we also performed analyses using patch clamp technique to study the electrophysiological properties of *C. gigas* cardiomyocytes cultured *in vitro*. The results revealed a calcium inward current in cells treated with OA, which was inhibited by verapamil and chelerythrine, a PKC inhibitor (Fig. 10). The studies showed that the sensitivity of macro patch clamp technique was several orders of magnitude higher than the sensitivity of other methods used.

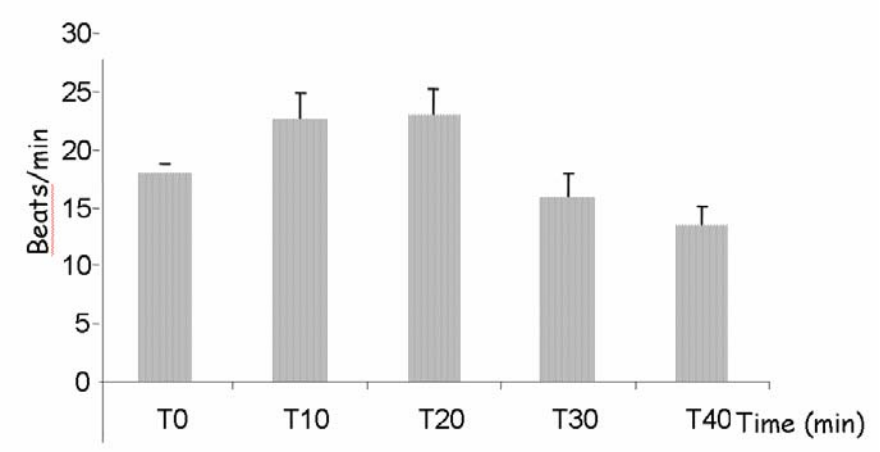


Fig. 9. Effect of 1000 nM OA on the spontaneous beating rate of *in vitro* cultured cardiomyocytes; results are given as means ± S.E.M.

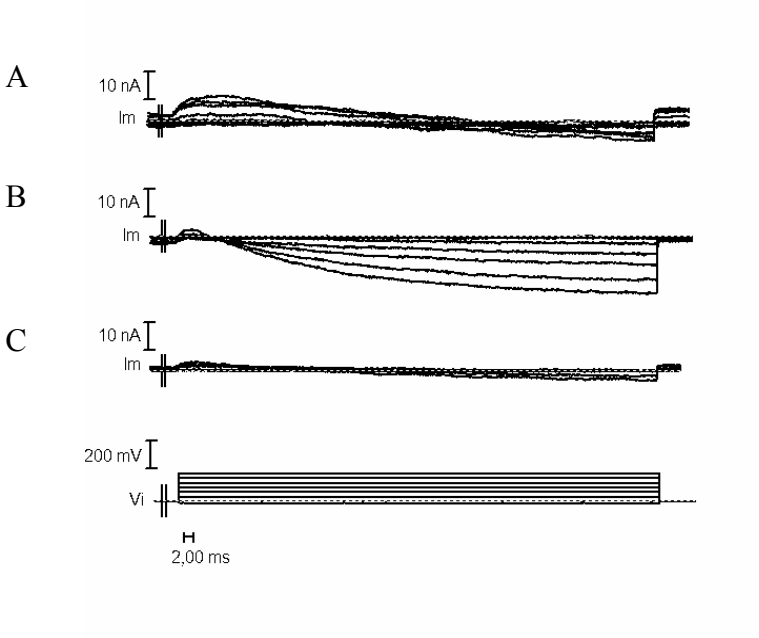


Fig. 10. (A) Control recording without OA showing both fast inactivating potassium current and calcium inward current. (B) Effect of the addition of OA (125 nM) on the currents: increase in the calcium inward current. (C) Inhibition of the calcium current after the addition of Verapamil (10^{-3} mol/L).

8. Appraisal of developed technologies against existing techniques

The methods and technologies developed in this project range from analytical chemistry, through biosensors to functional assays and from simple "dip stick" assays to quantitative LC-MS. Members of the project consortium also participated in trials of techniques run under related programmes (i.e. BIOTOX) or by national, community or private bodies. All of the partners were sent identical test materials prepared by Integrin to evaluate the performance of their methods. This material consisted of mussel homogenate that had been naturally contaminated with okadaic acid and DTX1 and posed a more realistic challenge to the various methods than simple standards. A comparison of the developed technologies with existing tests was performed.

PP2A assays have several advantages. Like the Integrin in-house assay, the commercial Toxiline assay is a fluorescence assay. This assay had very similar operational characteristics to the Integrin in house assay, though its limit of quantification was slightly lower compared to the Integrin kit. The commercial DSP Rapid kit is a colormetric and with a LOQ of 10 µg/100 g is comparable in cost to Integrin's in house assay.

In the BIOTOXmarin project, the OWLS system was explored as a potential toxin detection system. The detector of the biosensor OWLS system is considerably cheaper than the competing SPR systems. The biosensors themselves are expensive though they can be used 20 times. The technique performed excellently during the project. The OWLS system is 1-3 orders of magnitude more sensitive compared to ELISA. The results were very similar both to the reference PP2A technique and our LC-MS results. This also shows that the antibody used in the biosensor is working well. The antibodies were clearly not affected by the hydrolysation step that was used to ensure that „DTX3" toxins were converted into their native toxins (a problem that has been encountered in some commercially available ELISA techniques).

The dick stick assay developed in this project has any real potential in the short to medium term as a method suitable for use in the field. This assay is non-quantitative, but by setting appropriate limits of detection a useful tool for screening out negative samples is possible.

In this project, LC-MS was used by two of the partners on the ring trial material. Both detected okadaic acid and at roughly similar amounts.

9. Industrial perspectives

The products and techniques developed in frame of this project are considered to be of high commercial value. This has been evaluated in a market analysis performed by the SME partners. The best commercial possibilities of the techniques examined as part of the BIOTOXmarin project appeared to be the PP2A assay, the OWLS Biosensor and the dip stick system as these gave opportunities for the development of associated products or kits. For LC-MS, the only commercial opportunities are in the production of standards (which is essential for its success) and actually offering testing services.

The SME partners involved in this project will also play the leading role in the future exploitation - dissemination activities. We expect that the tests developed in this project will contribute to the replacement of animals in tests (mouse bioassay for the detection of marine biotoxins) by non-animal tests (tests based on antibodies and instructed synthetic polymeric receptors).

10. Table of deliverables

The following table summarizes the all deliverables from the beginning of this project, describing their status, including expected time of completion, as well as the dissemination level.

Table: Deliverables list

Deliverable No	Deliverable name	Date due	Actual delivery date	Lead contrac-tor	Status/expected time of completion	Level of dissem-ination
D1	Management Team	0	0	**P1**	**Delivered** - completed -	**PU**
D2	Steering Committee	0	0	**P1**	**Delivered** - completed -	**PU**
D3	Minutes of the Project meetings: 1st plenary meeting, 2nd plenary meeting, 3rd plenary meeting, 4th plenary meeting (Final symposium), Mid-term symposium, Workshops (several workshops), Summer School	1, 12, 24, 36	1, 8, 12, 24, 36	**P1**	**Delivered** - completed -	**CO**
D4	Reporting structure	3	3	**P1**	**Delivered** - completed -	**RP**
D5	Protocols for interactions with the EU	3	3	**P1**	**Delivered** - completed -	**RP**
D6	Electronic network including a data-warehouse	3	3	**P1**	**Delivered** - completed -	**PU**
D7	Strategy for the exchange of staff and materials	1	1	**P1**	**Delivered** - completed -	**RP**
D8	Consortium agreement (before contract signature)	0	0	**P1**	**Delivered** - completed -	**CO**
D9	Reports	0, 6, 12, 18, 24, 30, 36	0, 6, 12, 18, 24, 30, 36	**P1**	**Delivered** - completed -	**CO or PU**[1]
D10	Identification of known and new toxins contaminating Mediterranean edible bivalves	12, 24, 36	12, 24, 36	**P2**	**Delivered** - completed -	**PU**
D11	Isolation of the main known biotoxins from producer organisms	12, 24, 36	12, 24, 36	**P2**	**Delivered** - completed -	**PU**
D12	Development of LC-MS methods for monitoring the presence of toxins in edible mollusks	24	24	**P2**	**Delivered** - completed -	**PU**
D13	Data on effect of biotoxins on cell viability, $[Ca^{2+}]_i$ level and caspase activities	12, 24, 36	12, 24, 36	**P2**	**Delivered** - completed -	**PU**

[1] Publishable executive summaries and Publishable final activity report are PU.

D14	Polyclonal and monoclonal antibodies against selected marine biotoxins	6, 12, 24	6, 12, 24	**P1**	**Delivered** - Work to produce antibodies against further biotoxins or improved antibodies will be continued and completed at the end of 2008 -	**RE**
D15	ELISA for marine biotoxins (e.g. okadaic acid) based on highly specific antibodies and IR-fluorescence imaging system available	24	24	**P1**	**Delivered** - Commercialization planned at the end of 2008 -	**RE**
D16	Western blot for marine biotoxins (e.g. okadaic acid) based on highly specific antibodies and IR-fluorescence imaging system available.	24	24	**P1**	**Delivered** - Commercialization planned at the end of 2008 -	**RE**
D17	Validation of the developed test systems done	24	24	**P1**	**Delivered** - Validation with antibodies against further biotoxins or improved antibodies will be completed at the end of 2008 -	**RE**
D18	Polymer receptors for binding different marine toxins	6, 12, 18, 24, 30	6, 12, 18, 24, 30	**P6**	**Delivered** - Commercialization planned at > 2008 -	**PU**
D19	Polymers obtained by derivatization, co-polymerization, and cross-linking	6, 12, 18, 24	6, 12, 18, 24	**P6**	**Delivered** - Commercialization planned in 2009 -	**PU**
D20	Measurement of affinity and selectivity with different toxins	6, 12, 18, 24, 30	6, 12, 18, 24, 30	**P6**	**Delivered** - Commercialization planned in 2009 -	**PU**
D21	Chemical characterization of the new materials	6, 12, 18, 24, 30	6, 12, 18, 24, 30	**P6**	**Delivered** - Commercialization planned in 2009 -	**PU**
D22	Incorperated transducer elements in the receptor phases	12, 18, 24, 30	30	**P6**	**Delivered** - Commercialization planned in 2009 -	**RE**

D23	Immobilized receptor phases for sensor applications	18, 24, 30, 36	30, 36	**P6**	**Delivered** - Commercialization planned in 2009 -	**RE**
D24	Dip stick and card tests [Patent application]	18, 30	18, 30	**P4**	**Delivered** - Commercialization planned at the end of 2008 -	**RE**
D25	Determination of the criteria for the production of the Igs in commercial conditions	36	36	**P4**	**Delivered** - Commercialization planned at the end of 2008 -	**RE**
D26	Availability of novel immunosensor chips for marine biotoxins [Patent application]	24	24	**P8**	**Delivered** - The equipment is already commercialized. The commercialization for the specific application is planned from the beginning of 2009 -	**RE**
D27	Availability of novel sensor chips for marine biotoxins based on instructed polymers [Patent application]	30	30	**P8**	**Delivered** - see D26 -	**RE**
D28	Results from evaluation phase (calibration and samples from mussels and sera from patients) available	30	30	**P8**	**Delivered** - see D26 -	**RE**
D29	Prototypes of the developed biosensors	36	36	**P8**	**Delivered** - see D26 -	**RE**
D30	Purified marine molluscan and sponge PP2A	12	12	**P5**	**Delivered** - completed -	**RE**
D31	DSP positive and negative material **(new deliverable)**[2]	30	30	**P5**	**Delivered** - completed -	**RE**
D31	Colorimetric assay [Patent application] **(cancelled)**[2]	18	24	**P5**	-	
D32	Fluorescence SPI assay with improved sensitivity **(new deliverable)**[2]	36	36	**P5**	**Delivered** - completed -	**RE**
D32	Colorimetric assay incorporating stable standard inhibitors **(cancelled)**[2]	24	24	**P5**	-	

[2] See Periodic Activity Report 2.

D33	Characterization of MAP kinases in mollusks	18	24	**P3**	**Delivered** - completed -	**PU**
D34	p38 immunoassay	24	24	**P3**	**Delivered** - completed -	**PU**
D35	Appraisal of the developed methods against existing techniques	36	36	**P5**	**Delivered** - completed (the companies involved in the project will continue to compare further developments of their tests with existing techniques -	**RE**
D36	Manufacture of prototype kits	30, 36	30, 36	**P5**	**Delivered** - Manufacture of further prototype kits by the companies will be completed until the end of 2008 -	**RE**
D37	Determination of the commercial potential for the products	24	24	**P4**	**Delivered** - completed -	**RE**
D38	Plan for Using and Disseminating the Knowledge for the marketing of the products developed in frame of the project including a marketing analysis and the design of marketing strategies	36	36	**P4**	**Delivered** - completed -	**PU**
D39	A4 Leaflets (50 copies each)	6,36	6,36	**P1**	**Delivered** - completed -	**PU**

Part II: Dissemination and use

Exploitable knowledge and its Use

The following overview table presents exploitable results achieved in BIOTOXmarin project, which have a potential for industrial or commercial application.

Overview table

Exploitable Knowledge	**Exploitable product(s) or measure(s)**	**Sector(s)** of application	Timetable for commercial use	Patents or other IPR protection	**Owner** & Other Partner(s) involved
New health marker (14-3-3)	ELISA kit	Consumer protection	2005/2006	**European Patent 2005; National phases 2006; US Patent 2005**	**P1** and **P4** (owner)
Identification of known and new toxins contaminating edible bivalves	Development of assay kits; routinely used in **P2** lab	Consumer protection	2008		**P1** and **P4**
New LC-MS method	LC-MS method for monitoring the presence of toxins in edible mollusks; production of standards; routinely used in **P2** lab and by SME partner **P5**	Consumer protection	2008		**P2** and **P5**
New antibodies	Polyclonal and monoclonal antibodies against marine biotoxins; antibodies ready to be commercial-ized; routinely used by SME partner **P5**	Consumer protection; research	2008		**P1** and **P4** (owner)
New ELISAs	ELISAs for marine biotoxins; Poss. licensing to kit manufacturers	Consumer protection	End of 2008	**Patent application in preparation (2008)**	**P1** and **P4** (owner)
Instruction receptor phases	Polymer recap-tors for marine biotoxins; Poss. licensing to kit manufacturers	Consumer protection	>2008		**P6** (owner)

Exploitable Knowledge	**Exploitable product(s) or measure(s)**	**Sector(s)** of application	Timetable for commercial use	Patents or other IPR protection	**Owner** & Other Partner(s) involved
New immuno-detection method	Dip stick and card tests; Poss. licensing to kit manufacturers	Consumer protection	End of 2008	**Patent 2006**; further Patent planned for 2008	**P4** (owner)
Integrated Optical Waveguide Sensor Technology	Immunosensor chips for marine biotoxins; Poss. licensing to kit manufacturers	Consumer protection	>2009	**Patent application submitted in 2008**	**P4**, **P8** (owner)
New IOGC technology	Sensor chips for marine biotoxins based on instructed polymers; Poss. licensing to kit manufacturer	Consumer protection	>2009	Patent application planned at the end of year 2008	**P8** (owner)
Molluscan and sponge PP2A	Development of assay kits	Consumer protection	>2008		**P1**, **P5** (owner)
PP2A inhibition assay	Colorimetric assay; routinely used by SME partner **P5**; Poss. licensing to other companies	Consumer protection	2007	Patent application planned at the end of year 2008	**P5** (owner)
MAP kinases fom molluscs	p38 immunoassay	Consumer protection	>2008		**P3**

Patents/patent applications

In order to protect the intellectual property the results have been patented; 5 patents have been approved and more than 10 patent applications have been submitted.

1. **EPA Patent No. EP 0 983 377 B1.** Verfahren zum Nachweis von Umweltverschmutzung unter Verwendung von 14-3-3 Proteinen als Biomarker. (Method for detecting environmental pollution using 14-3-3 proteins as biomarkers). European Patent Office. Applicant and Inventors: W.E.G. Müller and H.C. Schröder (18.05.2005).

2. **United States Patent 20050009094**. Use of 14-3-3 proteins and a method for determining the same in the fluids or tissues of organisms. Inventors: W.E.G. Müller and H.C. Schröder.

3. **Patent Austria-Europe 0983377.** 14-3-3 Proteine als sensitive Biomarker für Pollution u. a. mit polychlorierten Biphenylen (PCBs) und (Xeno)Östrogenen sowie Verfahren zur schnellen Bestimmung der 14-3-3 Proteine. Inventors: W.E.G. Müller and H.C. Schröder.

4. **Patent Great Britain-Europe 0983377.** 14-3-3 Proteine als sensitive Biomarker für Pollution u. a. mit polychlorierten Biphenylen (PCBs) und (Xeno)Östrogenen sowie Verfahren zur schnellen Bestimmung der 14-3-3 Proteine. Inventors: W.E.G. Müller and H.C. Schröder.

5. **Patent Switzerland-Europe 0983377.** 14-3-3 Proteine als sensitive Biomarker für Pollution u. a. mit polychlorierten Biphenylen (PCBs) und (Xeno)Östrogenen sowie Verfahren zur schnellen Bestimmung der 14-3-3 Proteine. Inventors: W.E.G. Müller and H.C. Schröder.

Further patent applications concern the development of suitable matrices for the dip/stick assay.

1. **DE102004021230.9.** Enzym- und Template-gesteuerte Synthese von Silica aus nicht-organischen Siliciumverbindungen sowie Aminosilanen und Silazanen und Verwendung. Inventors: Müller WEG, Schwertner H, Schröder HC

2. **PCT/EP2005/004734.** Enzym- und Template-gesteuerte Synthese von Silica aus nicht-organischen Siliciumverbindungen sowie Aminosilanen und Silazanen und Verwendung. Inventors: Müller WEG, Schwertner H, Schröder HC

3. **EP1740707.** Enzym- und Template-gesteuerte Synthese von Silica aus nicht-organischen Siliciumverbindungen sowie Aminosilanen und Silazanen und Verwendung. Inventors: Müller WEG, Schwertner H, Schröder HC

4. **Japan No. 2007-509991.** Enzym- und Template-gesteuerte Synthese von Silica aus nicht-organischen Siliciumverbindungen sowie Aminosilanen und Silazanen und Verwendung. Inventors: Müller WEG, Schwertner H, Schröder HC

5. **Canada No. 2,565,118.** Enzym- und Template-gesteuerte Synthese von Silica aus nicht-organischen Siliciumverbindungen sowie Aminosilanen und Silazanen und Verwendung. Inventors: Müller WEG, Schwertner H, Schröder HC

6. **US 11/579,019.** Enzym- und Template-gesteuerte Synthese von Silica aus nicht-organischen Siliciumverbindungen sowie Aminosilanen und Silazanen und Verwendung. Inventors: Müller WEG, Schwertner H, Schröder HC

7. **DE10352433.9.** Enzymatische Synthese, Modifikation und Abbau von Silicium(IV)- und anderer Metall(IV)-Verbindungen. Inventors: Müller WEG, Schwertner H, Schröder HC

8. **PCT/EP2004/012668.** Enzymatische Synthese, Modifikation und Abbau von Silicium(IV)- und anderer Metall(IV)-Verbindungen. Inventors: Müller WEG, Schwertner H, Schröder HC

9. **EP1682658.** Enzymatische Synthese, Modifikation und Abbau von Silicium(IV)- und anderer Metall(IV)-Verbindungen. Inventors: Müller WEG, Schwertner H, Schröder HC

10. **US 10/578,959.** Enzymatic synthesis, modification and degradation of silicon(IV)- and other metal(IV)-compounds. Inventors: Müller WEG, Schwertner H, Schröder HC

11. **China 200480040186.8.** Enzymatic synthesis, modification and degradation of silicon(IV)- and other metal(IV)-compounds. Inventors: Müller WEG, Schwertner H, Schröder HC

Exploitable results will be used by the SME partners in the project. The plans for use and exploitation are briefly outlined below.

BIOTECmarin GmbH will use the antibodies developed in frame of the project as well as the Lateral Flow Assay technology. This company will further elaborate this technology to produce assays for the detection of selected biotoxins.

Integrin Ltd will exploit the new procedures for its in house PP2A assay. In addition, this company is also offering testing services applying the LC-MS method.

InstAction GmbH has developed and is patent owner of the Receptor-Phase technology and will exploit the new applications of this technology and the developed methods for the use of Receptor-Phases for the detection and isolation of specific biotoxins.

Microvacuum Ltd will exploit the novel biosensors based on the OWLS technology for the future commercial use. In addition, this company will further elaborate the biosensors based on the InstrAction Receptor-Phase in cooperation with InstAction GmbH.

Dissemination of knowledge

The following overview table summarizes the dissemination activities of BIOTOXmarin project in the past and future.

Overview table

Planned/ actual Dates	Type	Type of audience	Countries addressed	Size of audience	Partner responsible /involved
During the project period and thereafter	Publications	Research	All	Large	All
During the project period and thereafter	Conferences/Oral Presentations/Posters	Research	All	Medium	All
3/3	Project web-site	Research/General public	All	Large	P1;P4

Planned/ actual Dates	Type	Type of audience	Countries addressed	Size of audience	Partner responsible /involved
6/6	First Flyer	General public	EU	Medium	P1;P4
During the project period and thereafter	Press release	General public	EU	Large	P1
8/8	First Summer school	Research	EU	Small / Medium	P1
21/20	Second Summer school	Research	EU	Small / Medium	P1
33/33	Third Summer school	Research	EU	Small / Medium	P1
36/36	Second Flyer	General public	EU	Medium	P1;P4

Publications

2005

1. Ciminiello, P., C. Dell'Aversano, E. Fattorusso, M. Forino, S. Magno, L. Tartaglione, M.A. Quilliam, A. Tubaro and R. Poletti: Hydrophilic interaction liquid chromatography/mass spectrometry for determination of domoic acid in Adriatic shellfish. *Rapid Commun. Mass Spectrom.* **19**: 2030-2038 (2005).

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2. Ciminiello, P., C. Dell'Aversano, E. Fattorusso, M. Forino, S. Magno, F. Santelia and M. Tsoukatou: Investigation of the toxin profile of Greek *Mytilus galloprovincialis* by liquid chromatography-mass spectrometry. *Toxicon*, **47**: 174-181 (2006).

3. Ciminiello P., C. Dell'Aversano, E. Fattorusso, S. Magno, L. Tartaglione, M. Cangini, M. Pompei, F. Guerrini, L. Boni and R. Pistocchi: Toxin profile of *Alexandrium ostenfeldii* (Dinophyceae) from the Northern Adriatic Sea revealed by liquid chromatography-mass spectrometry. *Toxicon* **47**: 597-604 (2006).

4. Schröder, H.C., H.J. Breter, E. Fattorusso, H. Ushijima, M. Wiens, R. Steffen, R. Batel and W.E.G. Müller: Okadaic acid, an apoptogenic toxin for symbiotic/parasitic annelids in the demosponge *Suberites domuncula*. *Appl. Environ. Microbiol.* **72**: 4907-4916 (2006).

5. Ciminiello, P., C. Dell'Aversano, E. Fattorusso, M. Forino, G. S. Magno, L. Tartaglione, C. Grillo, N. Melchiorre. The Genoa 2005 Outbreak. Determination of Palytoxin in Mediterranean *Ostreopsis ovata* by a new liquid chromatography tandem mass spectrometry method. *Analytical Chemistry* **78**: 6153-6159 (2006).

6. Ciminiello, P., C. Dell'Aversano, E. Fattorusso, M. Forino, G. S. Magno, F. U. Santelia, V. I. Moutsos, E. N. Pitsinos, A. C. Elias. Oxazinins from toxic mussels: isolation of a novel oxazinin and reassignment of the C-2 configuration of oxazinin-1 and -2 on the basis of synthetic models. *Tetrahedron* **62**: 7738-7743 (2006).

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7. Müller, W.E.G., S.I. Belikov, O.V. Kaluzhnaya, S. Perović-Ottstadt, E. Fattorusso, H. Ushijima, A. Krasko and H.C. Schröder: Cold stress defense in the freshwater sponge *Lubomirskia baicalensis*: Role of okadaic acid produced by symbiotic dinoflagellates. *FEBS J.* **274**: 23-36 (2007).

8. Ciminiello, P., C. Dell'Aversano, E. Fattorusso, M. Forino, L. Grauso, S. G. Magno, R. Poletti, L. Tartaglione. Novel DesulfoYessotoxins from Adriatic mussels. A new problem for the seafood safety control. *Chem. Res. Toxicol.* **20**: 95-98 (2007).

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Web site

The BIOTOXmarin project web site contains a short introduction describing the BIOTOXmarin project and links to the different work packages and activities.

Website address: **www.biotoxmarin.de**

Summer school

The Summer school of BIOTOXmarin project was annually held in Rovinj, Croatia (Center for Marine Research, Ruder Boskovic Institute). This Summer school was organized together with the German Center of Excellence "BIOTECmarin" and the Marie-Curie Research Training Network "BIOCAPITAL". About 35-50 scientists (PhD students, postdocs, senior researchers) participated in the summer school. In the morning sessions, the participants presented the results of their work. In the afternoon, experimental courses on specific topics were organized, as well as catching/dredging excursions with the Institute boat to the islands around Rovinj and to the Limski Canal (location of a mussel farm).

Joint meetings

H.C. Schröder (Johannes Gutenberg Universität, Mainz; Partner 1) the results of the project were also presented in joint meetings of the three projects on marine biotoxins, for example during a Joint workshop of the XIIth International IUPAC Symposium on Mycotoxins and Phycotoxins, Istanbul, May 21 - 25, 2007.

Dissemination to the public

A number of measures has been undertaken to disseminate the results of the project to the public. These activities did not only include contributions to conferences or workshops but also contributions to exhibitions/fairs or the distribution of information of material to potential customers by the industrial partners involved in this project. BIOTOXmarin project was mentioned in Nature (Published online: 21 September 2006; doi:10.1038/news060918-8) and interviews of the coordinator about the topic "Marine biotoxins" have been published in several newspapers.