BRAAVOO

biosensors for real time monitoring of marine contaminants

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Executive summary

Marine environments are threatened by pollution through a variety of activities, both directly and indirectly. The varying types, sources, levels and impacts of pollution in marine environments make it very difficult to develop efficient monitoring tools. In addition, monitoring strategies need to be adapted depending on the "use" of the marine environment (e.g., aquafarming, tourism, transport) or for the quality of marine environments as natural ecosystems themselves. The major aim of the BRAAVOO project and its contribution to the Ocean of Tomorrow program (FP7-OCEAN-2013) is to develop innovative solutions for measurement of high impact and difficult to measure marine pollutants. In contrast to classical environmental analytics, which is based on site sampling, ex-situ sample extraction and purification, and high-end sophisticated compound detection, the strategy of BRAAVOO is to provide near real-time in-situ sampling and analysis.

The BRAAVOO concept of near real-time in-situ sampling and analysis is based on the use of three types of biosensors, to enable both the detection of a number of specific marine priority pollutants and also of general biological effects that can be used for early warning. The first type of biosensor uses label-free antibody-based immuno-sensing on innovative nano-optical platforms such as bimodal evanescent waveguides or asymmetric Mach-Zehnder interferometers. The second sensing platform consists of live bacterial "bioreporters," which produce bioluminescence in response to chemical exposure. Finally, the photosystem II fluorescence of marine algae is exploited to monitor changes induced by toxic compounds.

BRAAVOO has rigorously tested the three biosensor systems for their analytical performance, responding to a set of targeted pollutants that include algal toxins, heavy metals, organic compounds related to oil, and antibiotics. To enable low-cost real-time measurements, the three biosensors were miniaturized, multiplexed and integrated into biosensing instruments, which allow simultaneous multianalyte detection. The instruments include the optical elements for biosensor signal generation and readout, the microelectronics for data storage, and specific macro- and microfluidics to expose the biosensors to the aqueous samples or calibration solutions. The modules were tested as stand-alone instruments with manual operation (e.g., sample addition manually), and were integrated in a marine buoy and an unmanned surveying vessel (USV). Integrated sensor instruments could be operated autonomously and remotely, store and transmit data to a remote observer. The performance of the stand-alone biosensors and biosensors in their integrated form was tested at field sites in Italy and Ireland, and was further bench-marked using spiked marine samples with known target compound concentrations. Comparative chemical analytics showed reasonable agreement between the two types of measurements, although limits of detection in biosensor measurements without sample pre-treatment were generally (and not surprisingly) higher than in chemical analytics with extensive sample purification and concentration.

Overall, the developed biosensors and biosensor instruments allow flexible and innovative solutions for marine monitoring in terms of efficiency (sample analysis in hours instead of the days or weeks needed for standard sampling, transport to external labs and subsequent analyses) and cost. Further benchmarking on real samples and sites will be necessary to improve the robustness of the biosensor instruments and protocols, and to validate the biosensors' responses in comparison to classical analytics.

Project context and main objectives

As a result of intensive exploitation, a variety of source inputs and multi-usage of marine environments, there is a slow but steady degradation of the marine water quality both in terms of chemical as well as biological quality and safety. It is the explicit strategy of the EC and its member countries to protect the marine environment while at the same time maintain its multi-usage in the long term (sustainability). Pertinent for the impact of this particular call of projects under "The Ocean of Tomorrow 2013" frame are the early detection and cost-effective monitoring of the "status" of the marine environment. In particular this means detecting and monitoring of chemical assaults, monitoring biological diversity and consequences in the changes thereof (e.g., algal or phytoplankton blooms), and monitoring the well-being of living organisms in marine environments and potential effects on human health. Biosensors (in the widest sense of the word) are considered an important asset and tool that should permit easier, real-time, in-situ, more cost-effective but still highly reliable measurements of contaminants in the marine environment. This is a tremendous challenge, since, there are virtually thousands of individual chemicals that alone or in mixtures and in combinations with physical changes potentially threaten the marine environment. How exactly should "biosensors" be able to accomplish this?

The unique solution that the BRAAVOO project proposed is to use a concept in which different kinds of biosensors are combined, through which one could rapidly assay both specific priority chemicals, such as those that have been shortlisted in international or regional treaties, and more general toxicity or stress, that are representative for the "well-being" of organisms living in the oceans and seas. The biosensors are to be used as hand-held single use instruments or combined and integrated in autonomous vessels. This flexible solution provides numerous advantages for monitoring programmes, by focusing on cost-effective targeted and specific 'first-line' early warning strategies, and only when necessary move to more detailed individual compound analysis.

Table 1. Model compounds and compound classes to be targeted by the BRAAVOO biosensors

Class	Compound	Scenario		Expected concentration range	Biosensor type[1]	End-user[2]
		Monitoring	Accident			
Organohalogen	Pentabromodiphenyl ether (BDE-47)	x		fM-pM	NIS	EA
Antibiotics	Tetracyclin, Ampicillin	x	x	pM-nM	NIS, BB	AQ
Antifouling biocides	Irgarol	x		fM-pM	NIS	AQ, EA
Marine toxins	Domoic acid Okadaic acid	x	x	pM-µM	NIS, BB, AR	AQ, PH
Herbicides	Atrazin	x	x	fM-µM	BB, AR	EA
Oil	Alkanes	x	x	nM-mM	BB	AQ, OC, EA, PH
	Monoaromatics	x	x			
	Polycyclic aromatics	x	x			
Heavy metals	Hg, Cd	x	x	pM-µM	BB, AR	OC, HA, PH
General toxicity	(first line warning)	x	x	nM-mM	BB, AR	EA, PH, AQ
Stress response	(first line warning)	x	x	nM-mM	BB, AR	EA, PH, AQ

1) NIS, nano-immunosensor; BB, bacterial bioreporter; AR, Algal reporter; 2) EA, environmental agencies; AQ, marine aquaculture; PH, Public Health; OC; oil companies; HA, harbour authorities.

The main goals of the BRAAVOO project were thus to develop three types of bioassays into innovative self-sustained micro-biosensors that can rapidly target a number of marine priority compounds or compound classes and can measure general toxicity. Secondly, the project would integrate the three biosensor types into a **data buoy** and an unmanned surveying **vessel (USV)** that can perform **real-time on-site** sampling, sample analysis and surveying. Both devices communicate relevant data to a remote observer or control centre. The biosensor modules were to be designed so they can also be deployed as **standalone single-use instruments**, e.g., on ships or by harbour authorities. The biosensor units were to be tested throughout the lifetime of the project and calibrated to state-of-the-art chemical analytics..

Although all three biosensor types were to be specifically developed and extensively tested with prime marine priority pollutants, the lifetime of the BRAAVOO project only permitted to concentrate on and demonstrate **proof of principle of automated real-time and in-situ detection** by the buoy and USV of a few relevant model examples of toxic compounds frequently encountered in harsh marine environments. Initially these were **oil pollution and algal toxins**.

The specific technical **BRAAVOO objectives** were the following:

1. Develop and test miniaturized antibody-based biosensors (*nano-immunosensors*) with integrated optical sensors (**WP1**),
    - Six specific marine priority pollutants were to be targeted through new, surface-immobilized antibodies in nanophotonic, microfluidic devices with integrated electronics.
2. Develop and test miniaturized bacterial biosensors with different target chemical specificities but singular fluorescence output (**WP2**),
    - These biosensors will integrate living *Escherichia coli* lab strains expressing autofluorescent proteins in microdevices with optical excitation and detector elements. Strains would have different detection specificities, notably targeting oil-pollutants, polycyclic aromatic hydrocarbons, heavy metals and general toxicity.
3. Develop and test miniaturized algal photosystem assays (**WP3**), as sensors for general toxicity and specific photosystem inhibitors.
    - The biosensors would integrate whole living unicellular marine algae in microdevices with optical illumination and detector elements.
4. Integrate microfluidic elements and appropriate microelectronics into the three biosensor types to provide simple robust autonomously operating sampling and analysis modules (**WP4, 5**).
    - The three biosensor types were to be completely embedded and integrated in fluidic modules that allow passage of the samples over the sensors, automated readout recording and data analysis. This would produce three biosensor "modules" that can be operated as stand-alones or in integrated format.
5. Integrate biosensor modules in a buoy and in an autonomously operating marine USV with telemetric data exchange (**WP6, WP7**).
    - Demonstrate integration and successful operation of the biosensor modules in an autonomous device, in conjunction with regular physico-chemical sensor packs.
6. Provide intercalibration and quality assessment of the biosensor performance as individual modules and as surveying vessel for real-time and in-situ measurement of marine priority pollutants (**WP8**).
    - Demonstrate the analytical quality of the developed biosensors in comparison to chemical analytics. Demonstrate further the real-time, in-situ measurement potential in on site analysis, with stakeholders and in mesocosms.

Main S&T results and foregrounds

Foregrounds

1. Eight-channel bimodal waveguide (biMW) and three-channel asymmetric Mach-Zehnder Interpherometric (aMZI) nanophotonic platforms fabricated. Leak-free fluidic connectors for independent sample delivery on the nanophotonic platforms. Fabricated laser light incouplers to both platforms. Biofunctionalization protocols of both biMW and aMZI surfaces. Complete detection assay procedures for Irgarol, Tetracycline and Okadoic Acid with method of detection limits of 0.04-0.2 µg/L. Less efficient detection of Ampicillin and Domoic Acid (10-20 µg/L).

2. Coherent set of *Escherichia coli* bioreporter strains producing bioluminescence in response to organohalogens, antibiotics, marine toxins, oil (alkanes), oil (monoaromatics), oil (polycyclic aromatics), heavy metals (Hg, Cd, As, Zn), DNA damage, oxidative radical damage. Single-use functional multi-target cartridges (10-wells, 10×36 mm) with lyophilised bioreporters. Continuous-use single-target bioreporter chip fabricated: 1 week life-time, demonstrated for arsenic detection at 10 and 50 µg/L.

3. Marine water resistant algal photosystem II fluorescence sensor based on *Chlorella vulgaris* in symbiosis with *Tetrahymena pyriformis.* Demonstrated detection of herbicides at 0.5 µg/L. Specific six-well continuous flow-through cartridge with immobilize algae produced. Life-time several months at 4–20°C.

4. Standalone instrument implementing the aMZI nanophotonic platform, including optical sources and detectors, including automated sample delivery as well as reagent deliveries. Standalone fluorimeter implementing the *Chlorella vulgaris* algal sensor and cartridge, and controlling algal maintenance. Standalone bacterial bioreporter unit implementing the 10-well cartridge with lyophilized bioreporters, and reading out bioluminescence kinetics. Design and fabrication of specific microfluidic rotary valves for the standalone instruments.

5. Automated biosensor instruments in watertight cases for marine deployment with sample and reagent delivery. Bacterial biosensor: implementing 30 cartridges with automated displacement allowing 1 month operating time. Optimized measurement protocol including standards, negative control and spiking control. Nanophotonic aMZI sensor: three parallel line chip, with automated sample and reagent delivery, regeneration protocol tested. Algal sensor: six-parallel line format for continuous measurement, with automated sample and reagent delivery.

6. Marine monitoring buoy and autonomous operating trimaran vessel built. All biosensors implemented and tested. Data acquisition and telemetry systems installed. Guidance, navigation and control systems implemented. Sampling system fabricated and tested; connections to biosensor fluidics built.

7. Biosensors first benchmarked on blind ring test with unknown marine samples. Second benchmarking in mesocosm facilities on Sicily. Third benchmarking in harbour in Ireland.

Nanoimmunosensors

Development of the optical transducer platforms

Immunosensors are based on specific interactions between target molecules and classes of recognition biomolecules, which can be probed by a variety of optical or amperometric methods. Immunosensors are typically based on antibodies bound to functionalized surfaces, but other types of proteins or oligonucleotide aptamers have also been used to achieve biomolecular target recognition. Their advantage for environmental analysis is their potential **high sensitivity and selectivity**.

The project opted for two types of optical transducers; one called the bimodal waveguide (BiMW) platform; the other the asymmetric Mach-Zehnder interferometer (aMZI). In addition, much of the initial optimization of the immuno-protocols depended on the use of Surface Plasmon Resonance (SPR). The three optical transducers base detection on **evanescent waveguides**, which not only enable **highly sensitive** but also **label-free detection** of target molecules (fM-pM concentrations) in real time, with reduced non-specific binding. The principle is the following: waveguides with dimensions smaller than the free-space wavelength of light have a strong evanescent field that extends a few hundred nanometers beyond the waveguide's surface into the surrounding media. The biological receptor is immobilised onto the core surface of the waveguide. Exposure of the functionalised surface to the complementary analyte molecules and the subsequent biomolecular interaction, induces a local change in the optical properties of the waveguide. This change is detected via the evanescent field of the guided light, the amplitude of which is correlated to the concentration of the analyte and to the affinity constant of the interaction. As the evanescent wave decays exponentially while it penetrates into the outer medium, it only detects changes taking place on the surface of the waveguide.

In aMZI, the incoming light into the waveguide is split in two arms with different lengths (**hence *asymmetric* MZI**), which are finally brought together again and the light interference is measured. In the longer arm a sensor surface is created, which is functionalized for the immunoreaction. Any change in light properties as a result of a target molecule binding to the functionalized surface will be detected in the interference pattern after the light from the two arms is again joined. aMZI waveguides are created by alternating silicon nitride (Si_3N_4) and silicon oxide (SiO_2) layers forming a 'triplex' core of Si_3N_4-SiO_2-Si_3N_4 fabricated in the proprietary TriPleX™ technology of partner LioniX.

In contrast to the aMZI, dual-channel interferometric design, the **bimodal waveguide (BiMW) transducer** is a **single-channel** waveguide that operates via interference of two waveguide modes of the same polarisation. The BiMW transducer is fabricated by standard silica-nitride technology in the cleanroom. The simplicity of the design of the BiMW interferometer makes it potentially interesting for mass production. The sensitivity level achieved with BiMW is in the order of $2.5 \cdot 10^{-7}$ refractive index units, translating into a chemical sensitivity of, for example, 200 fM.

Various designs of the aMZI and BiMW transducers were made, fabricated and tested. The aMZI fabrication is in a more "professional" state, which made it more easy to produce robust versions (Figure 1). The BiMW transducer is still mostly fabricated in research lab cleanrooms. On the other hand, more experience existed with the biofunctionalization and detection in the case of the BiMW setup (Figure 2). Considerable time and efforts were spent, with multiple rounds of trial and error, to produce robust light coupling to the chip surface and to fabricate fluidic connectors that would be able to seal individual sensor channels while allowing introducing the various immunoreagents. The final versions allowed to use both the aMZI and BiMW optical transducer chips outside the laboratory setups in a compact dedicated instrument (see below).

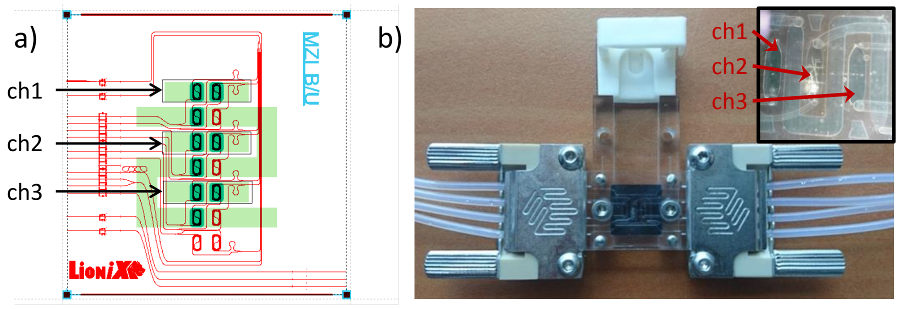

Figure 1 – Final layout of the aMZI optical transducer for the immunoreaction. a) The three-channel layout for immunosensing with a fourth reference channel. In red the optical paths, in green the fluidic overlay to introduce the sample and reagents. b) Connection of the aMZI chip (middle) to the incoming (left) and outgoing light waves (right). In the middle in overlay the fluidic connector. Shown in the right top inset the detail of filled channels on the microfluidic part, indicating the leak-tight seals between the different channels.

Development of the label-free competitive nano-immunoassays – optimizing the immunoreagents

On the biochemical side of things, the main focus of the work consisted of **identifying** the appropriate immunoreagents to allow label-free detection of the set of initial target chemicals (see Table 1 on p. 2) and **optimizing** the protocols for use with the various transducer platforms. Partner CSIC-CIN2 developed the assays in first instance on the SPR for the targets 2,2',4,4'-Tetrabromodiphenylether flame retardant

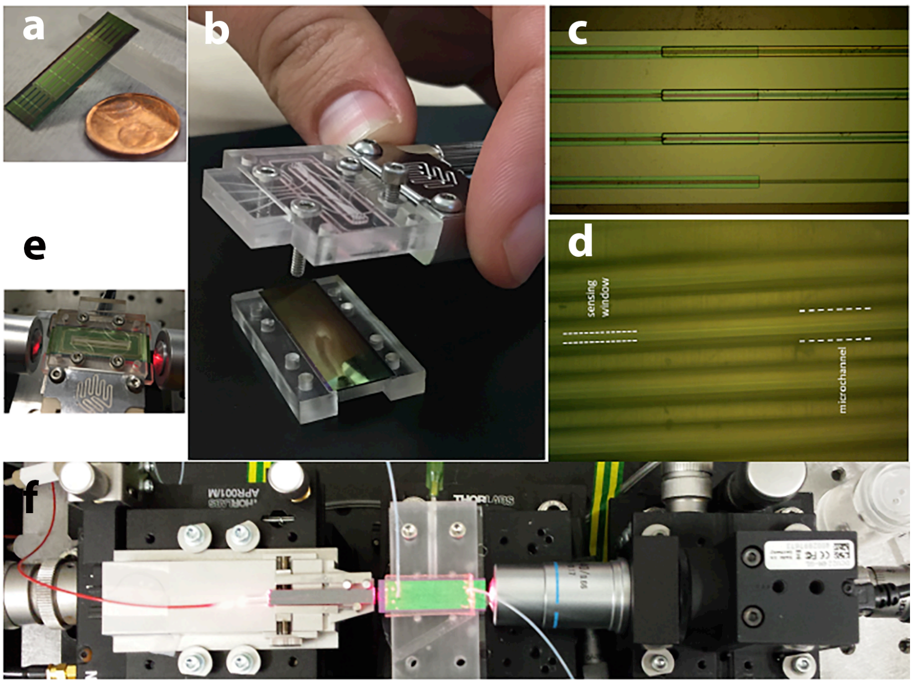

Figure 2 – Detail of the BiMW optical transducer assembly. a) Size of an 8-parallel line BiMW chip. b) Transversal fluidic connection to the BiMW surface, which is "opened" in specific regions of the waveguides for the immunoreagents (c). d) Detail showing the difficulty for fluidic sealing between two waveguides (sensor area), which needs an accuracy of 50 µm. e) Light incoupling (left and right) and fluidic coupling (from bottom to top) in laboratory setup with laser illumination. f) Top view of laser light coupling to the BiMW chip (middle) and connecting light waveguides to the detector.

(BDE-47), the biocide Irgarol, the two antibiotics tetracycline (TET) and ampicillin (AMP), and the two algal toxins domoic acid (DA) and okadoic acid (OA). All the selected chemical targets are low molecular weight molecules (smaller than 1000 Da). In the label-free competitive immunoassay format the antibody is mixed with the chemical target, the analyte present in the sample. Antibodies therefore compete with the chemical target in solution and the bioreceptor antibody binding sites on the surface. The bioreceptor, which can consist of the analyte itself or an analyte derivative, usually analyte-protein conjugates, is previously attached to the sensor surface. The more target chemical is in solution, the more it will titrate the antibodies away, which cannot bind the surface. Only antibody bound to the immobilized bioreceptor is finally measured, and, therefore, the signal is inversely proportional to the target chemical concentration in the sample.

The development of the immunoassays thus first required **specific antibodies for each target**, and, in addition, **suitable surface receptors** (Table 2). Only some of those could be acquired through commercial sources. In the other cases, the project established contacts with expert research groups in immunoreagent synthesis and antibody production. Three different collaborations were hereto started with: (1) **Prof. M.P. Marco** (Nanobiotechnology for Diagnostics Nb4D group, IQAC-CSIC, Barcelona, Spain, who is partner in the FP7 **Sea-on-a-Chip** Project, FP7-OCEAN-2013). Prof. Marco's group provided the immunoreagents for Irgarol detection. (2) **Dr. F. Rubio** (Abraxis LLC, USA), who provided an anti-BDE-47 antibody, and (3) **Prof. W.L. Shelver** (USDA-ARS Biosciences Research Laboratory), who provided a BDE-47-protein conjugate (BDE-BSA).

Table 2. Immunoreagents for nano-immunosensor development.

Chemical target	Analyte	Antibody	Receptor
Tetracycline	Tetracycline hydrochloride(1)	Sheep monoclonal Ab(2)	DoxAc10-AD (2)
Ampicillin	Ampicillin trihydrate(1)	Mouse monoclonal Ab(1)	BSA-penicillin G
Domoic acid (DA)	Domoic acid(1)	Rabbit polyclonal Ab(3)	Domoic acid
Okadaic acid (OA)	Okadaic acid(1)	Mouse monoclonal Ab(1)	Okadaic acid
Irgarol 1051	Irgarol 1051(5)	Rabbit polyclonal Ab(5)	4e-CONA and 4e-BSA(5)
BDE-47	BDE-47(4)	Rabbit polyclonal Ab(6)	BDE-BSA(7)

BDE-47: Pentabromodiphenyl ether; CONA: Conalbumin; BSA: Bovine Serum Albumin; n.a.: not commercially available. Purchased from (1)Abcam, (2)Fitzgerald, (3)Acris and (4)AccuStandard®, Inc. Provide by (5)Prof. Marco's group, (6)Abraxis LLC and (7)Prof. Shelver's group.

The specificities of the antibodies and real-time receptor-antibody interaction were first tested by SPR. Functionalized surfaces were prepared by covalently binding the protein conjugate receptors (tetracycline-BTG, 4e-CONA, 4e-BSA or BDE-BSA) to the SPR chip surface by amino coupling. The surface was previously activated with a terminal carboxylic-alkanethiol self-assembled monolayer. For ampicillin, domoic acid or okadaic acid detection, pure compound was covalently linked to the sensor surface, which was previously modified with an amine-dextran network (see Deliverable 1.1 for details).

Best results were obtained for the receptor/antibody pairs 4e-cona/anti-irgarol PAb, DA/anti-DA PAb and OA/anti-OA Mab (Table 2). Detection limits in the ng/L range were obtained on SPR for the Irgarol competitive immunoassay, and in the µg/L level for DA and OA. Regeneration of the bioreceptor layers by a 100 mM NaOH solution produced good results. In all three cases, numerous successive detection cycles followed by regeneration were obtained. Although preliminary results showed specific receptor-antibody interaction for the BDE-BSA/anti-BDE Ab pair, a high anti-BDE PAb concentration was necessary in order to achieve sufficient signal on the instrument. No specific receptor-antibody interaction was observed for antibodies acquired for tetracycline (two Tetracycline-BTG/anti-tetracycline Ab pairs evaluated) and ampicillin (ampicillin/anti-penicillin Ab pair). In second instance, other conjugates such as ampicillin-BSA or penicillinG-BSA were tested. An alternative antibody specific for the tetracycline antibiotic family was also tested (DoxAc10-AD/As256, see below).

Optimization of biofunctionalization protocols of Si_3N_4 surfaces

Because both BiMW and aMZI transducers are based on silicon nitride material (Si_3N_4), which is different from the SPR surface, the functionalization protocol for the immobilization of the receptors and conjugates had to be adapted. This included protocols for covalent attachment of the receptor on the surface, which would increase long-term stability and reusability of the receptor surfaces. Reusable surfaces are important for applications of the competitive immunoassays in the automized flow protocols for BRAA-VOO, where the chips would have to be used for multiple consecutive sample analysis. Protocols were mainly based on surface **silanization** and subsequent **covalent receptor binding**. Silanization efficiency was enhanced by a cleaning protocol to completely remove contaminants with minimal damage of the surface as well as oxidative pretreatment (see Deliverable 1.1 for details).

Three different silanizing agents were evaluated:

i) Carboxyethylsilanetriol sodium salt (CTES) which provides a surface functionalized with carboxyl groups, allowing the covalent immobilization of receptors presenting amino groups in their structure such as the hapten-protein conjugates (e.g. 4e-cona, Tetracycline-BTG, BDE-BSA) by EDC/NHS chemistry,

ii) 3-aminopropyltriethoxy silane (APTES), which leads to a surface functionalized with amine groups, allowing the covalent immobilization of receptors presenting carboxyl groups in their structure (e.g. domoic acid and okadaic acid), and

iii) ethoxy silane carboxylic acid-functionalized polyethylene glycol (Silane-PEG-COOH), which also provides a surface functionalized with carboxyl groups.

Functionalization yields were evaluated using raw Si_3N_4 samples and tetramethylrhodamine isothiocyanate-BSA conjugate (TRITC-BSA) as a model receptor, by measuring the fluorescence of the resulting functionalized surface. The best results in terms of sensitivity (signal inhibition in the presence of Irgarol or B/B_0 ratio, Figure 3a) were obtained for sensor surfaces immobilized with the “4e” Irgarol derivative onto an APTES-functionalized BiMW chip, or with the 4e-cona Irgarol conjugate onto a BiMW chip previously modified with a CM-dextran network. In parallel, the above described APTES silanization protocols were applied for the covalent immobilization of domoic acid (DA) as receptor using EDC/NHS chemistry via carboxyl groups present in the DA structure.

In the final protocol for **Irgarol 1051** immunoassay detection, the 4e-CONA-Irgarol derivative is immobilized onto an APTES-functionalized sensor surface. With this biofunctionalization protocol, the BiMW sensor even showed improved analytical performance as compared to the SPR biosensor, with a **LOD** of only **15 ng/L** (Figure 3). The BiMW sensor surface could be used in **30** successive measurement-regeneration cycles without loss of sensitivity (<30%). For the aMZI sensor, the chip was first salinized with APTES and next thermally cured. Afterward, the chip was fixed to the fiber array and placed into the microfluidic chamber.

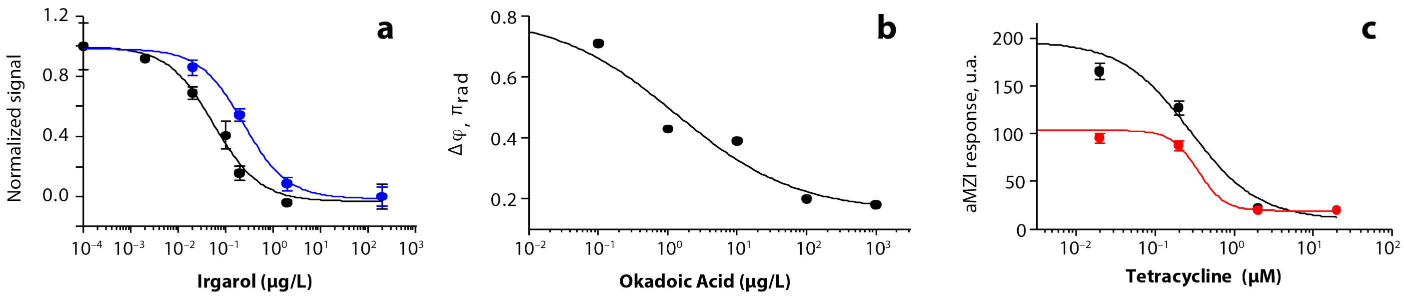

Figure 3 — Calibration curves for the nanoimmunosensor detection of (a) Irgarol, (b) Okadoic Acid, and (c) Tetracycline. a) black measurements: BiMW surface, blue: aMZI. b) BiMW surface; solutions prepared in PBS; anti-OA Ab at 0.5 µg/mL. c) BiMW surface, tetracycline Ab at 1:6000 diluted (red) or at 1:3000 diluted (black line).

Finally, the Irgarol 1051 derivative was linked on the APTES-modified surface by using stopped-flow incubation. The sensitivity to Irgarol in the aMZI was around 90 ng/L (Figure 3).

For the development of the protocol for **tetracycline detection**, a functionalization approach based on CTES silanization and subsequent covalent receptor immobilization was selected. CTES-modified BiMW or aMZI chips were positioned in their microfluidic cartridges. The DoxAc10-AD receptor was immobilized in an on-flow protocol with a continuous flow (20 µL/min) of deionized water, the carboxylic groups on the sensor surface were activated with 200 mM EDC and 50 mM sulfo-NHS solution in 100 mM MES buffer containing 0.5 M NaCl, at pH 5.0 (150 µL, 20 µL/min) followed by incubation with the receptor solution (10 – 20 µg/mL, 150 µL at 10 µL/min). Finally, an ethanolamine solution (1 M, pH 8.5) was flowed onto the sensor surface (25 µL/min, 120 s) to deactivate residual carboxylic groups and to remove electrostatically bound ligand. At the optimal As256 antibody dilution factor of 1:3000 in MES buffer (10 mM, pH 5), the **LOD** for tetracycline was 0.09 µM (**0.04 µg/L**), whereas the response curve showed an IC_{50} value of 0.43 µM (0.19 µg/L), with a dynamic range from 0.2 to 1.1 µM (Figure 3b, Table 3).

Table 3. Figures of merit for detection of marine pollutants using label-free nano-immunobiosensors.

Figure of merit	Biosensor	Irgarol 1051	Tetracycline	Ampicillin	Domoic acid	Okadaic acid
LOD, µg/L	SPR	0.024	0.04	5.9	11	1
	BiMW	0.015				
	aMZI	0.09	0.04			0.2
IC_{50}, µg/L	SPR	0.30	1.6	> 3500	17	50
	BiMW	0.17				
	aMZI	3.9	0.19			1.3
DR, µg/L	SPR	0.06 – 1.9	0.18 – 16	NA	13 – 23	
	BiMW	0.03 – 0.73				
	aMZI	0.4 – 35	0.09 – 0.5			0.08 – 20

LOD, Limit of detection. DR, dynamic range. IC50, inflection point at which 50% of the response is achieved.

For **ampicillin** detection, a penicillin G-BSA conjugate was used as receptor, which was covalently attached to the aMZI surface like for tetracycline. However, the immobilization yield was lower than expected probably due to a low CTES silanization yield and a high conjugation yield in the penicillin G-BSA conjugate synthesis (see Deliverable 1.3 for further explanations). Specific receptor-antibody interactions for the penicillin G-BSA /anti-penicillin Ab pair was then evaluated by flowing solutions of different antibody concentration onto the functionalized surface. A high anti-penicillin Ab concentration was necessary in order to achieve a response. Moreover, the reproducibility of the response of the three aMZI sensors within the same chip was poor (RSD up to 56 %). On the basis of these results, we decided to discard ampicillin as chemical target for the final multiplexed photonic immunosensor.

Detection of **Domoic Acid (DA)** in the BiMW and aMZI sensors, was achieved by direct binding of DA to a previously APTES-modified surface. However, no decrease of the signal was observed even in the presence of high DA concentrations (see Deliverable 1.2 for details). For **Okadoic acid (OA)** we also used covalent attachment to the sensor surface previously silanized with CTES but modified with a 70 kDa amine-dextran network (see Deliverable 1.3 for details). At an antibody concentration of 0.5 µg/mL, the sensor response for OA standard solutions in the range from 0 to 1 mg/L was evaluated (see Figure 3c). This resulted in a LOD of **0.2 µg/L**, an IC_{50} value of 1.3 µg/L and a dynamic range from 0.08 to 20 µg/L (Table 3). The BiMW sensor surface stability was not very good, leading to a ~30% decrease of the initial signal after 9 measure-regeneration cycles.

Unfortunately, the chosen immunoreagents for **BDE-47** detection did not yield a good quality assay, despite intensive efforts on optimize the parameters for BiMW and aMZI surface functionalization. Therefore, this pollutant was discarded in the multiplexed immunosensor. This system carries three parallel channels plus a reference (Figure 1). The targets of choice here were thus Irgarol, Tetracycline and OA.

Bacterial bioreporters

Engineering of the *Escherichia coli*-based set of bioreporters

Whole cell living bacteria have been frequently used to measure biological responses to toxic compounds. Of particular interest is the use of so-called bacterial (or eukaryotic) bioreporter systems, in which specific bioreceptors within the cell are coupled to a *de novo* synthesis of non-cognate but easily measurable **reporter proteins**. The use of specific bioreceptors enables target detection of individual or groups of related chemical compounds by the (bacterial) cell, under the production of a highly specific signal. This feat was mainly achieved by using existing transcription regulator proteins which **detect** a target chemical via biomolecular interactions, and **control** the *de novo* expression of the gene for the *reporter* protein (Figure 4). The measurement in this type of bioreporter assay consists of exposing the cells to the sample and detecting the **increase** in reporter signal over time.

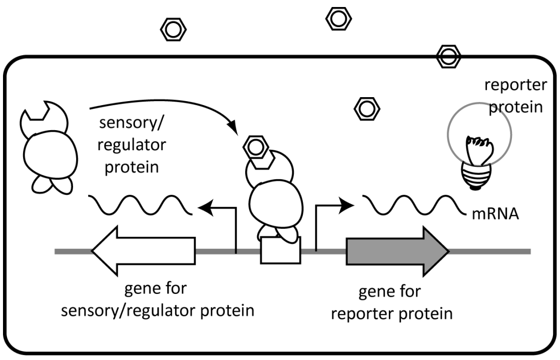

Figure 4 – Principle of a bacterial bioreporter. The bioreporter produces a sensory/regulator protein that can sense the incoming target compound and controls expression of a gene for a specific reporter protein, which is non-native to the cell to reduce any background. Upon target compound recognition, the reporter protein is produced by the cell, and its presence or activity can be measured non-invasively in kinetic or end-point modes. By using bioreporter species with different sensory/regulator proteins, target selectivity can be obtained while maintaining the same sensory output. This enables simultaneous multi-analyte detection. Since the bioreporters are living cells, they can be operated continuously.

Through **exploitation of a wide variety of regulator proteins**, it was possible to obtain bioassays for the detection of e.g., mercury (using the MerR regulator), cadmium and zinc (ZntR), naphthalene (NahR), phenanthrene (PhnR), alkanes (AlkS) or biphenyls (HbpR). Most importantly, bacterial bioreporter assays are quantitative within a range of environmentally relevant concentrations (nM-µM), rather precise (typical measurement error of up to 10 percent) and reasonably rapid (30 min to 3 h per measurement). Important for the underlying project, bacterial bioreporters enable quantitative assessment of both **very broad general sample toxicity** (without actually identifying the toxic compounds), as well as **specific**

compound classes (Table 1). Therefore, they are excellently suitable for incorporation in the BRAAVOO project, because they enable detection of a number of priority pollutants but also potentially uncover 'anything unknown' that causes toxicity. Two specific formats of bioreporter assays were investigated during the project: (i) lyophilised cells, which can be stored and subsequently activated by the addition of the sample, and (ii) continuously growing cells, which might allow real-time, in-situ measurements of multiple sequential samples.

The first task in this part of the project was to re-engineer a set of *Escherichia coli*-based bioreporters to produce coherent bioluminescence light output from the *luxCDABE* operon (Table 4). The bioreporters were calibrated in laboratory assays with known concentration ranges of target compounds both in standard buffers as well as seawater media.

Table 4. List of BRAAVOO target chemicals and corresponding bacterial reporters

Class	Compound	Bacterial reporter[1]	Reporter strain number
Organohalogen	Pentabromodiphenyl ether (BDE-47)	HbpR, *marR*	
Antibiotic	Tetracycline	*soxS*	5462
	Ampicillin	*sodA*	
Antifouling biocides	Irgarol	*micF*, *grpE*	
Marin toxin	Domoic acid	*micF*	
	Okadaic acid	(none)	
Herbicides	Atrazine	*micF*	
Oil	Alkanes (octane)	*alkB*	5150
	Monoaromatics (toluene)	*tbuT*	5147
	Polycyclic aromatics (naphthalene)	*phnS*	
Heavy metal	Hg	*merT*	4820
	Cd	*zntA*	5460
	Arsenite	*arsR*	2697
	Cyanide	*cydA*	
DNA damage	Nalidixic acid	*recA*	
Oxidative stress	Paraquat	*micF*	5458
Constutitive	NA	NA	5463

1) All strains, except *phnS*, are based on *Escherichia coli*. Numbered strains (fourth column) are those more extensively used in the project.

Development of a single-use multi-target bioreporter chip

After the initial testing and re-engineering of the *E. coli* bioreporter strains, several potential solutions were brought up to develop a single-use multi-target bioreporter chip. Such chip would contain lyophilised reporter cells, which could be exposed to the sample, after which the bioluminescence reaction would be recorded and compared to a set of calibrations. The final developed and fabricated biochip measures 10×36 mm, has 10 individual wells (cavities) of 50 µl volume, all with optically transparent bottom (Figure 5a). The biochip is fabricated in the proprietary layer-by-layer RPMD printing process of partner MicroTec. The transparent bottom is manually added afterwards.

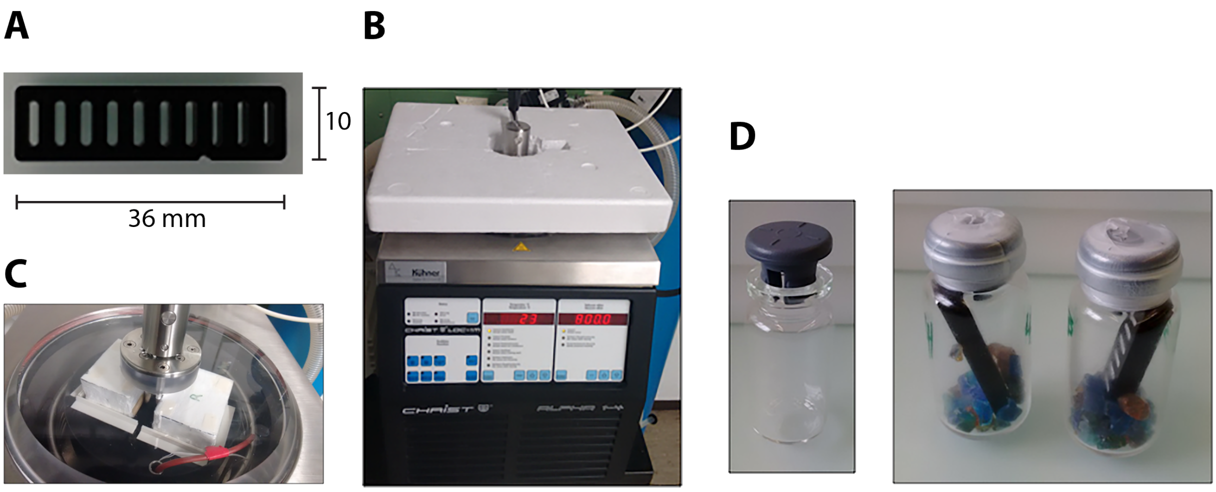

Figure 5 – Outline of the reporter strain lyophilization. A) The final biochip with 10 wells of 50 µl volume. B) The lyophilizer used for biochip freeze-drying. C) Handmade styrofoam blocks stuck to the lid of the lyophilizer in order to be able to stick a thin aluminium cover on top of each biochip after freeeze-drying. D) For longer preservation, individual biochips were placed in glass vials, which were again dried and stored under vacuum in presence of silica gel to avoid rewetting by air moisture.

In the final biochip concept, **five different bioreporter strains** are introduced in duplicate on the chip. One set of five strains is exposed to the sample; the other set is simultaneously exposed to a set of calibration standards, to compare the response of the unknown sample with. A single sample analysis uses a single chip, but up to five compounds can be targeted simultaneously.

Freeze-drying of bioreporter cells in the biochips was carried out using a lyophilizer (Figure 5b) with home-made Styrofoam blocks (85 x 32 x 62 mm), in order to enable closing the biochip after lyophilisation with an aluminium autoadhesive cover (Figure 5c). Bioreporter strains were cultured from freshly grown plates in LB complex liquid medium and grown until mid-exponential phase (culture turbidity of 0.3–0.4). Cells were then recovered by centrifugation and gently resuspended in cryoprotectant solution. Re-suspended cells were slowly transferred into the wells of the chip (20 µL per well). After filling, the bottom of the biochip was submerged in a dry ice/ethanol bath for 1 minute and placed on dry ice until all other biochips were frozen in dry ice/ethanol. All chips were then placed at –80°C, transported on dry ice to the lyophilizer, and quickly placed below the Styrofoam blocks inside the cardboard delineation (maximum 7 chips below each block). The chips were dried at –25°C and at 0.12 mbar overnight and for 6 additional hours at 0.001 mbar and +20°C, then sealed with the aluminium covers under nitrogen gas, and stored at 4°C in the dark in specific closed glass vials (Figure 5d, e).

After freeze drying, most of these bioreporters were able to distinguish between a blank and a target analyte concentration gradient (Figure 6a, b). This ability was maintained even after a month of storage at 4°C and in the dark (Figure 6c). The general BRAAVOO concept to detect multiple targets consisted of having multiple different bioreporter strains frozen in different wells of a single biochip. Depending on the choice of target chemicals one could adapt the biochip to the respective strains (see, for example, Table 4). One embodiment of this idea consisted of a biochip having five different strains, each positioned twice on the biochip. The biosensor strains which were included on this biochip for the final testing in the project included the mercury and cadmium sensors, the alkane sensor, the sensor for oxidative stress and a constitutive sensor (Table 4). This would allow measurement of an unknown sample and a clean sample at the same time, by which the signals could be compared (Figure 7). Such a chip was tested several times and also prepared for tests at the mesocosm and field sites (see below).

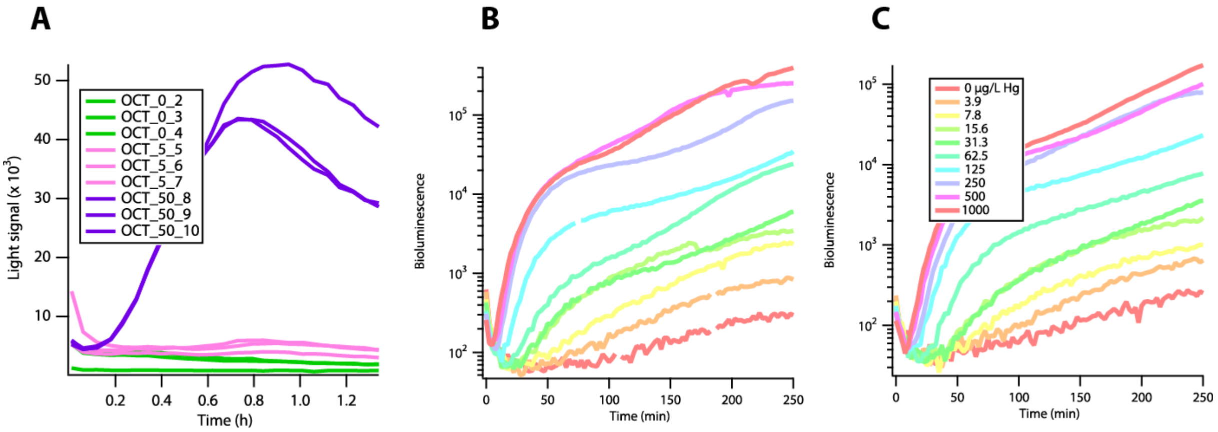

Figure 6 – Examples of freeze-dried biochip bioreporter performance. A) Replica testing of the 5150 reporter without (OCT_0–2/3/4) or with octane (5 µM, cyan lines; 50 µM magenta). Triplicates in neighboring wells. B and C) Mercury bioreporters on a single chip either directly after freeze-drying (B) or after 1 month at 4°C (C). Concentrations as indicated.

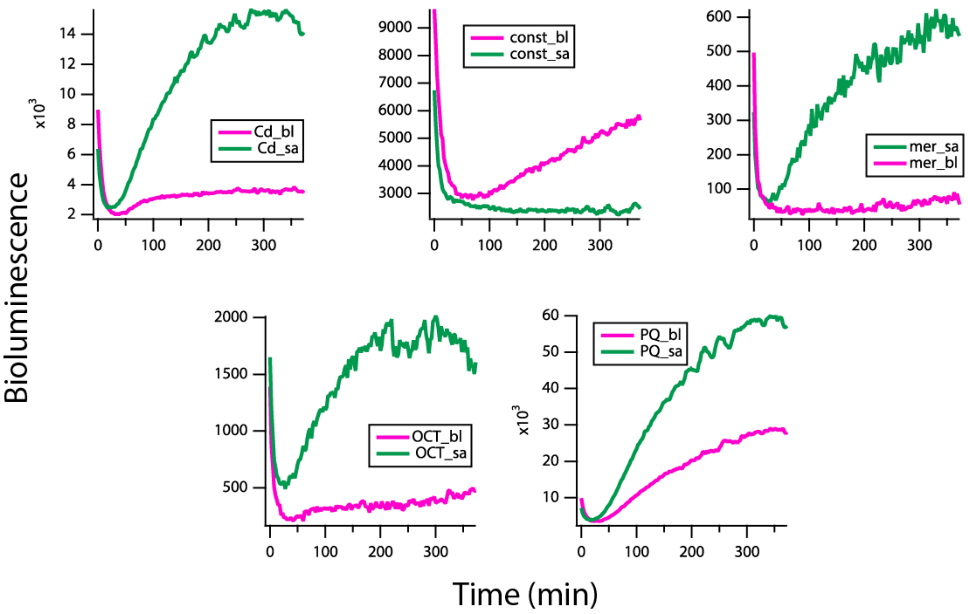

Figure 7 — Example of a biochip with five different bioreporters each positioned twice and freeze-dried on the chip, exposed to a clean sample (bl) and a contaminated sample (sa). Final concentrations of the contaminations in the sample: 1.5 mg/L Cd, 20 µg/L Hg, 5 mg/L paraquat and 1 µM octane. Cd, cadmium reporter; const, bioreporter with continuous light emission; mer, mercury bioreporter; OCT, octane reporter; PQ, oxidative stress reporter. Note that the response of the constitutive light reporter is "opposite": light will diminish in presence of a toxic sample.

Development of a continuous-use single-target bioreporter chip

The concept of the continuous-use single-target bioreporter chip was to create a mini-chemostat reactor with constantly growing and actively reacting reporter cells, from which they can be transferred automatically into a "zone" on a microchip, where they can be exposed to the sample and where the signal can be detected. Continuous growth of cells can be achieved in a closed reactor connected to an in- and an outlet, which open at regular time intervals in synchrony, to let in new medium and to flow out part of the growing cells. Under growth conditions, these outlet cells are directed to a waste. For every measurement, the reactor outlet is opened and cells are directed to the measurement chamber.

The design of the mini-chemostat reactor has been described in D4.3 (Figure 8a). The reactor was fabricated in PDMS and bonded on glass (Figure 8c). A second PDMS layered was manufactured, which contained all valve channels, to open and close the channels in the layer below with the cells. Valves were operated by air pressure on water-filled channels, connected to the chip. Time intervals and the sequence of valve opening and closing was controlled by a custom-written LabVIEW program. Reporter cells are inoculated and given fresh nutrients by opening the nutrient in- and outflow valves simultaneously (Figure 8a). The frequency of opening in- and outflow valves surrounding the microreactor while maintaining constant pressure-controlled flow determines the growth rate of the cells.

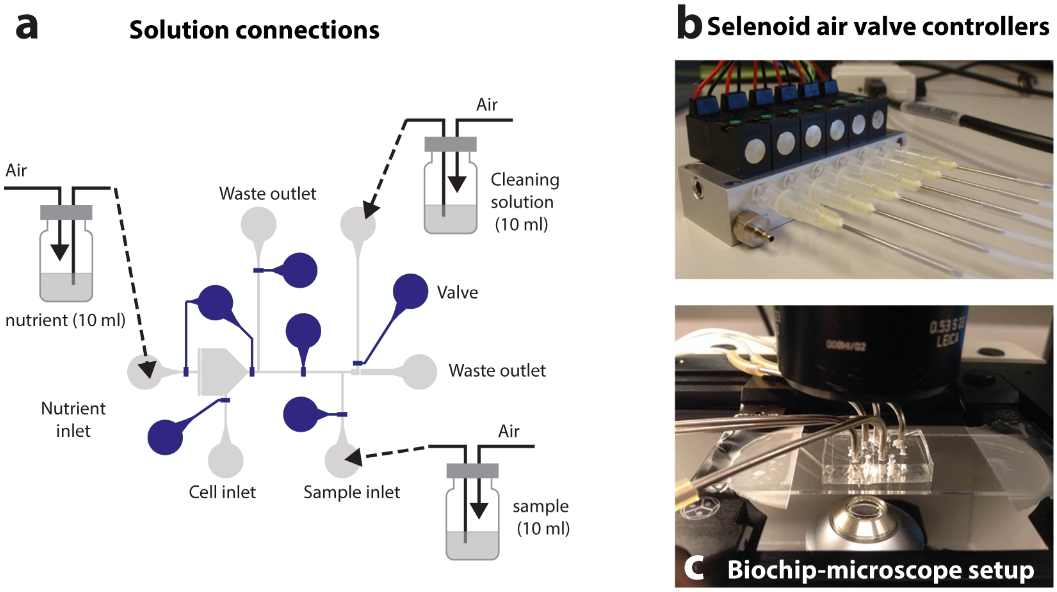

Figure 8 — Set-up of the microfluidic reactor for constant growth and biosensor monitoring. ***a*** *Sterile solutions and samples are connected to the respective inlets, and are driven by air-control overflow (0.3-0.5 bar air pressure). Other in- and outlets (apart from the cell inlet) are connected to 1 cm open tubes filled with 10 µl* H_2O *in order to avoid evaporation of liquid and formation of air bubbles in the channels.* ***b*** *A system of solenoid valves joined to pressurized air, which are connected by Teflon tubes and bent stainless-steel metal connectors to the biochip, controls the air pressure in the valves. The solenoid valves are operated by a LabView custom program.* ***c*** *Final set-up of the biochip reactor on a glass slide under an inverted epifluorescence microscope for continuous growth and measurements of biosensor fluorescence. Visible are the stainless-steel adapters inserted in the biochip inlets.*

In order to make a measurement, part of the cells from the chemostat is released during 30 min and accumulated into the measurement zone (Figure 9a). Cells in the measurement zone are exposed to the sample (Figure 9a). During the measurement period (180 min) the middle valve is closed and culturing in the reactor continues as before. After measurement, the cage and channels are cleaned by backflow from the second external reservoir, to remove accumulating cells. As shown in Figure 9, the bioreporter cells induced GFP fluorescence on three consecutive days between 6 and 9-fold after 180 min exposure to an arsenite solution of 50 µg As_{III} l^{-1} at a dilution rate of 0.12 h^{-1} in the 50 nl reactor compared to a sample without arsenite. The variation of mean GFP fluorescence after 150 min induction on different consecutive days was ~11.1%. The shortest exposure time reproducibly leading to a GFP signal development with 50 µg arsenite-As_{III} l^{-1} significantly different from a control without arsenite was 40 min.

This showed that it was possible to create an automated biosensor system allowing multiple consecutive measurements of a specific analyte in water samples with constantly growing *E. coli* biosensor cells on a single nl-reactor microfluidic biochip. Dividing cells maintain good physiological properties in the microreactor for at least one week to enable immediate reaction to the analyte. For every measurement a quantity of biosensor cells is removed from the microreactor and directed by on-chip flow control to a specific measurement cage, where they are exposed to the aqueous sample. One could imagine developing this concept further for the design of a compact biosensor instrument. Given the small size of the continuous growth chip (2x2 cm), one could also imagine creating different target specificities by multiplexing chips with different reporter strains.

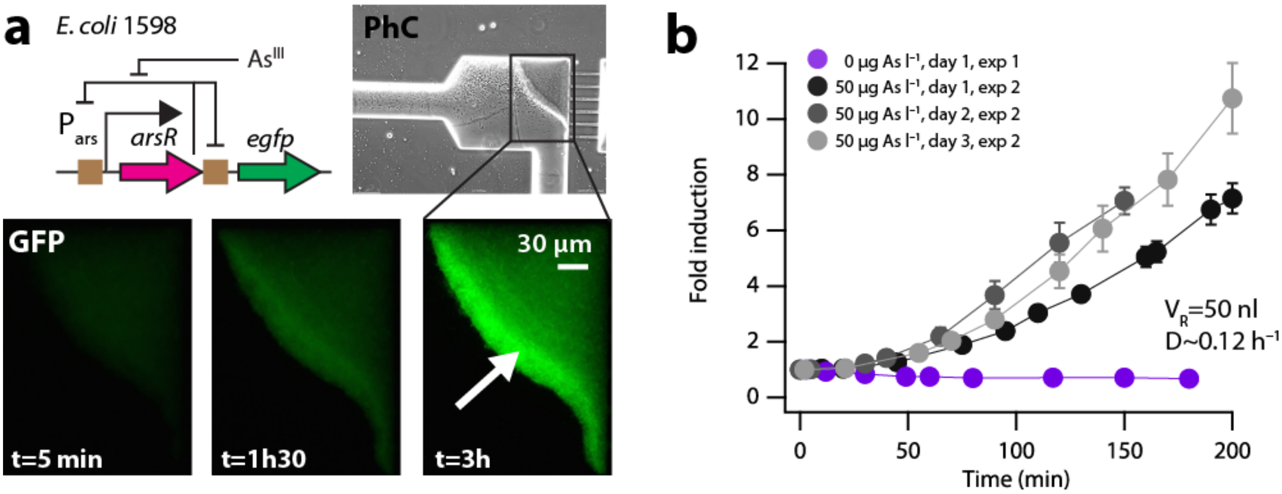

Figure 9 — Bioreporter cell induction to arsenite on the biochip. a) Fluorescence images (GFP, scaled to same maximal intensity, corresponding to experiment in panel c) at selected time points of E. coli 1598 arsenite biosensor cells in the microfilter measurement zone (PhC, phase contrast inset). b) Fold change of mean GFP fluorescence in the measurement zone over time to 50 µg arsenite-AsIII l^{-1} on three consecutive days (exp 2). Values normalized to a mean GFP fluorescence at t = 0 over all four series.

Algal bioreporters

Selection of an algal bioreporter insensitive to marine samples

As a third type of biosensor, the BRAAVOO project focused on algal photosynthesis. **Photosystem activation** in algae is a general physiological process that is delicately woven into the energy metabolism of the cell. Any disturbance in cofactor regeneration or the photosystem proteins themselves will lead to an immediate change in photosystem autofluorescence. By comparing photosystem II fluorescence before and after exposure, the sample toxicity can be inferred non-invasively and sensitively. The photosystem test is classically carried out in cuvettes in specialized fluorimeters with whole cells of the freshwater green algae (e.g. *Chlamydomonas reinhardtii*), requiring less than 5 min to detect the inhibition reaction. However, *C. reinhardtii* is not particularly resistant to the salt concentrations of marine samples and, therefore, one of the first important tasks in the project was to test and select alternative algae capable of withstanding the salt and showing a sensitive photosystem II fluorescence response to toxicants.

The photosystem II assay consists of (i) measuring baseline photosystem II fluorescence emission in absence of the analyte, and (ii) in presence of the analyte or sample. From the kinetic fluorescence curve, a number of important parameters are derived, which are used for calculating analyte effects (Figure 10):

- **F_0**: initial fluorescence. It is related to the activity of the PSII light harvesting structure. The F_0 level is reached a few ns after the start of light excitation.

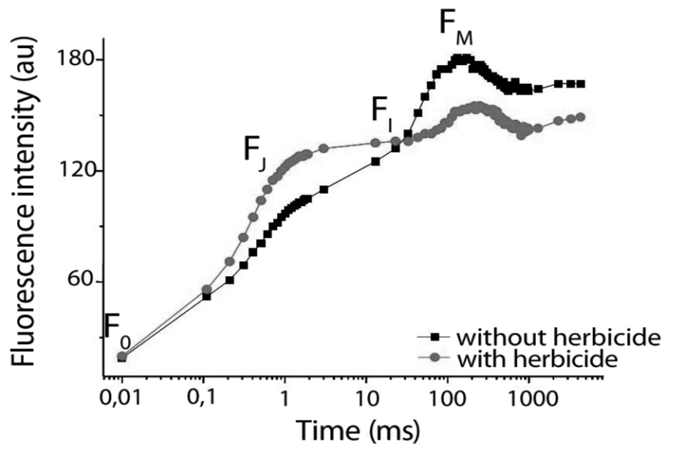

Figure 10 – Typical fluorescence kinetics of the photosystem II, as recorded on a Multilight Sensor (Biosensor srl.): 10 min dark, 11 sec of light at 127 arbitrary units, 10 sec recording. Samples or analytes were added directly to the free algal culture or to immobilized algae with prior exposition to light at 50 µmol/m^{2}s for 5 min. See main text for explanation of the major parameters F_0, F_J, F_I, F_M and derivative calculations.

- **Fm**: maximum fluorescence is the maximum intensity of the fluorescence transient, normally reached hundreds of milliseconds to several seconds after light excitation. The Fm level increases with higher excitation light intensities, until saturating excitation light intensities.

- **Fj**: fluorescence at the J region of the curve at about 2 ms (Figure 10).

- **Fv**: variable fluorescence (Fm – F_0).

- **Fv/Fm**: indicating the efficiency of PSII apparatus, useful for analysis of vitality of photosynthetic organisms.

- **Vj**: Vj= (Fj−F0)/(Fm−F0). Useful to detect pesticides acting on the quinone pocket binding site.

- Area above the curve and between $\mathbf{F_0}$ and **Fm** is also used for pesticide detection.

A variety of marine and freshwater algae were tested for their photosystem II fluorescence response to a series of analytes, and for their resistance to salt water.

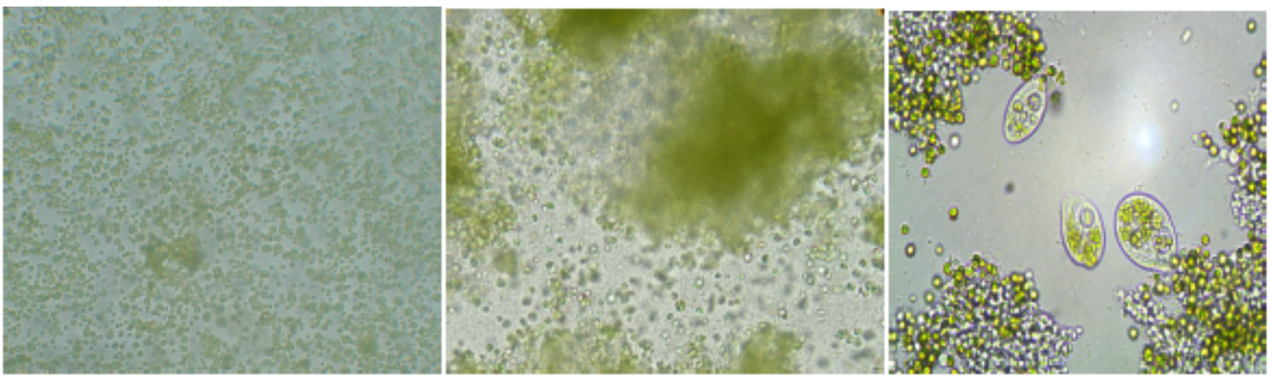

Figure 11 – Left) Microscopic analysis of cultures of Chlorella vulgaris *alone and (middle) in symbiosis with a ciliate paramecium. Right) detail of* T. pyiriformis *after symbiosis with the algal cells inside the paramecium body.*

The best strains were: i) specific mutants of C. *reinhardtii* strain IL obtained by selection through irradiation with UV light; ii) algae belonging to the genus Chlorella (*Chlorella minutissima*, *Chlorella vulgaris*, *Chlorella sorokiniana*, *Choricystis parasitica)* and iii) a symbiotic form of Chlorella with protozoa (notably *Tetrahymena* and *Colpodii* paramecia). The most stably performing combination for marine samples consisted of *Chlorella vulgaris* in symbiosis with *Tetrahymena pyriformis* (Figure 11). *T. pyriformi* is a free-living ciliate paramecium with a small cell size between 40 and 60 µm and an elongated form able to engage a symbiotic association with *Chlorella sp.* The symbiosis has various benefits for both the paramecium and the alga. For the symbiotic algae, the host can supply the algae with nitrogen and carbon dioxide.

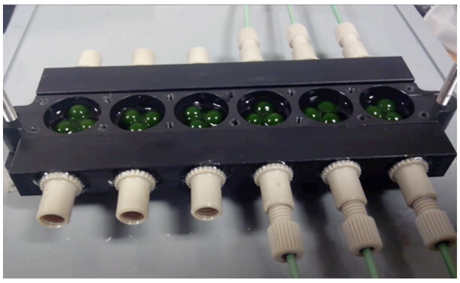

Figure 12 — Embedded and immobilized algae (green beads) in the photosystem II fluorescence sensor. The designed system has six parallel flow cells.

Table 5. Parameters of photosystem II fluorescence measured in the integrated sensor.

Addition	Dark	Fluo at t0	Fm	Tm	Fv	Fv/Fm	Vj2ms	V4ms	V8ms	V10ms	Area
PBS	**8**	**377**	**2749**	**479**	**2199**	**0.80650**	**0.21230**	**0.2233**	**0.24511**	**0.25647**	**59990**
PBS+Diuron	**8**	**12324**	**43600**	**10920**	**31881**	**0.73121**	**0.91022**	**0.9226**	**0.93601**	**0.93773**	**$2 \cdot 10^6$**
PBS+IPA 50%	**215**	**12148**	**41610**	**10910**	**30051**	**0.72220**	**0.92576**	**0.9345**	**0.94083**	**0.93947**	**$3 \cdot 10^6$**
IPA 100%	**112**	**11810**	**39883**	**10890**	**28534**	**0.71544**	**0.93912**	**0.9445**	**0.94907**	**0.94669**	**$4 \cdot 10^6$**

Diuron was added at 0.5 µg L^{-1}. Two alginate-algal beads were added per measurement well. PBS, phosphate buffered saline. IPA, isopropylalcohol (negative control, to inhibit photosystem II fluorescence).

In order to optimize the response of the algae-paramecium symbiosis in an automated instrument, it was decided to immobilize the cells in calcium alginate beads (2-3 mm). Those beads are easy to manipulate and can be embedded in a specifically designed and fabricated flow-through cell (Figure 12). The flow-through cell can be connected to an optical unit which provides basic illumination for the algae, and enables induction and detection of photosystem II fluorescence changes. The immobilized algae can remain alive and functioning for several months at temperatures between 4–20°C.

Biosensor instrument design and fabrication

Standalone instrument fabrication

The original idea in the BRAAVOO project was to build three biosensor instruments, which would contain either one of the biosensing elements described above (i.e., nano-immuno optical transducer platform - BiMW or aMZI, bacterial bioreporter cells in the biochips, immobilized algae). The instruments should be operatable as stand-alone units, being able to introduce the sample, the different necessary reagents, perform the readout and collect the data. In a later stage, these instruments would then be connected and included in the autonomous platform (buoy and unmanned surveying vessel).

For the **photosystem II fluorescence measurement** of the algal sensors a new fluorimeter was developed by partner BIOSENS, that was based on a newly designed electronic board controlling the optical elements, to which the algal flow chambers could be connected. The optical module for the fluorescence induction measurement consists of a microcontroller connected to a series of boards and six optical cells where the fluorescence measurements are carried out. Each cell contains two red and two white light LEDs, and an optical fluorescence detector that acquires the measurements (Figure 13). The instrument can work directly from a rechargeable battery at 12 volts in case of power line failure or for analyses on a buoy. The fluorimeter provides input of 500 µmol photons m^{-2} s^{-1} red light for 11 s at 650 nm and measures the fluorescence emission by a photodiode at 680 nm. The red light covers a surface of 1 cm^2, resulting in highly reproducible measurements averaged over 1000 points.

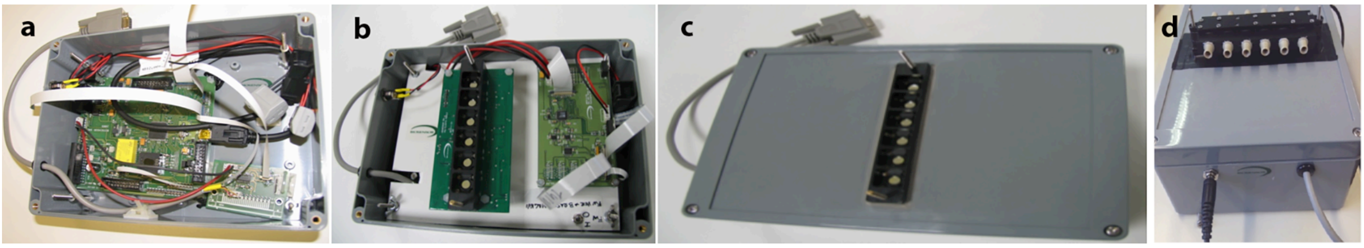

Figure 13 – Integrated fluorimeter instrument developed by Biosensor.srl to allow algal photosystem II fluorescence measurements. a) The lower electronic boards controlling the optical elements and data recording. b) The mounted illumination system, which goes underneath the six flow cells. c) System with cover leaving only the light units for the flow cells. d) Instrument with the mounted flow cells on top.

The white LEDs in each measurement cell ensure maintenance of the algae, and are switched on continuously for 7 h out of a 24 h period. This guarantees the necessary photoperiod for the survival of the algae. The immobilized algae are introduced into each of the six flow-through wells, which are mounted on top of the fluorimeter (Figure 14). In order to exclude ambient light, the flow cell is encased in a black box. Once a command run is executed, the measurement starts, and two integrated fluidic pumps allow automatic sampling into each of the six parallel flow cells.

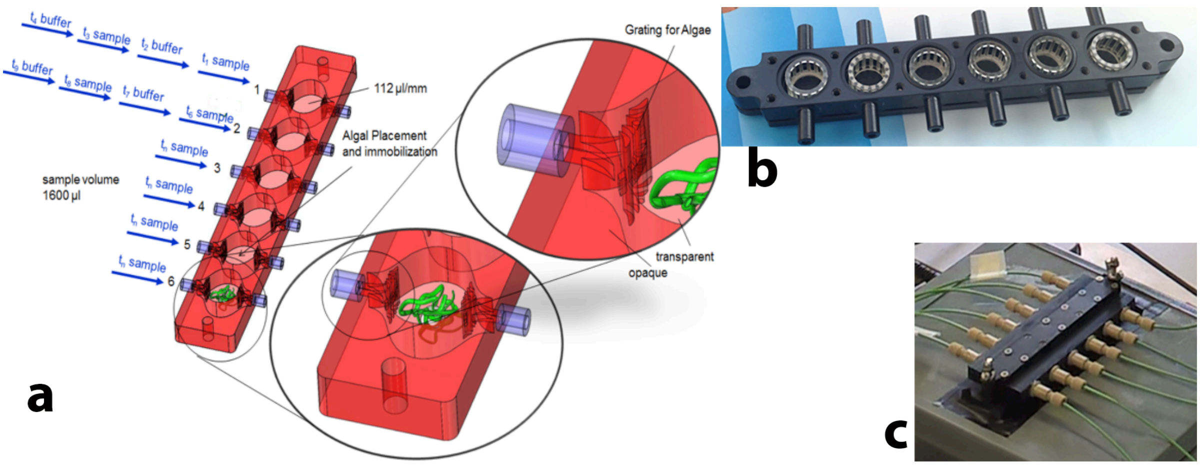

Figure 14 – The flow cell holder for the immobilized algae. a) Six parallel flow cells with internal filter structures enable the immobilized algae in calcium-alginate beads to remain inside while being exposed to a sample or regeneration solution. b) The RPDM-printed version of the six cages with the filter insets. c) The mounted closed flow cells with the fluidic connectors.

After sample analysis is finished, the data are either collected on a memory card or send to the central control unit of the buoy or vessel, in which the biosensor module is placed and connected. The immobilized algae can be regenerated after each measurement with the aid of the white LEDs and a regeneration buffer for long-time maintenance. The instrument allows determination of the main chlorophyll fluorescence parameters: F_0 is calculated using an algorithm that determines the line of best fit for the initial data points recorded at the onset of illumination, this line was extrapolated to time zero to determine F_0; F_M is the maximum value achieved during recording; and F_v is the variable component of fluorescence. Measuring fluorescence at 2 ms gives F_{2ms} and the corresponding $VJ = (F_{2ms} - F_0)/(F_M - F_0)$ that refers to the rate of reoxidation of QA^- with respect to its reduction (see also D3.2).

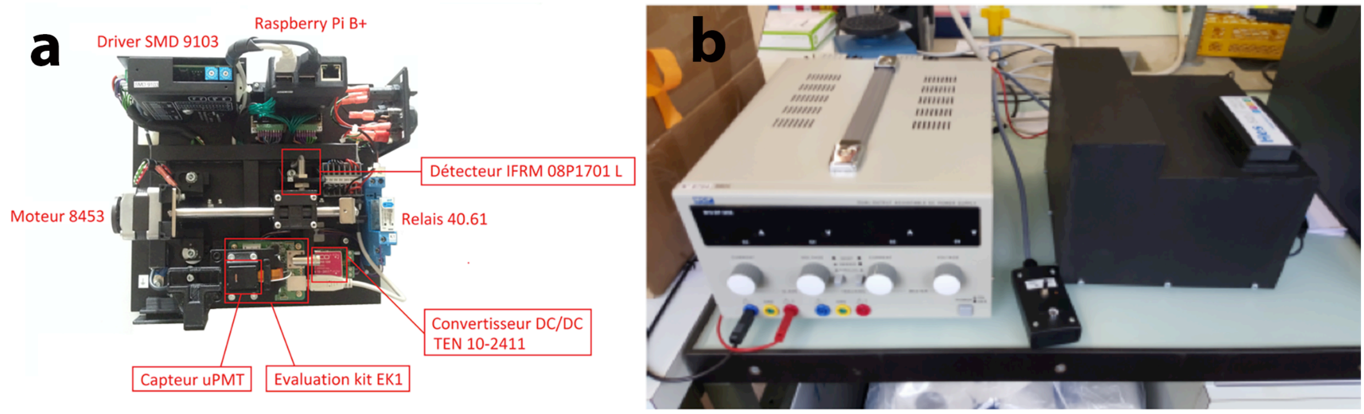

Figure 15 – The manual bacterial biochip bioluminescence reader produced for the BRAAVOO project. a) Inside stepper motor moving the 10 well biochip over the Hamamatsu microPMT detector. Raspberry PI controller for the motor, detector and data storage/transmission. b) Packed instrument on the right with power supply on the left.

A further specific stand-alone instrument for the **bacterial bioreporter unit** was designed and fabricated (Figure 15). The bioluminescence from the bacterial cells in the 10-well biochip is detected by a commercially available micro-photomultiplier (µPMT) from Hamamatsu. The sensor counts the number of photons passing through the measurement head, which has a dimension of 1 by 3 mm and fits below the transparent window in the biochip (see Figure 5a). The instrument has a stepper motor which moves each well above the detector in repeated cycles of around 2.5 min. The detector counts the emitted light from each well during 20 sec in each measurement cycle, which is repeated up to 150 times to obtain a kinetic measurement (Figure 6). The stand-alone instrument is powered with a 24 V battery. A 24/5V converter is included. In the manual protocol, the freeze-dried bacteria in the well of a biochip are reconstituted manually with sterile saline solution, after which the sample is added.

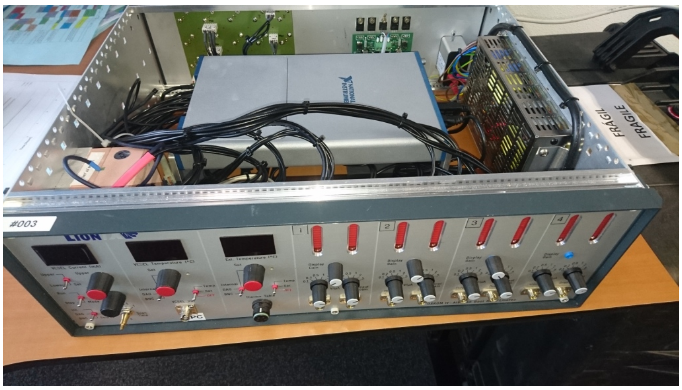

Figure 16 – The OSROM light source and detector unit for the aMZI and BiMW nano-immuno sensors.

The detector unit for the **nano-immuno sensor** is comprised of a so-called "OSROM"-system, to which the incoming and outgoing optical fibres to the aMZI or BiMW chips are connected (Figure 16). This system is described more in detail in deliverable D4.1, and is also used to control the VCSEL (optical source) current and temperature settings, and produces the light required at the input of the BiMW/aMZI sensor. The system also contains 8 photodiodes with amplifiers for read-out of up to eight sensors and a high end DAQ card for fast signal processing with high accuracy.

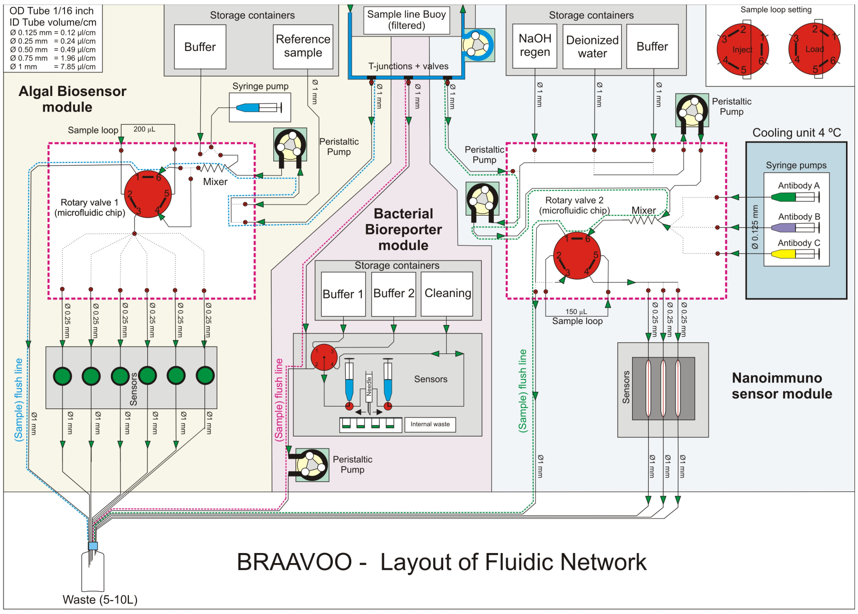

Figure 17 – General layout of the fluidic requirements of the three BRAAVOO biosensors.

Microfluidic units

All three sensor instruments depend to larger or lesser extent on integrated microfluidic systems to provide the reagents for the measurement and the sample. The algal and bacterial biosensor units as stand-alone allow manual filling of the flow cell and biochip, respectively. In their automated modes, they also depend on integrated fluidics and microfluidic systems. A general layout of the (micro-)fluidic requirements for each of the three systems is presented in Figure 17.

For the **algal biosensor** it is necessary to either provide the immobilized algae with regeneration (nutrient) buffer or with the sample. In addition, a reference compound may need to be added to calibrate the reaction from the algal photosystem.

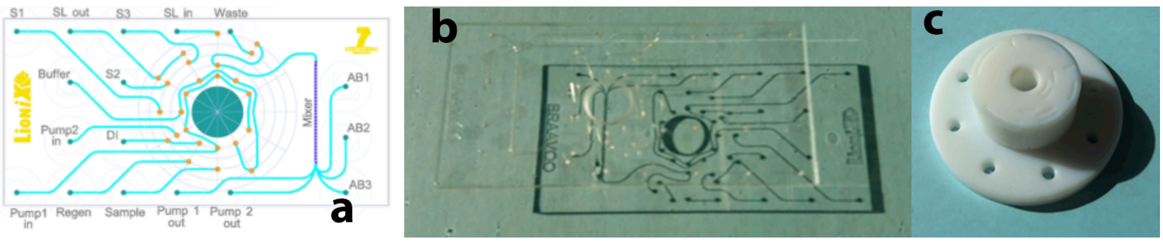

Figure 18 – Example of a rotary valve designed for the BRAAVOO biosensor microfluidics. a) Layout of the different possible fluidic connections. b) Glass chip with the etched channels. c) Teflon rotor with the connecting channels etched in its top, which fits the central hole in the glass chip.

Sample inflow is provided by a peristaltic pump integrated in the system. Other reagents are provided with a syringe pump, that can be mixed with the sample. The concept of the six parallel flow-cells with the algae is that the first flow-cell is used for as long as the algae hold out. If a toxicity is observed and the algae can no longer be regenerated, the system will switch to the second flow-cell, etc., until the last flow-cell is used and new algae have to be loaded. This requires a very complicated automated tasking of dividing and controlling fluidic streams, which was achieved by the fabrication of a specifically designed rotary valve system by partner LioniX (Figure 18).

The **nano-immuno sensor** was the most complicated in terms of different immunoreagents that are needed for the complete assay to be carried out on the BiMW or aMZI chip. In the final prototype system with three measurement channels and one reference (Figure 1), three different antibody solutions need to be provided, in addition to buffers, regeneration solution, sample and calibration solutions. The antibodies are provided in the system by three integrated syringe pumps, whereas samples and other reagents are provided by peristaltic pumps operating with specific sample loops.

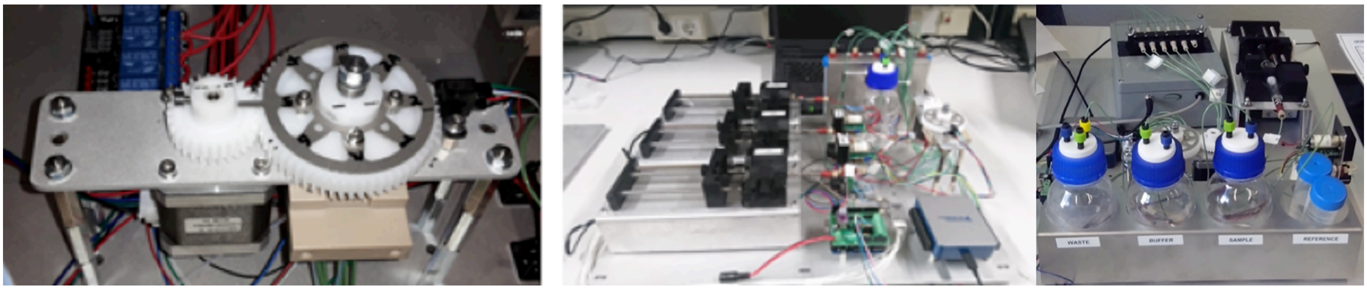

Figure 19 – Rotary valve control in the algal and nano-immuno sensors. Left, the stepper motor which controls the different positions of the rotary valve. Middle, integrated system with the syringe pumps, the reagents bottles, the fluidic connections and the Arduino control unit. Here shown for the nano-immunosensor unit. Right, Integrated fluidics for the algal sensor unit.

Mixing chambers are again integrated to allow sample dilution or reagent mixing, and this is conceptualized in another specific rotary valve (similar to that in Figure 18). Both rotary valves have 12 positions, which each represent one of the 12 different complex fluidic pathways required in the measurements protocol of the multi assay nano-immuno sensor. Setting the rotary valve on a certain position is performed by a stepper motor connected to the rotor of the valve by transmission gearings and is controlled by an integrated Arduino (Figure 19). For the stand-alone system a laptop was used as a major control unit running under Labview to enable parameter optimization and testing of the system.

The fluidic network for the bacterial biosensor unit (in its automated mode) was slightly different (Figure 20). Since for each sample measurement a new biochip (with lyophilized bacteria) is used, which is covered by a thin aluminium adhesive film, the system needed to be able to puncture this cover in order to inject the liquid. Furthermore, three different solutions, (i) a regeneration solution to rewet the dried cells (i.e., sterile saline), (ii) a calibration solution and (iii) a cleaning solution for all the fluidic lines needed to be provided by the system. In addition, the sample itself needed to be injected at the appropriate moment. To guarantee this, the system uses a more simple valve and two integrated syringe pumps (Figure 17).

The system is designed with the idea that the cavities with lyophilised bacterial cells are filled with a small volume (10 µl) of reviving buffer followed by sample injection (10 µl). A peristaltic pump is used to draw in the sample from the main source, from where a small volume is drawn in by the first syringe pump. A needle is driven through the aluminium adhesive cover on the biochip and the volume of the syringe is injected into the detection chamber a little after the second syringe has injected a small volume of reviving fluid. A third container with cleaning liquid is installed to rinse the syringes. Internal short lines avoid crosstalk between different samples and salt depositions on the needle points.

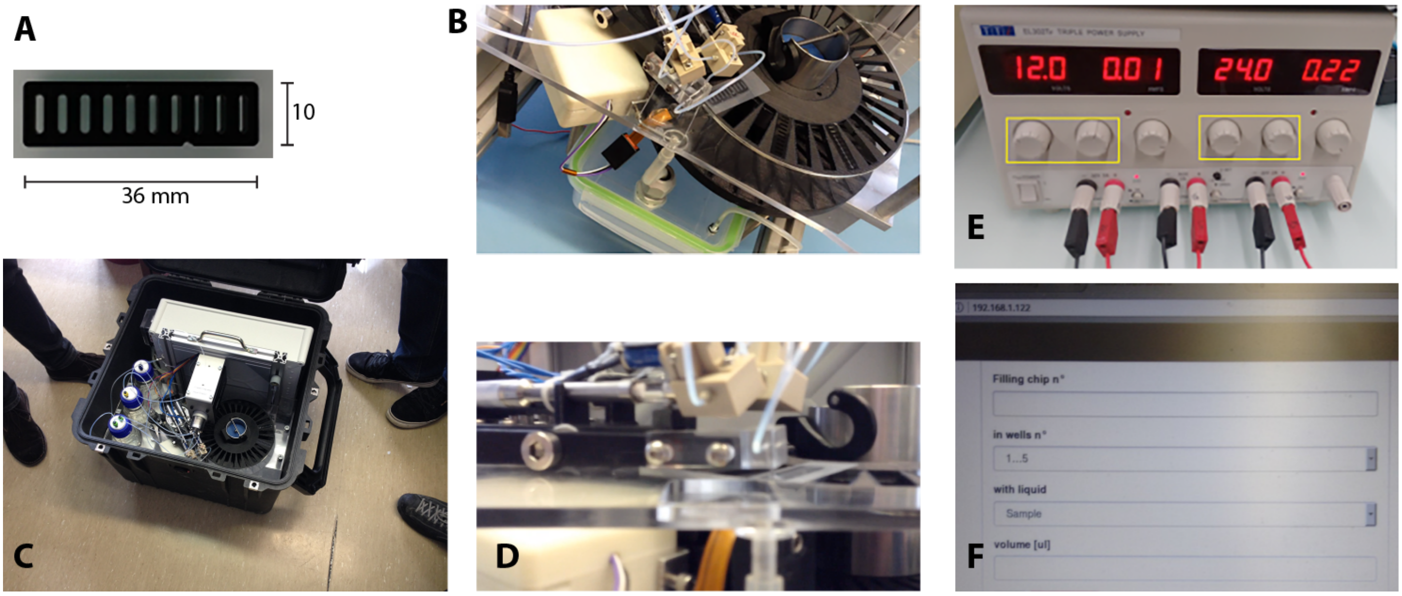

Figure 20 – The automated bacterial bioluminescence biosensor instrument ("Biolum"). The biochips with freeze-dried cells (A) are inserted into a carousel that provides space for 30 chips (B). C) the complete instrument can be placed inside a waterproof case for protection and implementation on the buoy or vessel. D) The syringes puncture the aluminium seal on the biochips and inject the solutions to the cells. The µPMT detector moves below and records the light coming from the cells. E) and F) The system is controlled by a 12 V power unit and operates under Labview.

A circular holder (the "carousel") was designed and manufactured, which can fit 30 individual biochips (Figure 20b). The carousel can automatically change to a new position after each measurement. A µPMT detector is mounted below the carousel, which detects the bioluminescence coming from the cells in each well of the chip (Figure 20c), like in the manual standalone system. The complete system uses 24 and 5 V and can be placed in a volume of 40 x 40 x 40 cm. Each well is first filled with saline solution to prevent contamination of the clean needle with sample water. The filling device moves above the biochips (Figure 20b). The vertical displacement of the needles is achieved by electromagnets pushing down when turned on, punching the seal, the resting upper position is achieved by springs.

The controlling system had to survey the measurement procedure and to communicate the results with the buoy. A dedicated electronic system including a processor was implemented. Because of its simplicity and the available interfaces, a powerful Raspberry Pi 2 computer was chosen as basic control for the instrument. It contains an Ethernet port to connect to the buoy and can act as USB master to control the PMT. The GPIO pins are used to drive the syringes, valves, pumps and all other mechanical devices. The measurement sequence can start by powering-up the system or by a specific command. If power-up serves as start-of-measurement command, the sequence in the script will be executed only once and the system powers-down automatically after its completion. The system can also start when the power is turned on by the main system (for example, the buoy). In that case, the main system can again shut down the instrument by powering off the voltage supply. At each new "power on" a new chip is placed and measured.

Final connectable sensor instruments

Based on the stand-alone systems further adaptations were made to enable their integration and communication to the buoy provided by IDS. Major improvements were needed in cabling, compensation for shocks (using springs on the ground plate), and in integration into water-tight Pelicases with flow inlines for the supply of filtered water samples taken from the lake or sea (Figure 21). All fluidic protocols were replaced on Arduino, without further need for Labview. Extended interface support was provided and the Datapod now emulates on a Laptop, with functionalities of sensing and fluidics integrated. The algal system is also housed in a Pelicase, supported by appropriate spring structures for shock absorption.

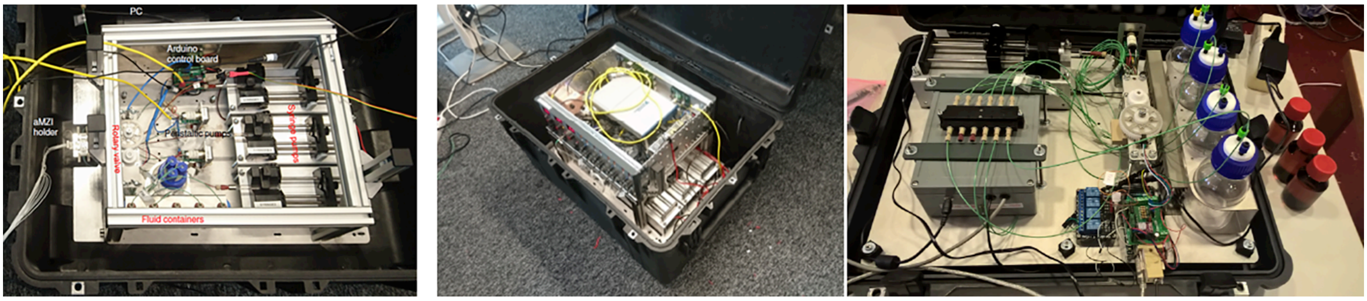

Figure 21 – The final connectable sensor instruments to the buoy and vessel. Left and middle, the nano-immuno aMZI platform with integrated fluidics and OSROM detector. Right, the algal sensor platform with integrated fluidics.

Autonomous marine deployment

Marine monitoring buoy

A key objective in the design of a marine monitoring buoy was to achieve a platform that was big enough to host the solar power and battery packs required to run the BRAAVOO system and at the same time have a system that can be packaged for easy transport. The platform was designed to be easily serviced, to providing a stable platform to facilitate safe boarding by an engineer during service visits in good weather; to provide large secure space. Providing significant space on the deck of the buoy to accommodate whatever is required. Finally, the platform needed to facilitate generation of required power. The prototype uses 10 batteries to provide 1200 Ah of capacity, which are charged with six 80W solar panels (Figure 22). The BRAAVOO data buoy prototype has been deployed in Loug Derg first in April 2015 and on several occasions after that in bays or lakes around Ireland.

A Data Acquisition and telemetry system was manufactured which allows communication to and from the platform, store data and other information transmitted by the sensor and to transfer (bio) sensor data from and to the platforms (data buoy and vessel). The Data Acquisition System is based on an adaptation of the existing IDS Monitoring DataPOD. This system has a much wider role than simply sensor data collection. It has several control functions, including switching on/off power to system components, sampling time control (including the Biosensors), winch control, monitoring of data buoy position and solar panels. The Data Acquisition System contains three power relays to switch on/off different sensors and the following interfaces: four RS232 ports, an RS485 Modbus Port, eight analog input channels, two digital pulse counting channels and an Ethernet port. The buoy is also fitted with a full single board computer. In addition, it includes two telemetry modes, including GPRS/GSM and ISM band radio (868 MHz). The system is capable of triggering alerts, and it is possible to use the system to achieve some level of remote control (e.g. reset the Biosensor on demand, take a sample, change a sampling interval, etc.). Data is delivered in the IDS Universal Long Format to a BRAAVOO DataLink Server.

In addition to the buoy, an autonomous stable movable platform (**the USV**) was developed that is large enough to accommodate the equipment required and not so large that it would be difficult to transport, launch propel and recover. The project opted for a small trimaran hull, based on a commercially available vessel components made by the WindRider company. The basic hull is stable with a plastic and aluminium structure that provides buoyancy that will allow install up to approximately 250 kg of equipment. A deck is built on top of the outrigger frame which provides additional space for equipment, and to install an outer frame to accommodate the ASV thrusters. The four thrusters that we have opted for are an adaptation of a Minn Kota thrusters and each can deliver about 36 Kg of thrust. The power system to run the thrusters and other systems on board has two main parts. The first part comprises a bank of deep-cycle sealed lead acid batteries, arranged in three separate battery systems delivering 24V and having a capacity of 300Ah. A secondary smaller battery will be implemented as a backup. This arrangement is necessary to achieve the endurance required.

The Vessel Control System Architecture is considered in two main parts. The first part of the system is the mobile command centre which remains onshore (or can be mounted inside a mobile vehicle) as the remote command and control centre and the second part concerns the systems onboard the vessel itself. At this control centre two separate linked Ethernet networks are implemented.

Figure 22 – The marine deployment vehicles for the BRAAVOO biosensor instruments, designed and fabricated by IDS Monitoring. Left and right, the data buoy. Middle, the USV in its "slave" configuration following a control boat.

The first one is the Control Network, which handles data coming from the ASV on board cameras, primary ASV navigation sensors, feedback from the propulsion systems and related control data. The navigation sensors include an IMU and a GPS, a 360 deg pan camera, plus backup GPSs. The Control Network is used as a transport media to read ASV navigation data from ASV-mounted sensors (positions, orientations, linear velocities, angular velocities, depth, sonar data, etc.), to watch and record live video feeds from on board cameras and to send control commands and mission files to ASV. The second network is the Sensors Network. This carries data from the BRAAVOO Biosensors, other real-time water quality sensors (temperature, pH, DO, etc) and also secondary radar/sonar data. The Sensors Network is used as a transport media to integrate BRAAVOO Biosensors with remote monitoring software, to analyse sampling and data acquisition in real time, to retrieve and store acquired data and to send and process radar/sonar data. The BRAAVOO Biosensors Computer in the command centre is connected to the Sensors Network.

Communication infrastructure at the shore side will include antennas and other network equipment including routers and switches. Two telemetry systems are integrated on the USV, a 5 GHz radio system and cellphone based 3G/4G network. The unmanned vessel is further capable of autonomous operation and is hereto equipped with a collision avoidance system. A prototype guidance, navigation and control (GNC) system compliant with the International Regulations for Avoiding Collisions at Sea, was developed. An obstacle avoidance module was implemented that uses a Fuzzy Logic Obstacle Avoidance Controller (FLOAC). **The Guidance system** continuously computes the reference (desired) position and velocity of the BRAAVOO USV to be used by the motion control system. **The Navigation system** is used to determine position, attitude, velocity, acceleration and course of the BRAAVOO USV and distance travelled. **The Control system** determines the necessary control forces and moments to be provided by the BRAAVOO USV in order to satisfy a certain *control objective*. The desired control objective is combined with the guidance system. The system has been implemented in the LabVIEW framework. Three main scenarios have been designed and validated in simulation environment: overtaking, head-on and crossing. Special “Master-Slave” Operation Mode (where Slave BRAAVOO USV “follows” Master Boat) has been designed to improve manoeuvrability in confined spaces and close proximity to obstacles. “Master-Slave” Operation Mode has been successfully tested in real-world environment in August 2016. The system is described in more detail in Deliverable D7.3.

Sampling system

A water sampling and filtering system was built by IDS that can sample from a depth of up to 20 m (considered to be normally sufficient for water depth in the coastal zone), and extract at least 100 ml of filtered sample within 10 minutes from this depth. The filter provided by IDS has a 15 micron mesh and is self-cleaning to avoid fouling and clogging of the filter. The extracted filtered sample is then made available to the BRAAVOO biosensor instruments. The system can run on the power available on the BRAAVOO Data Buoy and BRAAVOO Autonomous Vessel and is sufficiently compact to be hosted on both. The system includes four peristaltic sampling pumps (Cole-Palmer) and a compressor. These allow sampling near the surface, mid-water and near the bottom. The pumps require a 12 Volt power supply, and the system is controlled by the IDS Pump controller developed for this project. The tubing used on this first prototype is 6 mm in diameter. At the bottom of each intake pipe was fitted a foot filter with a relatively coarse mesh size (100 micron or smaller mesh size).

In the case of the data buoy the main structure was designed to provide space for relatively large prototype systems. The sensors were simply placed on the deck of the buoy and could be strapped in position as required and were then integrated easily with the power systems, the data network and the fluidic sampling system which were almost all wall mounted. Once a biosensor was installed on the platform there were three connects made. All biosensor instruments were operated on the 24V, 12V or 5Vdc as delivered by the buoy. The fluidic connection was provided by a connector main tube branching, from which all biosensors tapped into. The waste from the sensors was kept within the sensor instrument box. The more sophisticated element of the integration was the data interface. The bacterial biosensor and the nano-immuno sensor instruments connected via Ethernet, whereas the algal biosensor instrument connected via RS232. A specific communication protocol was developed between devices, starting the measurement sequence when the main DataPod clock decides. The connections worked well because of the main integration already achieved within the platforms themselves and it was not difficult to build on this.

Biosensors and system benchmarking

Benchmarking scenarios

The biosensor instruments were "benchmarked" during various stages of development, and in their final integrated stage with the marine monitoring buoy for their technical functioning and, more importantly, for their deployment in a near real-life situation. However, in order to sensibly calibrate the biosensor outputs it would be important to deploy them under conditions in which to expect the concentrations at which the targeted contaminants would be present. Unfortunately, only very rarely permission can be obtained to cause a contamination in real-life that would enable testing the biosensor in the field with "known" contaminant concentrations. Three types of alternative scenarios were thus created to test, calibrate and compare the output from the biosensor instruments to external reference methods such as environmental chemical analytics.

In the first scenario, the biosensor instruments were kept under well-defined laboratory conditions but were given blind a set of marine samples to which known concentrations of target compounds were added. In the second scenario, a contamination was created in **mesocosm** facilities, which meant an upscaling of the contamination size, but with still a good level of control on the expected contaminant concentrations. In this case, the biosensor instruments were transported to the mesocosm facilities (in Messina, Italy) to work on-site. In the third scenario, the biosensor instruments were shipped to a marine site with possible but unknown contaminations. On-site sampling and analysis was tested during a week. In all cases, samples were taken and shipped back to the analytical labs for verification. In the comparative tests, it should further be noted that in all these scenarios, the biosensors worked directly on the sample without any prior purification, concentration or extraction (except simple dilution). The chemical analytics standard procedure involves a set of purification, extraction and concentration steps, which drastically lowers the method detection limits for most target compounds. Of note also that the logistics of sending the instruments and bioreagents around was complicated and challenging for the experiments, which sort of limited the number and duration of the occasions for deploying the biosensor instruments under field conditions.

Examples of ring laboratory testing

Six marine water samples were spiked with Irgarol 1051 at environmentally relevant concentrations in IDAEA-CSIC. After being spiked, the seawater samples were labelled as blind samples and sent to CIN2-CSIC, where they were analysed with the nano-inmunosensor (BiMW platform) for Irgarol. Overall, a very good agreement was obtained between the nano-inmunosensor and the nominal concentrations (Pearson index of $r=0.998$ and a $p<0.001$). The nano-immunosensor results thus showed good promise as a monitoring tool for this biocide.

For testing the algal biosensor, marine samples were spiked with diuron, simazine and atrazine. The obtained concentrations by the algal bioreporter were in good agreement with the spiked concentrations ($R^2>0.9985$, $p<0.001$, $\alpha=0.05$ %) for both diuron and simazine. The sensor overestimated the response of these two biocides: the slopes of the correlations were 0.592 ±0.013 ($p<0.001$) and 0.698±0.006 ($p<0.001$), for diuron and simazine, respectively, instead of 1.0. The reason for this may be a synergistic activity between the fortified analyte and other toxic substances, to which the algae are sensitive.

Mesocosm experiments

The mesocosms experiments were carried out between 9 and 13 May 2016 in Messina at the facilities of

CNR (see Figure 23). The first day the tank was filled with seawater and after two hours an oil spill was simulated using 2 L of Pier-oil. After 4 days of sampling, oil was collected with CASTALIA floating barriers and adsorbent material, simulating the action of the Italian national emergency plan in case of oil spill. Fourteen samples were taken in total, two in each sampling time (t=0 h, 4 h, 22 h, 29 h, 46 h, 70 h and 77 h), one on the upper layers of the mesocosms (Figure 23b) and another in the bulk of the mesocosms (Figure 23c). An additional sample was taken 1 hour prior to the oil spilling as a control of the background contamination.

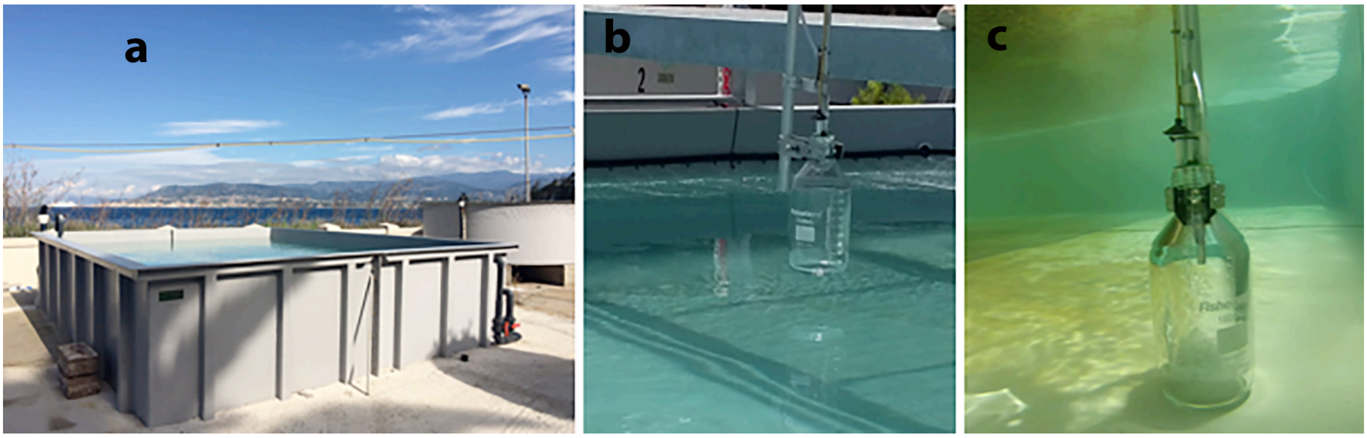

Figure 23 – Mesocosm facilities at Messina (a) used for the artificial oil spill sampling (b and c) to test biosensor's responses.

Table 5. Bacterial biosensor (standalone instrument) data on samples from the oil mesocosm.

Sample	Sensor					
	OCT-eq (nM)	MICF (mg/L)	SOX (mg/L)	ARS	ZNT	TOL (nM)
t=0-0	20	0.5	0	0	0	10
SA0U 20 cm	150	1	0	0	0	30
SA0U 80 cm	150	1	0	0	0	20
AU3 - 22h	0	0	0	0	0	0
AU4 - 29h	0	0	0	0	0	0
AU5 - 46h	0	0	0	0	0	0
AU6 - 70h	0	0	0	0	0	0
AU7 - 77h	0	-	0.4	-	-	0

The bacterial biosensors in the standalone instrument picked up signals of alkanes and BTEX in the early samples of the spill, as well as some signs of oxidative stress (Table 5). Measurements by GC-MS indicated individual PAHs ranged from the limit of detection to 0.8 µg/l. These concentrations were not high enough to induce a response in the bioreporters. Measured concentrations of BTEX were in the ng/l and sub-ng/l levels and did not result in a positive reaction of the bacterial bioreporters. These concentrations rapidly decreased through time probably because of their high volatility. The most concentrated sample was the initial one (SA0U), similar as for alkanes. In this case, SA0U and SA1U did induce a positive response to the bacterial bioreporter, demonstrating its functionality as an early warning tool.

Field-buoy deployment

Using the freeze-drying workflow, biochip batches were prepared with five strains in duplicate: the mercury, the alkane, the cadmium, the oxidative stress and the constitutive bioreporter. Their proper functioning was tested upon arrival at the site after air-freight delivery to artificially contaminated seawater. The most optimal measurement protocol for the test was to, (i) re-hydrate the cells by injection of 10 µl 0.9 % NaCl, and (ii) inject 10 µl of artificial clean seawater to wells 1-5 (controls) and 10 µl of seawater sample to wells 6-10 (sample). At this point the light emission from all wells was measured 50 times in series (taking around 2 h). Afterwards, 10 µl of calibration solution containing Cd^{2+} (4.5 mg/l), Hg^{2+} (60 µg/l), paraquat (15 mg/l), octane (3 µM) was added to all wells and the biochip was measured for another 100 cycles.

The figure below (Figure 24) gives an example of how the bioreporters responded to a seawater sample (of unknown composition) in comparison to clean ONR7a. In particular, the cadmium and the alkane bioreporters reacted to the seawater sample since the slopes of the bioluminescence kinetic response were higher than in the clean sample. This was not the case for the mercury and oxidative stress (PQ) reporters. All reporters also reacted to the addition of the calibration standard after 120 min by an increase of the light emission, indicating that they respond correctly and are not inhibited by the sample. This showed the usefulness of working with this approach based on a duplicate set of reporters on a biochip, one part of which is exposed to a controlled blank sample and the other to the sample.

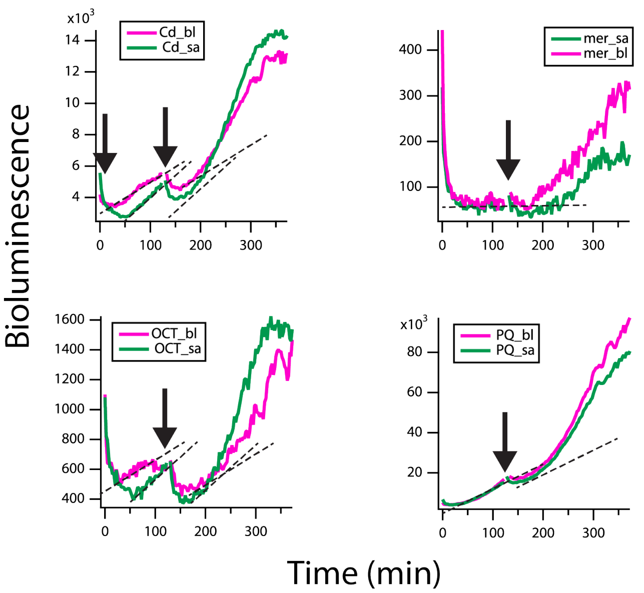

Figure 24 – Real sample analysis (encoded as "_sa") in remote mode using the five-strain bacterial biosensor biochip with duplicate mode, in comparison to clean ONR7a medium (encoded as "_bl"), plus injection of the calibration standard (at time 120 min.) Cd: cadmium biosensor. OCT: short-chain alkanes. PQ: oxidative stress. mer, Hg^{2+} sensor.

Analysis of the harbour water in Kilrush by the integrated algal biosensor showed no contamination by herbicides. The immobilized algae were first kept in the dark for 30 min, then exposed to light for 11 s, after which the fluorescence response was recorded and transmitted back to the remote observer.

The nano-inmunosensor was fully integrated in the buoy, it was operational and communicated successfully with the buoy command pod (more details are given in deliverable 8.4). Unfortunately, the connection between the optical fibre and chip deteriorated during the field tests causing the loss of the measurement signal after the initialisation measurements. This problem could not be repaired in-field because of the lack of necessary materials. Therefore, the performance of the nano-immunosensor at chemical level could not be assessed in the field deployment.

GC-MS analysis confirmed different alkanes with higher molecular masses, which may explain the reaction of the bioreporter cells to the harbour sample. ICP-MS analysis could not find traces of Cd, therefore, the bacterial reporters may have reacted to another metal. Neither mercury nor herbicides were detected, which is in agreement with the bacterial and algal reporters. The *Alivibrio fischeri* test also showed no toxicity of the sample in terms of acute toxicity. The occurrence of PAHs was relatively low in the analysed samples. Irgarol 1051 was not detected.

Socio-economic impact

The BRAAVOO project expected main three expected impacts in relation to the call topic:

- Enable early detection and more effective monitoring of the marine environment and its status and implementation of appropriate management actions in line with the Marine Strategy Framework Directive (MSFD);
- Improve sustainable management and exploitation of marine resources (such as fisheries and aquaculture) in particular the monitoring of quality of shellfish waters and minimise risks to human health;
- Provide competitive advantage and leadership to European industry, for example within the fields of biotechnology, sensor development, diagnostic technologies and nanotechnology.

Early detection and more effective monitoring

One of the actions to achieve long-term maintenance of marine environments is early detection and effective monitoring of its chemical, physical and biological "status". Biosensors (in the widest sense of the word) are considered an important asset and tool that can permit more easy, real-time, in-situ, more cost-effective but still highly reliable measurements. It cannot be realistically expected that biosensors will measure the 250,000 or more individual chemicals that potentially may threaten the marine environment in a cost-effective manner. Instead, by **focusing on individual chemicals, compound classes** as well as **general parameters of biological relevance** (e.g., broad toxicity or stress), the BRAAVOO project attempted to cover a wide range of potential impacts and assaults. From the onset, we focused on a dozen important individual chemicals, which were chosen to match as much as possible a number of priority chemicals mentioned in the Marine Board / ESF Position Paper, while concentrating on the other hand on compounds which it can realistically target through existing and new R&D. This was important, because the project wanted to build on numerous existing laboratory proofs of concept of biosensors[1], and start tackling the question of how they can be integrated in easy deployable and perhaps automized instruments.

[1] see, for example, Brussaard CP, Peperzak L, Beggah S*, et al.* (2016) Immediate ecotoxicological effects of short-lived oil spills on marine biota. *Nat Commun* **7**: 11206. Siegfried K, Hahn-Tomer S, Koelsch A*, et al.* (2015) Introducing Simple Detection of Bioavailable Arsenic at Rafaela (Santa Fe Province, Argentina) Using the ARSOlux Biosensor. *Int J Environ Res Public Health* **12**: 5465-82.

In the end, we can conclude that BRAAVOO has delivered a number of extremely tangible results and exploitation possibilities for biosensors. Based on the consortium's technical expertise, a successful embodiment was created for the three types of targeted biosensors: nano-immunosensors, bacterial whole cell sensors and algal sensors, in suitable electronic instruments, which were able to carry out label-free sample measurements. Humbly, the project had to realize how **difficult** such R&D transition actually is, and how much time and energy needs to be invested to find, build and test engineering solutions. This was particularly relevant, since solutions had to be found between partners that were geographically widespread. Despite the fact that the project would have like more actual measurement time with the completed biosensor instruments, the important result was a demonstration that the biosensor reactions, the assays and the signals could be picked up with the automated instrument versions. This thus showed, in essence, that the project's goal of using three types of sensors to achieve overlapping detection specificities, is possible. Only by deploying the three biosensor types together, we think one could achieve a system that can cover both **single priority compounds** as well as have an appropriate **first-line warning** system against unknown toxicants. Not surprisingly, the nano-immunosensor in its embodiment with the aMZI interface was the most sensitive (pM-nM range, depending on the quality of the immunoreagents), followed by the bacterial bioreporter and algal photosystem sensors (nM-µM range, depending on the sensor type and the target chemical). Since the last two systems detect biological **effects** they arguably cover the most likely scenario of a pollution spill. Without further optimizations, the sensors could thus be deployed as a **first-line alarm system**, which, when raised, could be followed up by more specific sampling using high-end chemical analytics to possibly identify the nature of such compounds.

The final instruments in their automated versions are still sort of bulky in their water proof Pelicases. In a way, this is ironic, because the biosensor systems themselves are very small (chips of a few centimetres), but it is the accompanying fluidics, electronics and pumps that make the prototype systems still quite big. This is not uncommon for technological developments; when gaining more experience, the sensors could successively be further miniaturized. For incorporation into the buoy or onto the USV, this size was not a limiting factor, whereas robustness was.

The project also showed that the biosensors can be deployed in a hand-held version of a smaller instrument. In this case, samples have to be introduced manually and the readout is performed automatically. The performance of the hand-held instruments was actually a little better than for the automated instruments, possibly because particularly for volatile analytes, some analyte is lost in the tubing in the instrument. We estimated that production cost for the biosensor parts (antibodies, bacterial and algal cells) per test is quite low (~1 € per test), and although the detector instruments were in the order of 10,000 to 50,000 € to produce (as prototype), this would be competitive compared to other instruments.

One of the potential advantages for the deployment of biosensors, which we initially emphasized, was their relatively short analysis time: from seconds (nano-immunosensors) to minutes (algal photosystem) and 1-2 hours (for most bacterial bioreporters). When automated sampling, initial filtration and fluidics is included, all three biosensors can perform a sample analysis within 1-3 h. As far as our experience goes and upon discussion with potential stakeholders, this analysis time would be largely sufficient for semi-continuous real-time and on-site sampling. We also showed that this can be combined by multi-sample analysis in parallel, for example, three simultaneous measurements for the nanoimmuno-sensor, five simultaneous targets for the bioreporter, by which the net individual time per analysis becomes more favorable. Also, for typical monitoring by a marine buoy, one or two samples per day may be sufficient to guard against accidents and spills. Even with the instrument prototypes and the preliminary field data, the BRAAVOO project raised sufficient interest from potential stakeholders, as a meeting in May in Messina with Port Authorities, local governmental agencies and military officials testified. Therefore, we can conclude that the basic foundation for such biosensor usage has been led. What is now needed are (i) more field data, (ii) further instrument optimization and (iii) detailed discussions with regulatory agencies for approval of biosensor analytics (since one of the major questions for monitoring labs is whether a test is legally binding!).

Improve sustainable management and exploitation of marine resources

Since it is widely considered that regular monitoring of critical chemical and biological parameters helps to recognize constant threats (e.g., low concentrations of dangerous bioaccumulating chemicals), early diagnosis of accidents with large impact (e.g., oil spills, other types of spills, algal blooms), would be a major advance if the time between sampling and analysis could be drastically shortened. In addition, it would be advantageous if more frequent and spatially more diverse samples can be analysed, which will increase the confidence of obtaining an appropriate measurement result and making suitable decisions or action plans. In addition to focusing on very few parameters, the BRAAVOO combinations of biosensors permit additionally more **global** parameters, which may ease on site interpretation. As example, oil analysis by advanced GC-MS/MS may reveal thousands of compounds each with individual importance but which need high expertise to interpret. On the other hand, a bacterial bioreporter assay for alkanes or for mono-aromatic compounds will give a combined signal for a complete compound class as it is perceived by the cells. This singular biological signal is therefore much easier to interpret and can, if need be, further detailed by chemical analysis.

Initially, we warned for care against too much "erosion" of analytical quality, for example, by false “self-analysis“ of environmental samples through individuals with a limited analytical background. We showed that biosensor-type measurements can be standardized and internally calibrated to such an extent that the “analytical“ output can be more easily interpreted, but at this point, we would definitively need more time and field-studies to **benchmark** the actual meaning of the biosensors output. BRAAVOO thus concludes that it should be possible to have sensors working directly in the sample at the required concentration range. However, we also acknowledge that pretreatment is an excellent option to further optimize target detection, and we hope that developments in other OoT-projects will enable such pretreatment options.

BRAAVOO showed that our devices can provide analytically correct data (albeit sometimes grouped as **biologically response data**), that help to make data interpretation much easier. We also demonstrated that the **modularity of the devices** and of the **individual sensor elements within** the devices make for extremely flexible and point-of-care solutions. Future projects can build on our experience to continue developing biosensors for real-time use. There is probably not a single area where R&D and competition is so fierce as in bioanalytics, particularly in the areas of human health and diagnostics. Importantly, it is not the absence of a market (in the sense of there being no need for sample analysis), but the cost and ease, and successful demonstration of the value of biosensor sample analysis on real-life samples that limits further market introduction. Our data help to obtain a "change of mind/perception" among analytical chemists that biological systems can produce useful analytical results and are not too complicated to be meaningful.

The second hurdle has been the use of genetically modified bacteria for the bacterial bioreporter tests. Given the strong negative public opinion against GMO plants and animals, this has long 'doomed' practical applications of bioreporter tests. In contrast, and again through pioneering work by HUJI, UNIL and related collaborating laboratories such as HZI-UFZ, bacterial bioreporter tests generally face a very **positive** attitude in public, and have been highlighted in numerous popular science contributions. Through the use of non-pathogenic and crippled laboratory strains (such as the *E. coli* host in the proposed bioreporters in BRAAVOO), and through the consequent use of completely closed systems with minimal handling of the bacteria as we demonstrated within the BRAAVOO project and instruments, the risks for escape and environmental survival of the bioreporter bacteria are minimized (though they will never be zero).

The BRAAVOO project worked as closely as it could manage with relevant stakeholders. Local and international stakeholders were invited to our meetings and to accompanying activities. The echo was not in all cases enormous, but we had fruitful discussions at the General meeting in 2014 in Barcelona with Spanish stakeholders, and in Messina in 2016 with a large group of Italian stakeholders. In addition, some members from other OoT projects were present during some occasions at the BRAAVOO General Meeting. Also, our external advisors were present on several General Meetings to give input. The mesocosm experiments in Messina in 2016 was an occasion to work with other projects, notably FP7 KILL-SPILL, and other FP7/Horizon2020 project members attended the final BRAAVOO Workshop in Villars in November 2016.

Regulatory issues and accreditation

One of the recurring aspects in stakeholder's discussions was the legal aspect of accredited assays. Governmental and commercial standardized laboratories are bound to certified methods and protocols for the analysis of compounds in sampling matrices, and, so far, biosensor assays are not part of this. This needs to be taken seriously, since much commercialized chemical analysis is performed under DIN or OECD standards. Without such standards, biosensor-type analysis will not be accepted. BRAAVOO has initiated this discussion, but was not able to progress much further than that, as a result of the focus on the technical aspects of the sensors. Its point of view was primarily to show first that biosensors can perform excellent assays and only then start a process of certification. This is a weak point but a single project like BRAAVOO is unfortunately not very capable to advance much on the certification of bioassays. This is an aspect that hopefully can be addressed after all OoT projects have finished and a better summary view of bioassay and biosensor analytics can be obtained.

Main dissemination activities and the exploitation of results

Dissemination

The main dissemination actions of the project focused on (i) setting up and maintaining an active BRAAVOO project website, which would be both aesthetically appealing for visitors as well as informative about the project and its partners, (ii) to publish scientific results in peer-reviewed journals, as a proof for the quality of its research, (iii) to disseminate results and concepts to both the wider public and a network of potential end users, (iv) to develop a general exploitation strategy for BRAAVOO results and technological developments and (v) to productively collaborate with other Ocean 2013.1 projects.

The full BRAAVOO public website (http://www.braavoo.org**)** was opened online in October 2014, and is fully described in the deliverable D10.1. The public area provides a generic overview of the project and its objectives, also for networking purposes, and includes a scrolling 'latest news' board, with information about meetings, publications, activities and other items of note (for instance, the exhibition of one partner's biosensor chip at a museum). Viewers can get access to further FP7 Ocean of Tomorrow projects also focusing on marine biofouling, acoustics, oil spills and biodiversity. One can also 'dive down' into the site for further information about the project, project outcomes and its participants, and also find the various contact information. Of interest for the public are: the project Final Public Workshop page, the project video from the BRAAVOO special edition of the Euronews Futuris feature (http://www.euronews.com/2016/05/30/monitoring-sea-pollution), the technical sheets summarizing the BRAAVOO biosensor instruments, the project publishable summary showing the achievements of BRAAVOO, and the short BRAAVOO flyer.

In addition, the website is the main entrance for the project partners, where they can find all the project's documents, plus all dissemination activities are recorded on the Final PUDF (D10.5) and on the restricted access (Members section) PUDF webpage: http://www.braavoo.org/members/pudf/index.php. All BRAAVOO related dissemination activities have also been recorded online on the European Commission participant portal SESAM.

Scientific publications always take a little longer to appear, but publications of project research results in the peer-reviewed literature are appearing, as described fully in D10.3, with manuscripts in Hydrocarbons and Lipid Microbiology Protocols, Marine Genomics, Microbial Biotechnology, Lab on Chip, or Biosensors and Biodetection. Numerous conference presentations were given by project partners on various aspects of the BRAAVOO findings and developments. Finally, integrated dissemination efforts included a special issue on Marine Biosensors in Current Opinion in Biotechnology (which will appear in 2017 with several contributions from BRAAVOO partners and from other OoT projects) and a Frontiers in Marine Sciences topic (also to appear in 2017; again with several contributions of OoT projects including BRAAVOO). These efforts should help increase overall impact of the project results. We are expecting several more peer-reviewed publications, but this may await further validation or lab verification experiments that have to be concluded after the project is finished.

Two specific stakeholders meetings were organized. A first 'stakeholder session' was held during the third consortium meeting in Barcelona, Spain, as mentioned in a section above (project flyer). A second stakeholder forum was held during the sixth consortium meeting in Messina, Italy. The second forum attracted 43 participants, primarily from local and national authorities, industries and research partners working on the field of marine environment monitoring. This was a particularly interesting and fruitful meeting to get feedback on the biosensor concepts and ideas.

The other OCEAN 2013-1 projects have been invited to the final BRAAVOO Workshop on November 25 and 26, 2016, which was organized in Villars (CH). The Scientific Committee of the Workshop was co-organized by the Sea-on-a-chip, Schema and SensOcean. The goal of the Workshop was to present sensor developments of different Ocean-of-Tomorrow projects and to be able to demonstrate the BRAAVOO instruments. Talks were given both by invited speakers, allocated speakers and young scientists. Some 30 participants attended the Workshop, which included two dozen interesting talks. All participants had optimal interactions and discussions.

UNIL as coordinator of BRAAVOO participated further in the common OoT-project activity during the OCEAN 2016 International Exhibition in London, where all projects were briefly presented in an oral communication (5 min) by the coordinator, followed by posters. UNIL as representative for BRAAVOO further participated in the European Science Open Forum (ESOF) 2016, which was held in Manchester. This was the occasion for a number of OoT projects to participate in a session about marine sensors and pollution monitoring, which was organized by the DG Marine. UNIL presented a 10 min talk on the concepts and results of the BRAAVOO project.

Further fruitful links and exchanges were maintained with other OoT projects. Notably, a 2017 issue of Current Opinion in Biotechnology was focused on environmental and ocean sensing, with various scientific contributions of other OoT projects. Furthermore, a topic issue of Frontiers in Marine Sciences was initiated, for which again several OoT teams were invited for contributions. This, we hope, guarantees further scientific dissemination of OoT (and BRAAVOO) activities.

Finally, UNIL as coordinator of BRAAVOO, organized **two one-week workshops for the general public**. The first was organized in collaboration with the Swiss project "Envirobot" and the organization "Biodesign for the real world". This "winter"-school was held from January 31 - Feb 6, 2016 at the University of Lausanne, CH. The course consisted of two main parts: building of an electronic fluorescence detector and construction of a fluorescence producing bacterial sensor. 20 Participants attended, from all over the world (including, US, India); surprisingly, many artists attended. Additional contacts were made to the Hackarium open science centre in Renens, near Lausanne.

The construction of a bacterial biosensor was repeated in Summer 2016 with 22 high-school students in the framework of a BRAAVOO-SCNAT summer school "Synthetic Biology for Beginners". For simplicity, in this case we refrained from the handling the electronic parts and only concentrated on the biological engineering. Also this school was a great success and it was heartening to see so many enthusiastic high-school students working during summer on such a project.

Exploitation

The BRAAVOO exploitation manager defined a general exploitation strategy, implementing the results of a survey of consortium members, for development of the planned use and dissemination of foreground (PUDF). Additionally, a market survey was compiled from specific stakeholder meetings and questionnaires, which allows some ideas on the potential for commercialisation of future biosensor products and their expected market volumes. An exploitation webinar was recorded by IDS in order to provide critical information to BRAAVOO partners about exploitation, about protection of IPR and about strategies for best use of project outcomes, as described in Deliverable 10.3 (M18). The major conclusions are:

- The market needs for biosensors for water analysis in marine environments are clearly present. For example, pesticides, herbicides and heavy metals were considered of importance and given the priority contaminants identified by EU legislation. There is also a genuine interest in automated monitoring systems for priority pollutants that can be operated on a site, and that may be used for maritime security protection of shallow waters and ports, and the need for ocean data and data mapping

- Stakeholders highly appreciated the objectives of BRAAVOO, the technical solutions developed. Various stakeholders and end-users showed potential interest to test BRAAVOO biosensor solutions and compare them with either gold standard analytical methods or other solutions currently on the market. Some solutions were found to be hard to adopt because the biosensor protocols are not standardized accepted assays, and as such have no legal value at the moment.

- There is still a long way to go for biosensor assays and instruments, in particular for reliability tests in harsh environments. Stakeholders see a significant growth in Unmanned Surface Vehicle (USV) market due to their potential benefits, cost effectiveness, and ability for operations in hazardous conditions. In particular advanced USVs with higher precision and reduced weight will become trendy. Other segments of the USV market in terms of payload such as sonars, sensors, camera, visual systems, etc. will also be influenced.

- The sensor segment is projected to grow at the highest CAGR (Compound Annual Growth Rate) during the forecast period (2016-2021), primarily driven by the increased use of sensors in scanning, detecting, mapping, remote sensing and the measurements of the various elements, compounds, absorption and presence of microscopic life.

- The increasing growth of the global environmental sensing and monitoring market will surely have an impact on demand for floating platforms. One of the key attractions of the BRAAVOO platform over many others is that the systems operate autonomously collecting data 24/7. Different ranges of buoys are used in coastal sites in support of academic research, coastal monitoring and operational applications such as fish farming or hydrographic studies.

A number of key issues still remain. Specifically, the cost is an issue - the larger platforms can be very expensive. It is also recognized that power is always a consideration on data buoys and as offshore broadband becomes more of a reality, new demands will further stress the existing, relatively low-power systems. Those challenges are currently being addressed.

Final conclusions

1) All three biosensor types produced suitable stand-alone versions, with integrated fluidics, optics and electronics. In one case, there is a clear commercial follow-up, because partner structures enable this. The algal biosensor produced by BIOSENSOR Srl is commercially available. Furthermore, CSIC-CIN2 has links to spinoff companies to potentially commercialise the nanoimmunosensor tests.

2) All three biosensor modules were integrated into an automated device, that connects to either buoy or USV, or both. Biosensors showed multi-target and multi-sample capacity as demonstrated by benchmarking proof-of-concept experiments. Through the modular design of the sensors (e.g., different strain - different target), one could then extend this easily.

3) A functional prototype of the complete system was constructed during the project's lifetime, which was capable of autonomously taking a sample, dispatching this over the sensors and measuring its output. This is the first deployment of biosensors on an automated vehicle in a marine environment.

4) A robust marine deployable buoy as well as an unmanned surveying vessel were constructed, which enable biosensors instruments and other sensors to be implemented. The marine buoy and vessel are autonomous in power usage, telemetry, navigation and can be remotely operated.

5) Ring-testing and calibration, plus testing in mesocosm facilities under realistic scenarios showed reasonable comparison between the biosensor measurements and classical high-end analytics. Indeed, some sensors were more accurate and sensitive than others, in particular when considering that biosensor measurements were carried out in the raw seawater sample, and the chemical analytics on highly purified and concentrated samples. Despite all the testing, further validation, particularly of the automated instrument, is necessary to convincingly demonstrate its robustness and usefulness.

This could not be completed during the project, because it was logistically too demanding.

6) The project has led to a larger variety of useful exploitable instruments that can be applied in newer project settings or in other formats, such as biochip designs, fluidic designs, optical designs, automated instruments, rotary valves, sampling devices, strains of bacteria and algae, immunoreagents and measurement protocols.

7) The project convincingly demonstrated that biosensors can be extremely useful tools for monitoring of the chemical quality in difficult environmental matrices, such as marine systems. Both accurate quantification and also "first-line warning" properties are attractive, and the combination of nano-immunosensors, bacterial bioreporters and algal photosystem II tests may provide a powerful combination to target a number of different compounds or "qualities" (such as toxicity of the water). The demonstration of both standalone versions and measurement protocols, plus automated modular devices was disseminated to potential stakeholders and interested parties, and can hopefully be followed up in future environmental monitoring exercises. The gain in time and cost may make a big difference in the strategies currently available for such monitoring.

Major achievements and further information can also be found in the website: http://www.braavoo.org.