BRAAVOO

biosensors for real time monitoring of marine contaminants

Second Periodic Report

Publishable Summary

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A summary description of the project context and objectives

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

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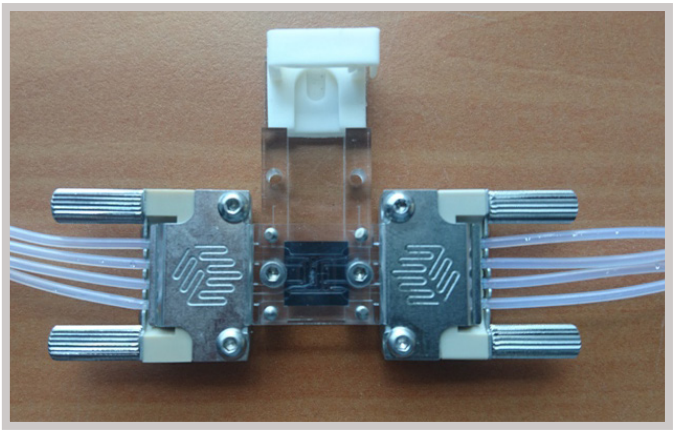
performance of the stand-alone biosensors and biosensors in their integrated form was tested at field sites in Italy and Ireland, and was further benchmarked using spiked marine samples with known target compound concentrations. Comparative chemical analytics showed reasonable agreement between the two 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 would 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 bench-marking 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.

Description of the work performed in the second period of the project and the main results achieved

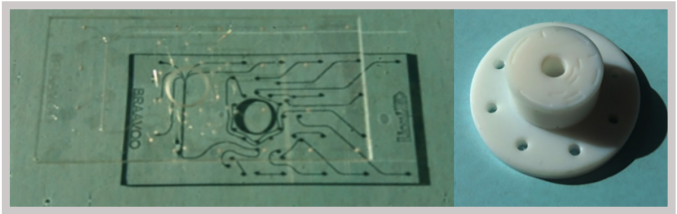
The protocols for the competitive immunoassays were further optimized and adapted for use in the bimodal waveguide (BiMW) and the asymmetric Mach-Zehnder interferometer (aMZI) nano-immuno sensor formats. The best immunoreagents allowed label-free detection of the biocide Irgarol at 5 ng/L in seawater in the BiMW sensor with an estimated 30 times regeneration possibility, and 30 ng/L in the aMZI sensor (that needed five-fold diluted seawater). Tetracycline could be detected at 40 ng/L in phosphate-buffered saline (PBS) in the aMZI, and okadaic acid at 0.2 µg/L in PBS in the BiMW platform with 9 regeneration cycles being possible without signal loss. Optimization of the protocols from the previous Surface-Plasmon Resonance platform to either BiMW or aMZI was not successful for detection of



↑ *Figure 1 — The final multiplex aMZI nanoimmunosensor chip (middle) with connecting optics (right and left) and the superimposed microfluidics connection (middle to top).*

2,2',4,4'-Tetrabromodiphenylether flame retardant (BDE-47), domoic acid and ampicillin. Mostly, this was caused by poor antibodies or poor surface binding efficiencies. This could not be further improved during the project's lifetime. A multiplexed aMZI chip with four parallel lines (incl. one control) was fabricated to measure simultaneously Irgarol, tetracycline and okadoic acid (Figure 1). Surface receptor binding was further optimized to allow parallel competitive immunoassays. A new microfluidic connector was designed and fabricated that allows isolated delivery of samples and the different reagents to the aMZI four-line chip. Furthermore, a new optical connector was fabricated that connects the light input to the chip. Finally, a complete new miniaturized solution in form of a rotary valve (Figure 2) was designed and fabricated which allows the simultaneous provision of more than a dozen different reagent and washing solutions, and samples to the chip. This was integrated in a single fluidic-sensor module.

↓ *Figure 2 — The final rotary valve: glass fluidic connections (left) and the Teflon-polished rotor fitting in the middle hole (fluidic connections in the top; right).*

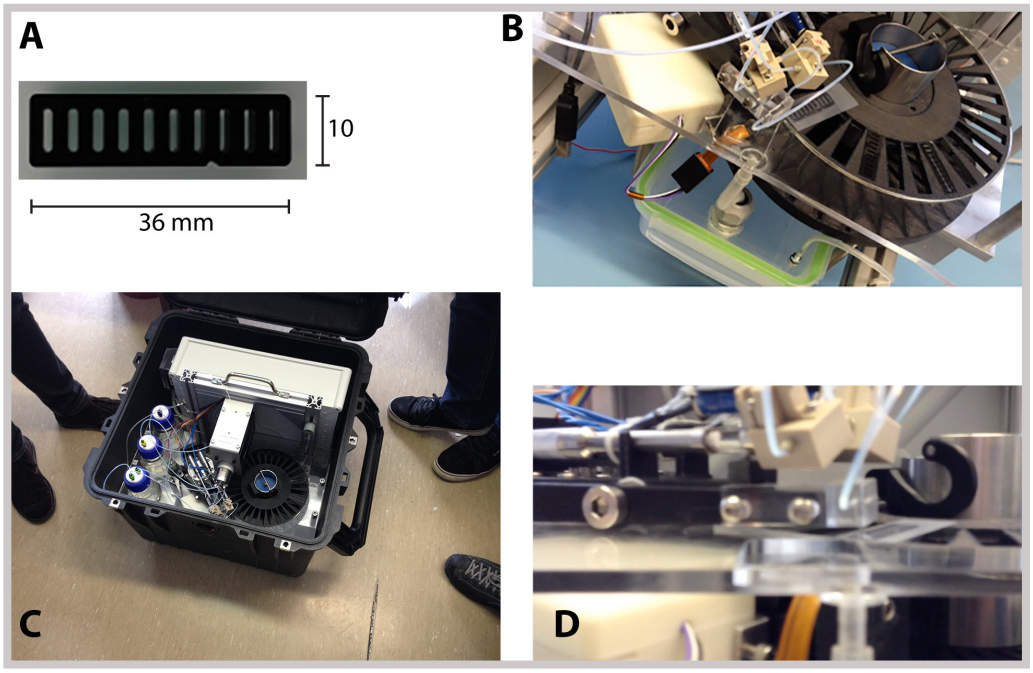


The set of *Escherichia coli*-based bioreporters that had been re-engineered to produce coherent light output during the first project period was intensively tested for target-induction properties after freeze-drying and in the appropriate final biochip format. The freeze-drying protocol was adapted such that it allowed preservation of the bacterial strains at 4°C, and permitted immediate activation of the bioluminescence development upon contact of the bacteria to their target in a seawater sample. A new biochip with 10 parallel 50 µl volume cavities and transparent bottoms was fabricated, which holds 20 µl lyophilized bacterial suspension. Up to five strains with different specificities are applied twice on the chip, one for the sample assay and one for the simultaneous calibration. The bacteria are activated by addition of sterile saline solution, after which the sample is added and measured for 2 h. After that period, a small volume of calibration solution is added to verify for sample toxicity, and the output is again measured for 2 h. A specific instrument was constructed, based on a commercial microphotomultiplier tube, which can read the light output from the cells in kinetic mode. This was then further integrated in an automated instrument, which can inject sample and reagents, record light emission from the cells, and replace the biochips for each new measurement (up to a total of 30 chips; Figure 3).

↑ *Figure 4 — Embedded and immobilized algae (green beads) in the photosystem II fluorescence sensor. The system allows six parallel flow cells.*

The final algal biosensors improved for marine samples consisted of *Chlorella vulgaris* in symbiosis with *Tetrahymena pyriformis*. This symbiotic consortium was immobilized in calcium alginate beads (2-3 mm) and embedded in a specifically designed and fabricated flow-through cell (Figure 4). The flow-through cell is connected to an optical unit which provides basic illumination for the algae, and enables induction and detection of photosystem II fluorescence changes. A final instrument was built that controls the algal illumination, records the fluorescence changes and controls the inflow of reagents and samples, again through an embedded rotary valve as in Figure 2.

↓ *Figure 3 — Remote Control Tests of the USV: a tight turn.*



Two autonomous devices, a man-sized buoy and a trimaran unmanned surveying vessel, have already been built that the project deployed for marine tests. The three biosensor instruments were enclosed in waterproof cages and connected within the buoy and on the trimaran. Communication, data storage and transmission to and

from the sensors were successfully established. The buoy and vessel can take and roughly filter the sample, after which it is dispatched to the various sensor instruments. Benchmarking tests in Italy and Ireland showed successful sensor operation. Comparative testing of the sensors with classical chemical analytics was reasonably in agreement, given that the biosensors operated on non-purified and raw samples.

On-line services, including customised tools, allowed consortium members to stay in contact and share results; while bi-annual Consortium meetings and ad-hoc technical meetings provide the opportunity for essential exchanges key to success of the project. Two specific field campaigns were organized to test the sensor setups in real time; a week-long harbour and mesocosm test in Messina, Italy, and a week-long harbour-dock test in Kilrush, Ireland. The logistics of instrument and vessel transport across Europe was a bit challenging.

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.

The BRAAVOO project was represented at the OCEAN International Meeting in London and the European Science Open Meeting in Birmingham in 2016, and organized a final symposium at the End of November 2016 in Villars, Switzerland. Fruitful exchanges with stakeholders and sister projects took place, whose feedback was integrated. Integrated dissemination efforts included a special issue on Marine Biosensors in Current Opinion in Biotechnology (which will appear in 2017) and a Frontiers in Marine Sciences topic (also to appear in 2017). These efforts should help increase overall impact of the project results.

Final results and potential impacts

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) 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.

5) 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.

6) 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 on the website: www.braavoo.org. Notably, the Outcomes pages summarize the BRAAVOO findings as presented in two workshops, a video, technical sheets and pubic documents and demonstrated in two series of field studies.