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PII: S0039-9140(18)30276-5
DOI: https://doi.org/10.1016/j.talanta.2018.03.036
Reference: TAL18470

To appear in: *Talanta*

Received date: 18 January 2018
Revised date: 12 March 2018
Accepted date: 14 March 2018

Cite this article as: Ana Sanchis, J.-Pablo Salvador, Katrina Campbell, Christopher T. Elliott, Weilin L. Shelver, Qing X. Li and M.-Pilar Marco, Fluorescent Microarray for Multiplexed Quantification of Environmental Contaminants in Seawater Samples, *Talanta,* https://doi.org/10.1016/j.talanta.2018.03.036

Fluorescent Microarray for Multiplexed Quantification of Environmental Contaminants in Seawater Samples.

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ABSTRACT

The development of a fluorescent multiplexed microarray platform able to detect and quantify a wide variety of pollutants in seawater is reported. The microarray platform has been manufactured by spotting 6 different bioconjugate competitors and it uses a cocktail of 6 monoclonal or polyclonal antibodies raised against important families of chemical pollutants such as triazine biocide (i.e. Irgarol 1051®), sulfonamide and chloramphenicol antibiotics, polybrominated diphenyl ether flame-retardant (PBDE, i.e. BDE-47), hormone (17β-estradiol), and algae toxin (domoic acid). These contaminants were selected as model analytes, however, the platform developed has the potential to detect a broader group of compounds based on the cross-reactivity of the immunoreagents used. The microarray chip is able to simultaneously determine these families of contaminants directly in seawater samples reaching limits of detection close to the levels found in contaminated areas (Irgarol 1051®, 0.19 ± 0,06 μg L^{-1}; sulfapyridine, 0.17 ± 0.07 μg L^{-1}; chloramphenicol, 0.11 ± 0.03 μg L^{-1}; BDE-47, 2.71 ± 1.13 μg L^{-1}; 17β-estradiol, 0.94 ± 0.30 μg L^{-1} and domoic acid, 1.71 ± 0.30 μg L^{-1}). Performance of the multiplexed microarray chip was assessed by measuring 38 blind spiked seawater samples containing either one of these contaminants or mixtures of them. The accuracy found was very good and the coefficient of variation was < 20% in all the cases. No sample pre-treatment was necessary, and the results could be obtained in just 1h 30 min. The microarray shows high sample throughput capabilities, being able to measure simultaneously more than 68 samples and screen them for a significant number of chemical contaminants of interest in environmental screening programs.

KEYWORDS: Fluorescent microarray chip, multiplexed analysis, immunoassay, antibody, environmental analysis.

1. INTRODUCTION

Over the last decades, marine pollution has been recognized as a worldwide problem and the state of the oceans has become a great concern to governmental agencies [1, 2]. Chemical contamination of estuarine and coastal areas, not only affects the biodiversity of the ecosystems, but also the oceans capacity to provide natural resources raising human health risks through the food chain [3, 4]. Main concerns for certain pollutants derive from the amounts generated by humans, their extremely negative effects on the environment or because of their accumulation in water, sediments, or biota. Included are endocrine disruptor chemicals (EDCs), a group of substances that may interfere with the endocrine and hormonal system of fishes and other non-vertebrate's organisms [5-7]. Some antibiotics, massively used in aquaculture facilities to combat fish infections and infestations, affect not only the ecosystem equilibrium but also human health spreading drug-resistant bacterial strains [8]. Other groups of chemicals, such as persistent organic pollutants (POPs), are of concern due to their persistence, bioconcentration and biomagnification effects [9]. Finally, a growing concern about the relation between pollution, and toxic algal communities has arisen. Recently, there has been a global increase of harmful algal blooms associated to elevated levels of marine biotoxins [10]. Those toxic compounds cause ecological and economic issues due to their effects on coastal marine resources and aquaculture facilities. The possibility of serious problems has resulted in the European legislation on regulations such as

the Marine Strategy Framework Directive (MSFD), which focuses on the need to evaluate the negative effects of contaminants in aquatic organisms and the marine environment [11]. Within this frame, the European Commission establishes environmental quality standards (EQS) to limit the concentrations of certain chemical substances that pose a significant risk to the environment or to human health in surface waters in the European Union (EU).

With this scenario, it is imperative to develop new approaches aimed to provide an accurate picture of the marine environmental health status in real time. Within this context, on July of 2012 the EC launched a call (FP7-OCEAN-2013) for proposals aimed at developing innovative real-time, *in situ* biosensors for monitoring the environmental status of marine water quality and provision of early-warning systems, in order to ensure sustainable management and exploitation of the seas and their resources. The technologies developed had to offer unique features for highly specific and precise measurements, including under multi-stressor conditions, by combining technological elements (including nanotechnologies) and bio-receptors in a single measurement device and remotely provide information on the environmental status.

One of the challenges to accomplish the required aim was to be able to simultaneously detect and quantify different families of chemical contaminants with minimum or without any sample preparation, at very low concentration levels, on a device able to work autonomously *in situ* (ocean, aquaculture facilities, etc). In the last years a variety of multiresidue chromatographic methods, usually coupled to tandem mass spectrometry showing high specificity and sensitivity have been reported (LC-MS/MS or GC-MS/MS) [9, 12-17]. However, these analytical approaches can hardly be implemented to work autonomously *in situ* in the middle of the ocean. The mass spectrometry technologies require highly sophisticated equipment supervised by qualified personnel and often, complex sample treatment procedures, often specific for one type of pollutant. As an alternative,

immunochemical techniques based on antigen/antibody interaction have demonstrated to offer high levels of specificity sometimes for a class of compounds and detectability with no need or very simple sample pretreatment. In addition to their recognized high sample throughput capabilities, their flexibility have allowed their implementation in a variety of formats and devices (i.e. biosensor) with exceptional multiplexed capabilities if combined with class specific antibodies [18-21]. From enzymatic assays [20, 22], fluorescent immunoassays [23, 24] to biosensors [25-27], several approaches to develop multiplexed analysis for environmental pollutants have been described.

The aim of the work presented here has been to develop and assess performance of a multiplexed immunochemical platform to detect and quantify, directly in seawater, six relevant families of potential marine contaminants including herbicides, sulfonamides and chloramphenicol antibiotics, frame retardants, hormones and algae toxins. For this purpose, a fluorescent microarray chip has been manufactured and used to develop the multiplexed immunochemical assay. As it will be shown, in spite of the variety of chemicals determined simultaneously, the technology shows very good analytical properties for its future implementation on an *in-situ* biosensor platform for monitoring the contamination of the ocean waters.

2. MATERIALS AND METHODS

2.1. Chemicals and Biochemicals

Domoic acid was purchased from Sigma Chemical Co. as well as the estradiol hapten; β–estradiol 6-(O-carboxymethyl)oxime (6E2). The preparation of the bioconjugate competitors and antibodies has been performed with the support of the U2 of the ICTS "NANBIOSIS", more specifically by the Custom Antibody Service (CAbS, CIBER-BBN, IQAC-CSIC). The immunoreagents for Irgarol 1051 (Irg)[28, 29], sulfonamide (SA)[18, 30] chloramphenicol

antibiotics have been developed previously by our group [29, 31]. The immunoreagents used for the chloramphenicol assay will be described elsewhere. For the case of BDE-47, the immunoreagents preparation has been described in Shelver et al. 2005 [32]. The monoclonal antibody for estradiol was purchased from Fitzgerald Industries International (North Acton, Massachusetts, USA) and the monoclonal antibody for domoic acid (DA) was generated according to Traynor et al. 2006 [33]. The preparation of the corresponding bioconjugate competitors $6E2_4$BSA and DA_5BSA is described below. The analytes used in this platform were Irgarol (Irg), Sulfapyridine (SPy), Chloramphenicol (CAP), BDE-47, 17β-estradiol (E2), and Domoic acid (DA), purchased from Sigma Chemical Co. (St. Louis, MO, USA), and BDE-47 which was kindly provided by Prof. Readman from Plymouth University (UK). Stock solutions of each analyte were prepared at 10 mM concentration in DMSO and stored at 4ºC until its use. Artificial seawater (aSW) was purchased from Sigma Chemical Co. (St. Louis, MO, USA) and prepared at 40 mg/mL in Ultrapure water.

2.2.Equipment and General Procedures.

The matrix-assisted laser desorption ionization time-of-flight mass spectrometer (MALDI-TOF-MS) used to estimate the hapten densities (δ) of the bioconjugates was a Bruker autoflex III Smartbeam spectrometer (Billerica, Massachusetts). For this purpose, 2 μL of the freshly prepared matrix (trans-3,5-dimethoxy-4-hydroxycinnamic acid, 10 mg mL^{-1} in ACN/H_2O 70:30, 0.1% HCOOH) was mixed with 2 μL of a solution of the native protein or the bioconjugates (10 mg mL^{-1} in the same solvent). Hapten densities were calculated according to the following equation: [MW(conjugate)-MW(protein)]/MW(hapten). The pH and the conductivity of all buffers and solutions were measured with a pH-meter pH 540 GLP and a conductimeter LF 340, respectively (WTW, Weilheim, Germany). Dilution plates were

purchased from Nirco (Barberà del Vallés, Spain). The plain microscope slides (75 x 25 mm) were purchased from Corning Inc (Corning, NY, USA). The slide printing was done using a BioOdissey Calligrapher™ MiniArrayer (Bio-Rad Laboratories, Inc., Hercules, CA, USA). All microarrays assays were performed on an ArrayIt® holder (Arrayit Corp, Sunnyvale, CA, USA). Microarray measurements were recorded on a ScanArray Gx PLUS (PerkinElmer, Waltham, MA, USA) with a Cy3 optical filter with 10-µm resolution. The laser power and photomultiplier tube (PMT) gain were set to 95% and 80%, respectively. The spots were measured by deducting the mean Cy3 background intensity to the mean of Cy3 foreground intensity using ScanArray Express v 4.0 (Microarray Analysis System, PerkinElmer, Waltham, MA, USA). The competitive curves were analyzed with a four-parameter logistic equation using the software GraphPad Prism v 5 (GraphPad Software Inc., San Diego, CA, USA)]. The standard curves were fitted to a four-parameter equation according to the following formula: $Y = [(A-B)/1-(x/C)^D]+ B$, where A is the maximal fluorescence, B the minimum fluorescence, C the concentration producing 50% of the difference between A and B (or IC_{50}), and D the slope at the inflection point of the sigmoid curve. The limit of detection (LOD) was defined as the concentration producing 90% of the maximal fluorescence (IC_{90}).

2.3.Buffers.

Unless otherwise indicated, phosphate buffer saline (PBS) is 0.01 M phosphate buffer in a 0.8% saline solution at pH 7.5. PBST is PBS with 0.05% Tween 20. The printing buffer (PrB) was PBST with 0.005% Tween 20 at pH 7.5. PBT2x is PB (0.02 M phosphate buffer, no saline solution) with 0.1% Tween 20 at pH 7.5. Borate buffer (BB) is 0.2 M boric acid/sodium borate at pH 8.7.

2.4.Bioconjugate competitors

The preparation of the bioconjugate competitors for Irgarol 1051 [29], sulfonamides [18, 30], and BDE-47 [32] have been reported, while that of the chloramphenicol will be described elsewhere. The preparation of the bioconjugates for estradiol ($6E2_4BSA$) and DA (DA_5BSA) was performed using the following procedures**.** Briefly, a solution of N, N'-dicyclohexylcarbodiimide (DCC; 50 µmol) in anhydrous DMF (50 µL) was added to a solution of the hapten (100 µL) in the same solvent followed by a solution of N-hydroxysuccinimide (NHS, 25 µmols, 50 µL) also in DMF [34]. The mixture was stirred for approximately 3 hours at RT until a white precipitate appeared. The suspension was then centrifuged (10000 rpm for 10 min) and the supernatant (25 µL for 6E2 and 50 µL for DA_5) was added dropwise to a solution of bovine serum albumin (BSA, 10 mg, 1.8 mL borate buffer) and the mixtures kept under gently stirred for 4 hours at RT. The protein bioconjugates were purified by dialysis against 0.5 mM PBS (4×5 L) and ultrapure water (1×5 L) and finally stored frozen at -40 °C. Unless otherwise indicated, working aliquots were stored at 4 °C in 0.01 M PBS at 1 mg mL^{-1}.

2.5.Manufacture of the microarray chip

Plain glass slides were first cleaned by immersing them in piranha solution (H_2SO_4:H_2O_2, 70:30, v/v, 30 min), rinsed with Ultrapure water, activated with 10% NaOH (30min) and then rinsed again with ultrapure water. Once the slides were cleaned, they were dried with N_2, and the chemical functionalization of the slides was achieved using undiluted 3-glycidyloxypropyltrimethoxysilane (GPTMS) (300 µL/slide, 30 min). Afterwards the slides were washed with ethanol, dried and stored in the desiccator until use. Biofunctionalization was performed by spotting solutions of the bioconjugate competitors (in PrB, 0.5-1nl $spot^{-1}$) under controlled temperature (20ºC) and humidity (65%) conditions and maintaining them for 1 hour inside the microarrayer chamber. The biofunctionalized slides could be stored at RT

in a desiccator until use. Up to 24 microarray chips could be printed in a single slide. Microarray chips for single analyte analysis had 5 spots, while for multiplexed analysis consisted on a matrix of 5 x 6 spots (6 analytes, 5 replicates) according the scheme shown in Figure S1a.

2.6.Fluorescent microarray

General considerations

The slides were placed on an ArrayIt® holder provided with a silicon gasket defining 8x3 wells on each slide. Before starting the assay, the slides were washed three times with PBST. Standard solutions used to calibrate the microarray were prepared in aSW (7 concentration points plus zero: 0.064 to 1000 nM for Irgarol 1051®, chloramphenicol and 17-β estradiol; from 0.128 to 2000 nM for sulfapyridine; and 0.64 to 10000 nM for BDE-47 and domoic acid). All the calibration curves were prepared in DMSO, and diluted 1 to 200 with aSW prior the assay. For the multiplexed microarray assays, a cocktail solution of antibodies was prepared. Optimum concentrations of the bioconjugates spotted and antisera/antibodies dilutions (see Table 2) were chosen by performing two-dimensional titration assays (2D-assay) and selecting those conditions able to generate a signal around 10.000 – 20.000 RFUs (Relative Fluorescence Units) at 70-80% of the saturation curve. For this purpose, the binding of serial dilutions of the antisera/antibodies (zero and 1/1000 to 1/64000, using 100 μL $well^{-1}$) to different concentrations of the bioconjugates spotted (zero and 200 μg mL^{-1} to 0.025 μg mL^{-1}) were analyzed. Unless otherwise indicated, the results of single-analyte microarray assays correspond to assay performed in one-day using at least 3 spot replicates of each concentration whereas for the multiplexed microarray the results given were usually performed in 3 microarray chips performed on, at least 3 different days and using five-spots replicates.

Fluorescent microarray assay

Solutions (50µL/well) of the standards or the samples were added to the wells followed by solutions of single antibody or the Ab cocktail (in PBT2x, 50µL/well). After 30 min of incubation at RT, the slides were washed 3 times with PBST, and a solution containing a mixture of anti-mouse and anti-rabbit IgGs-TRITC conjugates (1/250 in PBST, 100 µL/well) was added. After another incubation of 30 min at RT in the dark, the slides were washed (3 x PBST + 1 x ultrapure water), dried with N_2 and read with the microarray scanner as described above (see Figure S1b).

3. RESULTS AND DISCUSSION

A variety of chemicals have been identified as potential marine pollutants. Activities in offshore aquaculture resources involve the use of some of these substances, others from various human activities can produce detrimental effects in the aquatic ecosystem, placing public health at risk. Chemical discharges and runoff can affect aquaculture facilities. Hence, different pesticides, veterinary drugs, industrial residues, hormones or toxins have shown to be able to produce a strong negative impact in the marine environment particularly if bioaccumulation and/or persistence are involved [35, 36].

To assess the environmental health of the ocean waters, we have addressed the development of a multiplexed bioanalytical system able to simultaneously detect and quantify a wide variety of contaminants. Based on their relevance and impact we have selected distinct representatives of some of the most important families of pollutants including herbicides, antibiotics, flame-retardants, hormones and algae toxins (see **Figure 1** and **Table 1** for chemical structures and reported levels of contamination). A key challenge was to be able to determine all of them directly in seawater in a single run, independently from their chemical nature and physico-chemical properties (see **Table 1**). Because of the diversity of chemical

structures, these chemicals show significant differences in their hydrophilic/hydrophobic nature. While the herbicide Irgarol (ACD/LogP, 3.27) or the antibiotic chloramphenicol (ACD/LogP, 1.02) are considered to be hydrophilic, the brominated flame retardant BDE-47 (ACD/LogP, 7.39) is highly hydrophobic showing opposite properties regarding its solubility in water or behavior in respect to the extraction procedures or analytical conditions (see **Table 1**).

Immunochemical analytical tools offer the possibility to analyze a wide variety of substances under similar conditions due to the high sensitivity and specificity dependent on the antibodies. In this work we have been used immunoreagents previously developed or commercially available for triazines (i.e. Irgarol 1051®) [28, 29, 37], sulfonamide [18, 30], chloramphenicol, PBDEs (i.e. BDE-47) [38], and domoic acid [33]. Dependent on the class specificity shown by some of these immunoreagents (i.e >10 sulfonamide antibiotic congeners are recognized by the immunoreagents used in this work), a microarray platform able to detect a large number of chemical substances [33] can potentially be developed.

3.1. Single-analyte microarrays for seawater analyses

Prior to its implementation in the final multiplexed microarray platform each single-analyte microarray was developed and used to assess its performance in seawater. This is a matrix characterized by its particular mixture of salts which provided a pH of 8.1 ± 0.2 (*N=20)* and a conductivity of 49.6 ± 1.5 mS cm^{-1} (*N=20*), much higher than the conductivity of the PBS buffer used in most of the assay (10 mM PBS, 16.0±0.5 mS cm^{-1}). The six fluorescent microarray assays demonstrated good analytical features (LODs in the ppb or sub-ppb level) with similar performance in buffer and seawater; although in some cases (sulfonamides and 17β-estradiol) it was necessary to slightly modify the concentration of the antibody to accomplish the same analytical features as in PBST (see **Table 2**).

A challenge of monitoring contamination of marine water is the low concentration of the pollutants. Half of the analytes detected in this work have no EQS value set in the actual legislation; however, the EQS established by the Marine legislation are far below the ones achieved in this work (see **Table 1**) and by most of the reference analytical techniques proposed, unless a sample treatment/preconcentration step is introduced [39]. It should be noticed that the detectability values achieved by the single-analyte microarrays are close to the reported environmental levels (see **Table 1**).

3.2.Fluorescent Multiplexed Microarray

The possibility of cooperative phenomena or shared reactivity (recognition of a bioconjugate competitor by more than one antibody) was assessed to ensure that the signal recorded on each spot is only due to the binding of the corresponding specific antibody. Although the chemical structures of the selected targets are very different, the use of the same bioconjugation procedure could have led to undesired common epitopes [40] (see **Table S1**). With this purpose, experiments were carried out to test the binding of each antibody to the different bioconjugates spotted on the glass slides. As shown in **Figure 2**, the antibodies only recognized their corresponding antigen, ensuring the absence of shared reactivity.

Ensuring the lack of cooperative phenomena recognizing the target analytes was accessed by comparing the response of the assays when the antibodies were used individually compared to a cocktail composed of 6 antibodies (Irg: As87, diluted 8000 times; SPy: As155 diluted 1000 times; CAP: As226 diluted 12000 times, BDE-47: As122 diluted 2000 times, E2: MAb_E2 diluted 25000 times, DA: MAb_DA diluted 2000 times; all in PBT2x). **Figure 3** shows the comparison of the standard curves of each analyte on each situation. Although there was observed slight differences in shape, the analytical features summarized in **Table 3** show that most of the assays maintain the same parameters once have been multiplexed (and being analyzed with the antibody cocktail), demonstrating that the use of the antibody

cocktail does not affect the detectability of the selected analytes and that those can be quantified individually in the multiplexed platform. Thus, Irgarol 1051®, sulfapyridine, chloramphenicol, BDE-47, 17β-estradiol and domoic acid can be detected in the multiplexed format at 0.190 ± 0.06, 0.17 ± 0.07, 0.11 ± 0.03, 2.71 ± 1.13, 0.94 ± 0.30 and 1.71 ± 0.30 µg L^{-1} (N=3), respectively, also close to the reported environmental levels for these pollutants (see **Table 1**).

3.3.Accuracy of the multiplexed microarray

The objective of these experiments was to assess accuracy and to prove that the presence of more than one contaminant in the sample did not affect the quantification. For this purpose, 30 blind spiked samples were prepared in seawater containing the selected analytes at different concentrations, including zero and 5 different concentrations for each analyte. All the samples were analyzed during three different days using five-spot replicates of the multiplexed microarray chips. As it can be observed in **Figure 4**, the linear regression studies provided slopes near 1 in all cases (being m=1.00 the perfect correlation) with very good regression coefficients (R^2 *>0.95*), indicating the excellent assay accuracy. The coefficients of variation (**Table S2**) were below of the 20% pointing to a good microarray assay precision.

Finally, blank seawater samples and spiked with mixtures of analytes were also quantified, to demonstrate the reliability of the platform. **Table 4** summarizes the results obtained showing that when several analytes were present it was possible to obtain good levels of accuracy and precision. The coefficients of variation are almost in all cases below 20% and never greater than 25%. No false positives were observed since all the blank samples were negative for the six families of analytes.

CONCLUSIONS

A fluorescent microarray for the multiplexed determination and quantification of six different families of potential pollutants of the ocean has been developed and its suitability to analyze seawater samples has been evaluated. The microarray chips consisted on a matrix of 6x5 spots, which allow quantification of each pollutant using five-spot replicates. Seawater samples can be measured directly without any sample treatment in just about 1h 30 min, being possible to analyze simultaneously several samples using the necessary multi-well holder, which allows running 96 microarrays in parallel. Moreover, due to the wide selectivity of some of the immunoreagents used in this study is very much likely that the present microarray chip would be able to detect a significant number of chemical congeners of the families selected. Despite of the different chemical structures and properties, herbicides, different antibiotic families, hormones, industrial contaminants, and toxins can be simultaneously quantified with a good accuracy and precision. Moreover, although the EQS (environmental quality standard) values set by the Water Framework Directive 2000/60/EC are not reached, the detectability achieved without any sample treatment or preconcentration step is very close to the concentrations found in the environment for most of the pollutants selected. The multiplexed platform here presented could be a suitable complementary analytical technique for screening and alarm purposes. The work presented here is the starting point for developing an autonomous multiplexed biosensor platform able to provide real time data of the contamination of the ocean. Further work will report the implementation of such multiplexed immunochemical assays on a biosensor device.

ACKNOWLEDGMENT

This work has been funded by SEA-on-a-CHIP project (FP7-OCEAN-2013, nº614168). The Nb4D group (formerly Applied Molecular Receptors group, AMRg) is a consolidated research group (Grup de Recerca) of the Generalitat de Catalunya and has support from the Departament d'Universitats, Recerca i Societat de la Informació de la Generalitat de Catalunya (expedient: 2014 SGR 1484). CIBER-BBN is an initiative funded by the Spanish National Plan for Scientific and Technical Research and Innovation 2013-2016, Iniciativa Ingenio 2010, Consolider Program, CIBER Actions are financed by the Instituto de Salud Carlos III with assistance from the European Regional Development Fund. The ICTS "NANOBIOSIS", and particularly the Custom Antibody Service (CAbS, IQAC-CSIC, CIBER-BBN), is acknowledged for the assistance and support related to the immunoreagents used in this work.

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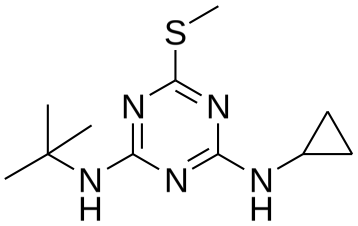
Fig 1. Chemical structures of the chemical contaminants selected on this study. For additional information on the environmental levels or physico-chemical properties see **Table 1**.

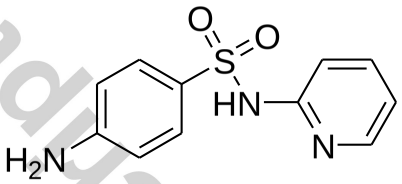
Fig 2. Specificity of the antibodies used towards all the bioconjugate competitors spotted on microarray chip. The results are the average and SD of measurements made on microarrays with five spots replicates

Fig 3. Calibration curves obtained when using single antibody solutions (colored curve) or the cocktail of antibodies (black curve). As it can be observed, no significant differences were found. See table 3 for the analytical parameters of the calibration curves of the multiplexed microarray assay. For the multiplexed data, each analyte concentration, was measured using five-spot replicates on each chip. The results are the average of 3 assays performed on 3 different days. The standard curves obtained using single antibody solutions are experiments recorded using at least 3 spot replicates for each concentration value.

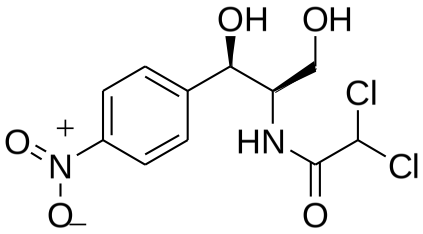
Fig 4. Results from the accuracy studies performed in artificial seawater in the multiplexed microarray format. The graph shows the correlation between the spiked and measured concentration values. The dotted line corresponds to a perfect correlation (m = 1). The data correspond to the average of at least three-well replicates from 3 different days.



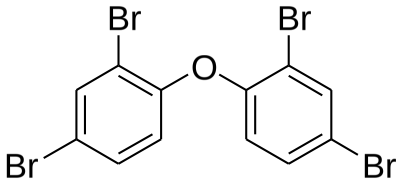
Irgarol



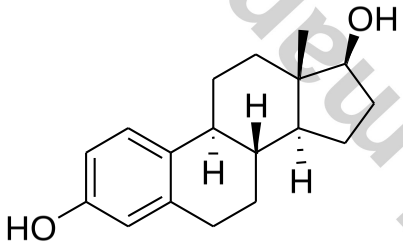
Sulfapyridine



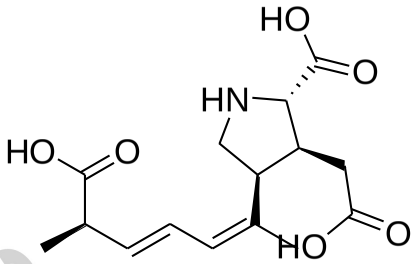
Chloramphenicol



BDE-47



17β-Estradiol



Domoic acid

Figure 1

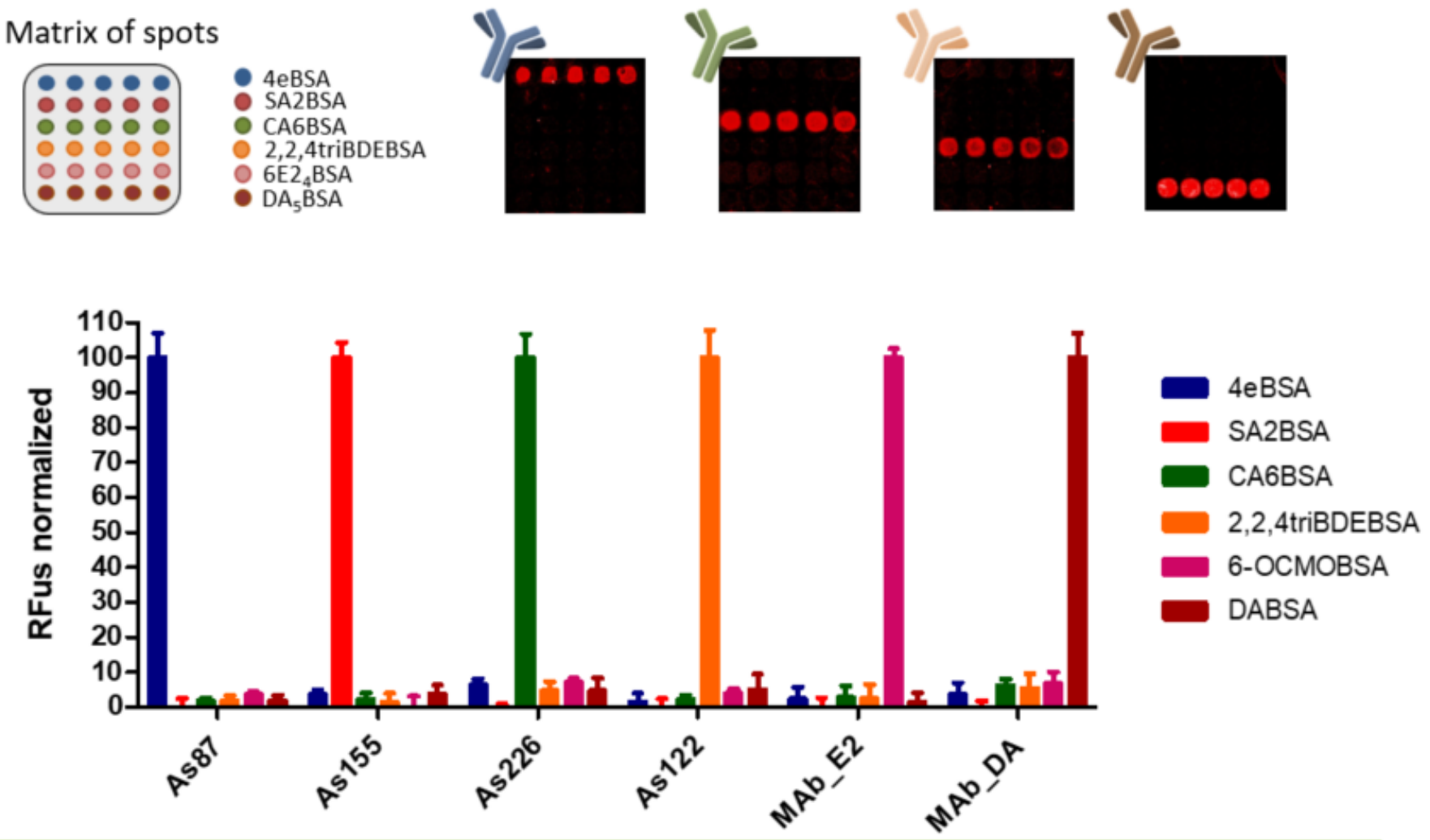


Figure 2

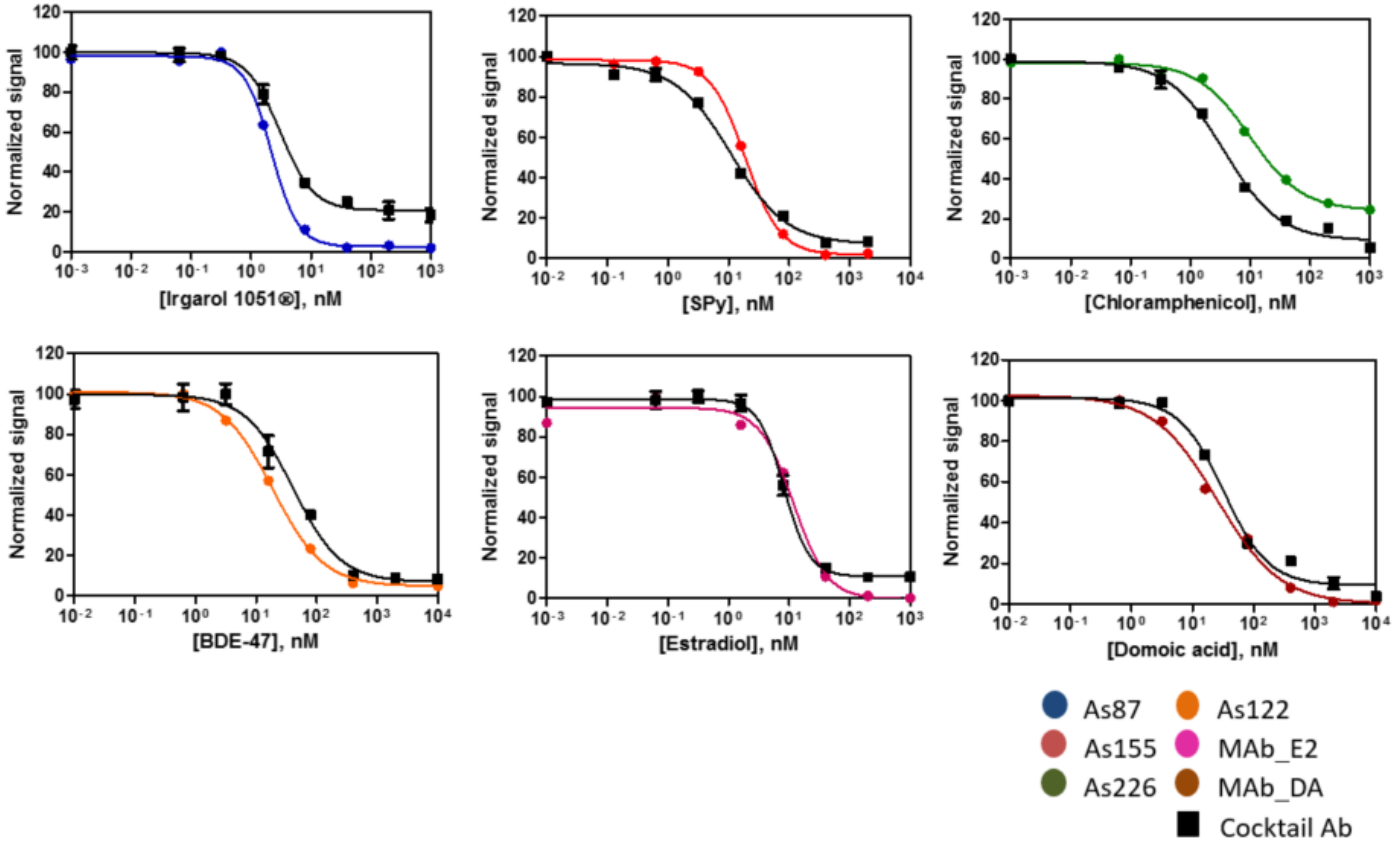


Figure 3

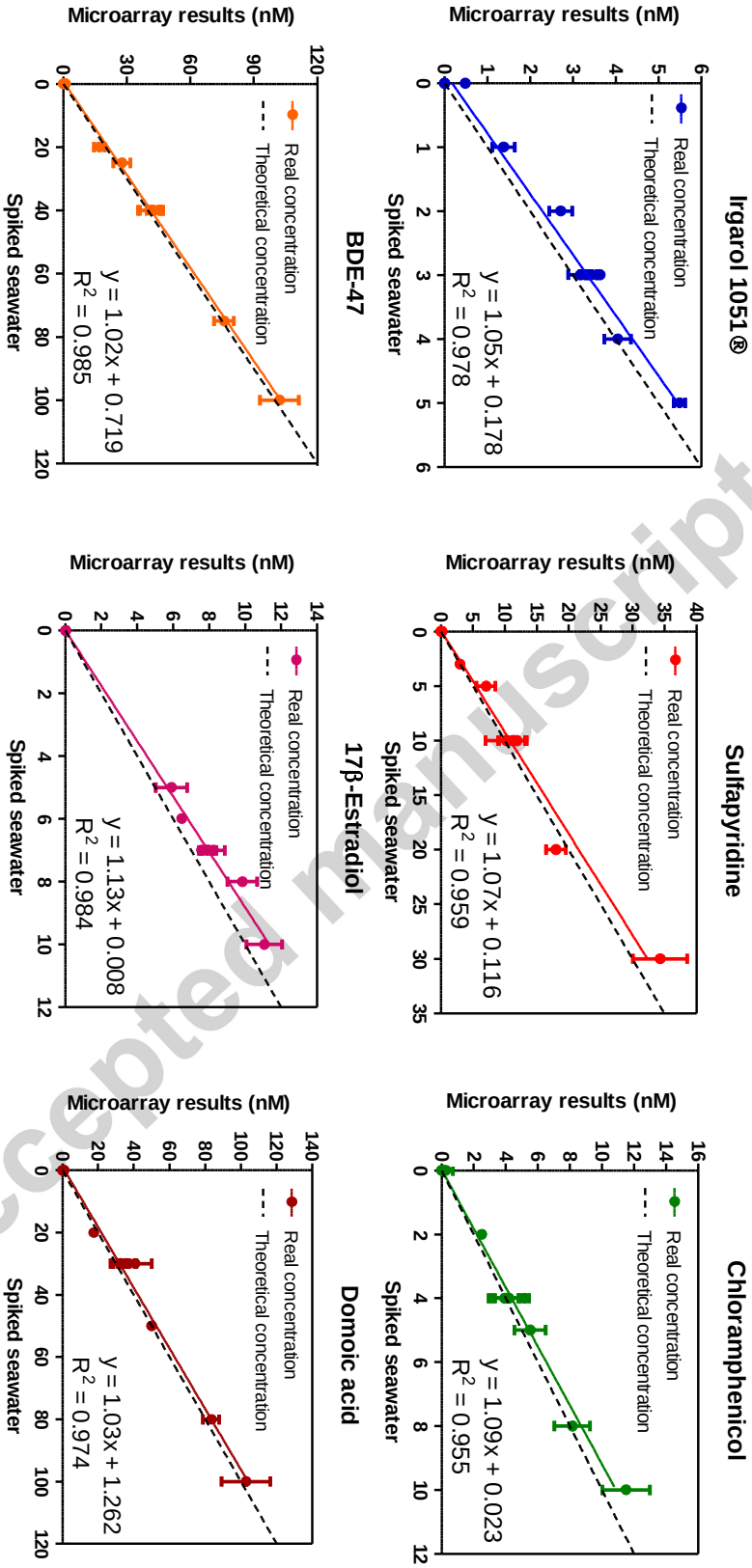
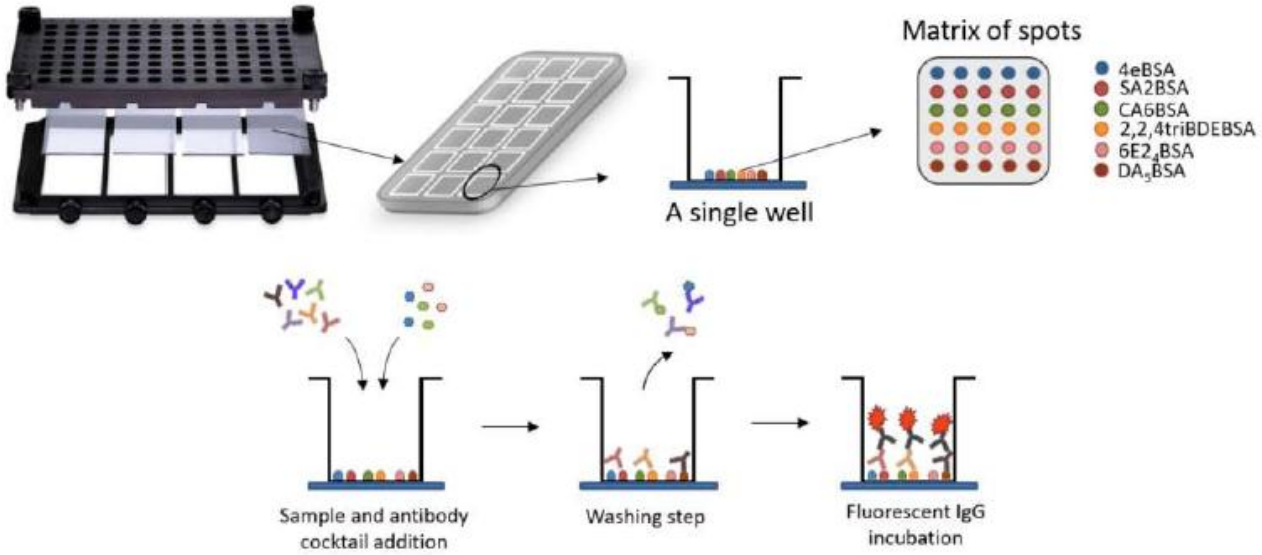


Figure 4



Graphical Abstract

Table 1. Analytes selected for the final multiplexed platform, their environmental quality standard (EQS), their logP value and their reported levels in aquatic environments.

Analyte	Contaminant type	EQS[b]	ACD/LogP	Levels reported (µg L^{-1})
Irgarol 1051®	Herbicide	2.5 ng L^{-1}	3.27	0.013 – 2 [41, 42]
Sulfapyridine	Antibiotic	-	0.03	0.05 - 0.3 [43, 44]
Chloramphenicol	Antibiotic	-	1.02	0.001 - 0.2 [45, 46]
Polybrominated diphenyl ether: BDE-47	POP[a]	2.4 fg L^{-1}	7.39	0.004 - 0.11 [38, 47]
17β - Estradiol	Hormone	80 pg L^{-1}	4.13	0.004 - 0.016 [48, 49]
Domoic acid	Algal toxin	-	0.61	0.02 – 13 [50, 51]

[a] Persistent Organic Pollutant. [b] Set by Water Framework Directive 2000/60/EC (WFD).

Table 2. Analytical parameters of the fluorescent microarrays for single analyte measurements.

	Irgarol		**Sulfapyridine**		**Chloramphenicol**	
Bioconjugate/antibody	4eBSA / As87		SA2BSA / As155		CA6BSA/ As226	
Condition	PBST	aSW	PBST	aSW	PBST	aSW
[CA], µg/mL	25	25	12.5	12.5	25	25
[As]/dilution	1/8000	1/8000	1/2000	1/1000	1/12000	1/12000
RFUmin	331.8	276.7	344.9	215.8	3643	2772.0
RFUmax	10855.0	9753.0	13223.0	11226.0	12529.0	11323.0
Slope	-1.449	-1.952	-1.138	-1.513	-1.189	-0.997
IC_{50} (nM)	2.29	2.19	15.23	18.90	10.25	9.89
IC_{50} (µg/L)	0.579	0.554	3.79	4.71	3.00	3.19
LOD (µg/L)	0.135	0.162	0.397	0.991	0.453	0.268
R^2	0.997	0.998	0.983	0.999	0.988	0.995
	BDE-47		**17β-Estradiol**		**Domoic acid**	
Bioconjugate/antibody	2,2,4triBDEBSA / As122		$6E2_4$BSA /MAb_E2		DA_5BSA /MAb_DA	
Condition	PBST	aSW	PBST	aSW	PBST	aSW
[CA], µg/mL	25	25	25	25	50	50
[As] dilution	1/2000	1/2000	1/16000	1/25000	1/1000	1/1000
RFUmin	459.0	971.4	13.65	97.11	328.3	6.99
RFUmax	13825.0	15748.0	25671.0	24704.0	14810.0	20004.0
Slope	-0.733	-1.072	-1.239	-1.547	-1.197	-0.829
IC_{50} (nM)	7.96	18.71	9.671	11.84	19.21	25.30
IC_{50} (µg/L)	3.87	9.09	2.63	3.22	5.97	7.88
LOD (µg/L)	0.216	1.24	0.362	0.463	1.26	0.709
R^2	0.995	0.998	0.998	0.989	0.989	0.996

The assays presented correspond to assays performed in one-day using at least 3 replicates of each concentration value, both in buffer and in seawater conditions. Microarray chips for single analyte analysis were contained 5 spots of the specific bioconjugate for each assay.

Table 3. Analytical parameters of the multiplexed fluorescent microarray for each of the target analytes

	Irgarol	**Sulfapyridine**	**Chloramphenicol**
Microarray	4eBSA / Cocktail antibodies	SA2BSA / Cocktail antibodies	CA6BSA / Cocktail antibodies
[CA], µg/mL	25	12.5	25
[As]/dilution	1/8000	1/1000	1/12000
RFUmin	2214.7 ± 481.9	854.5 ± 467.3	877.2 ± 257.6
RFUmax	10662.0 ± 459.8	11985.3 ± 186.0	9477.3 ± 257.6
Slope	-1.62 ± 0.38	-0.944 ± 0.080	-0.951 ± 0.040
IC_{50} (nM)	3.05 ± 1.01	11.14 ± 1.61	3.66 ± 0.45
IC_{50} (µg/L)	0.773 ± 0.257	2.775 ± 0.404	1.184 ± 0.147
LOD (µg/L)	0.190 ± 0.06	0.171 ± 0.071	0.105 ± 0.035
R^2	0.995 ± 0.005	0.994 ± 0.003	0.992 ± 0.001
	BDE-47	**17β-Estradiol**	**Domoic acid**
Microarray	2,2,4triBDEBSA / Cocktail antibodies	$6E2_4$BSA /Cocktail antibodies	DA_5BSA /Cocktail antibodies
[CA], µg/mL	25	25	25
[As] dilution	1/2000	1/25000	1/2000
RFUmin	907.4 ± 161.8	2281.3 ± 417.0	1016.0 ± 212.7
RFUmax	13025.0 ± 1111.7	20653.0 ± 941.9	11197.0 ± 193.5
Slope	-1.12 ± 0.22	-2.17 ± 0.50	-1.18 ± 0.05
IC_{50} (nM)	40.77 ± 4.46	8.22 ± 1.30	32.40 ± 0.98
IC_{50} (µg/L)	19.81 ± 2.17	2.95 ± 0.47	10.08 ± 0.30
LOD (µg/L)	2.71 ± 1.13	0.936 ± 0.298	1.71 ± 0.30
R^2	0.991 ± 0.005	0.998 ± 0.001	0.990 ± 0.003

The assays presented were performed directly in seawater, and correspond to assays performed during three different days using at least 3 replicates of each concentration value. Each analyte was detected using a cocktail antibody following the concentrations described in Table 2. Microarray chips for multiplexed analysis were spotted in a matrix of 5 x 6 spots (6 analytes, 5 replicates).

Table 4. Complex samples and blanks analyzed by the final multiplexed microarray platform.

	Irgarol 1051®		Sulfapyridine		Chloramphenicol		BDE-47		17β-estradiol		Domoic acid	
Sample	Multiplexed quantification	CV (%)	Multiplexed quantification	CV (%)	Multiplexed quantification	CV (%)	Multiplexed quantification	CV (%)	Multiplexed quantification	CV (%)	Multiplexed quantification	CV (%)
M1	3.44 ± 0.14	4.01	10.77 ± 2.23	20.67	4.25 ± 0.92	21.73	42.25 ± 5.21	12.33	7.92 ± 0.33	4.22	35.74 ± 7.26	20.30
M2	3.29 ± 0.32	9.78	11.75 ± 0.61	5.2	4.14 ± 1.04	25.11	43.14 ± 4.35	10.08	7.96 ± 0.47	5.85	35.58 ± 3.78	10.61
B1	<LOD	-	<LOD	-	<LOD	-	<LOD	-	<LOD	-	<LOD	-
B2	<LOD	-	<LOD	-	<LOD	-	<LOD	-	<LOD	-	<LOD	-
B3	<LOD	-	<LOD	-	<LOD	-	<LOD	-	<LOD	-	<LOD	-
B4	<LOD	-	<LOD	-	<LOD	-	<LOD	-	<LOD	-	<LOD	-
B5	<LOD	-	<LOD	-	<LOD	-	<LOD	-	<LOD	-	<LOD	-
B6	<LOD	-	<LOD	-	<LOD	-	<LOD	-	<LOD	-	<LOD	-

Two complex samples containing all the analytes at different concentrations (Irg:3nM; SPy:10nM; CAP:4nM; BDE-47:40nM; E2:7nM and DA:30nM) were analyzed by the multiplexed platform. Each sample was detected using a cocktail antibody following the concentrations described in Table 2, during three different days using at least 3 replicates for each concentration value. Microarray chips for multiplexed analysis were spotted in a matrix of 5 x 6 spots (6 analytes, 5 replicates).

Highlights

- A fluorescent microarray for the detection of 6 families of pollutants is proposed.
- The platform has been developed for the analysis of seawater samples.
- No cross-reactivity has been detected between immunoreagents.
- Up to 96 samples in parallel can be measured directly, in less than 1h 30 min.
- Good values of accuracy were found for the 6 target pollutants selected (CR<20%).