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In vitro bioanalytical assessment of toxicity of wetland samples from Spanish Mediterranean coastline

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Abstract

Background: Fresh water bodies represent less than 1% of overall amount of water on earth and ensuring their quality and sustainability is pivotal. Although several campaigns have been performed to monitor the occurrence of micropollutants by means of chemical analysis, this might not cover the whole set of chemicals present in the sample nor the potential toxic effects of mixtures of natural and anthropogenic chemicals. In this sense, by selecting relevant toxicity endpoints when performing in vitro bioanalysis, effect-based methodologies can be of help to perform a comprehensive assessment of water quality and reveal biological activities relevant to adverse health effects. However, no prior bioanalytical study was performed in wetland water samples from the Spanish Mediterranean coastline.

Methods: Eleven samples from relevant water bodies from the Spanish Mediterranean coastline were collected to monitor water quality on 8 toxicity endpoints. Aryl hydrocarbon receptor (AhR), androgenicity (AR+ and AR−), estrogenicity (ER+ and ER−), oxidative stress response (Nrf2) and vitamin D receptor (VDR+ and VDR−) reporter gene assays were evaluated.

Results: AhR was the reporter gene assay showing a more frequent response over the set of samples (activated by 9 out of 11 samples), with TCDD-eq in the range 7.7–22.2 pM. For AR, ER and VDR assays sporadic activations were observed. Moreover, no activity was observed on the Nrf2 reporter gene assay. Wastewater and street runaway streams from Valencia could be responsible for enhanced activities in one of the water inputs in the Natural Park 'L'Albufera'.

Conclusions: Water quality of relevant wetlands from the Spanish Mediterranean coastline has been evaluated. The utilization of a panel of 5 different bioassays to cover for different toxicity endpoints has demonstrated to be a good tool to assess water quality.

Keywords: Surface water, In vitro bioassay, Aryl hydrocarbon receptor, Androgen receptor, Estrogen receptor, Oxidative stress, Nrf2, Vitamin D receptor, Coastal lagoon, TCDD-eq

Introduction

The International Convention on Wetlands estimates that surface water bodies such as lakes, rivers, marshlands, estuaries and aquifers serve as natural reservoir for the majority of available freshwater worldwide. However, that only represents < 1% of the overall amount of water in the aquatic system [1]. On 2010, the General Assembly of the United Nations recognized the access to freshwater

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as a human right [2] although more than 2200 million people do not yet have access to salubrious freshwater to cover their necessities [1]. Additionally, the World Health Organization establishes access to safe freshwater as indispensable for health [3]. Consequently, substantial efforts should be made to ensure water quality of natural freshwater reservoirs.

Over the recent decades, chemical analysis has been widely used for the assessment of water quality and the detection of new chemicals of emerging concern [4–9]. However, wide-scope chemical analyses still have many limitations and it may only cover a small part of the pollutants, mainly related to human activities (industry, agriculture and urbanization), that might potentially be present in water bodies. In addition, no information is provided on potential toxic effects of mixtures of natural and anthropogenic chemicals [10, 11]. In this sense, effect-based methodologies can be of help by revealing biological activities that could be relevant to adverse effects in freshwater organisms [11–13]. Thus, the utilization of bioanalytical methodologies to assess water quality can bridge the gap between chemical analysis and real environmental status [10, 14]. As a complementary tool to chemical analysis, effect-based methodologies can, therefore, detect toxicity posed by untargeted compounds or chemical mixtures [14]. Several studies have used bioassays to assess toxic activities in surface water bodies [15–20].

Selecting relevant toxicity endpoints based on the nature of the water samples is key for a comprehensive assessment of water quality [21]. The in vitro bioanalytical approach used in this work consisted of a panel of 5 bioassays as markers of relevant toxicity endpoints in surface water samples due to the potential to detect combined effects of organic micropollutants. Aryl hydrocarbon receptor (AhR) has diverse physiological functions related to chemical and microbial defense, reproduction, development, energy metabolism, immunity and inflammation [22] and AhR activity is often observed in surface water samples based on, e.g., elevated presence of aromatic hydro alkyl substances in the environment. Although this might not be an adverse effect per se, it highlights the presence of bioactive chemicals in the water sample [23, 24]. The presence of endocrine disruptive chemicals and hormones can be evaluated by means of androgen receptor (AR) and estrogen receptor (ER) activation or inhibition. The control of such types of chemical species in surface water ecosystems is relevant to prevent from intense distress of the normal physiology of exposed organisms [24, 25] due to the relevant role of androgens in the development and function of the immune, musculoskeletal, reproductive, cardiovascular and neural systems, and estrogens being essential for bone strength, cardiovascular function, reproduction, cognitive behavior and gastrointestinal systems [26, 27]. Additionally, oxidative stress response, measured by means of the nuclear factor erythroid 2-related factor 2 (Nrf2) activity, is a good indicator of environmental pollution [23, 24, 28] since this receptor is often triggered by the presence of organic micropollutants in aquatic samples. Oxidative stress can result in different adverse outcomes such as carcinogenicity, tissue damage or teratogenicity [29, 30]. The cellular defense mechanism against oxidative stress, regulated by Nrf2, can be used to assess the presence of oxidative stress inducing compounds in water samples. Finally, vitamin D endocrine signaling, transduced via the vitamin D receptor (VDR), plays important roles in multiple physiological systems. A disruption of this signaling pathway would be defined as an endocrine disrupting effect, which could potentially have serious effects to both humans and the ecosystem [31].

In this work, we aimed to assess the toxic activities of different water bodies along the Spanish Mediterranean coastline. In this region, water scarcity has become of major concern. It is expected that climate change will exacerbate this phenomenon; therefore, it is essential to evaluate water quality of significant natural reservoirs. Selection of wetlands was based on the list of Wetlands of International Importance [32] along with other locally relevant water bodies. Among the sampled locations, Ebro River and Albufera Natural Park (Valencia, Spain) are of special interest as they might be affected by effluent wastewater streams of big cities and industries. This work reflects the first comprehensive study covering a broad set of samples from Mediterranean Spanish coastline and evaluating their water quality by using a set of 5 bioassays for 8 different toxicity endpoints.

Materials and methods

Collection of samples

In this study, 11 water samples were collected from relevant water bodies along the Spanish Mediterranean coastline (Fig. 1). The sampled locations, including both river water and coastal lagoon water, were selected based on regional significance and/or their inclusion in the list of Wetlands of International Importance (8 out of 11 samples) [32]. Table 1 summarizes the relevance of the sample as well as the potential impact of surrounding activity. Briefly, samples CL2 and CL7 were collected in areas surrounded by rice agriculture, even CL2 having instream water from irrigation of rice camps. Samples CL5, CL6, CL10 and CL11 were collected in areas with a prominent level of citrus-fruit agriculture with the associated likelihood of impact by pesticides in those samples. Otherwise, samples CL3,

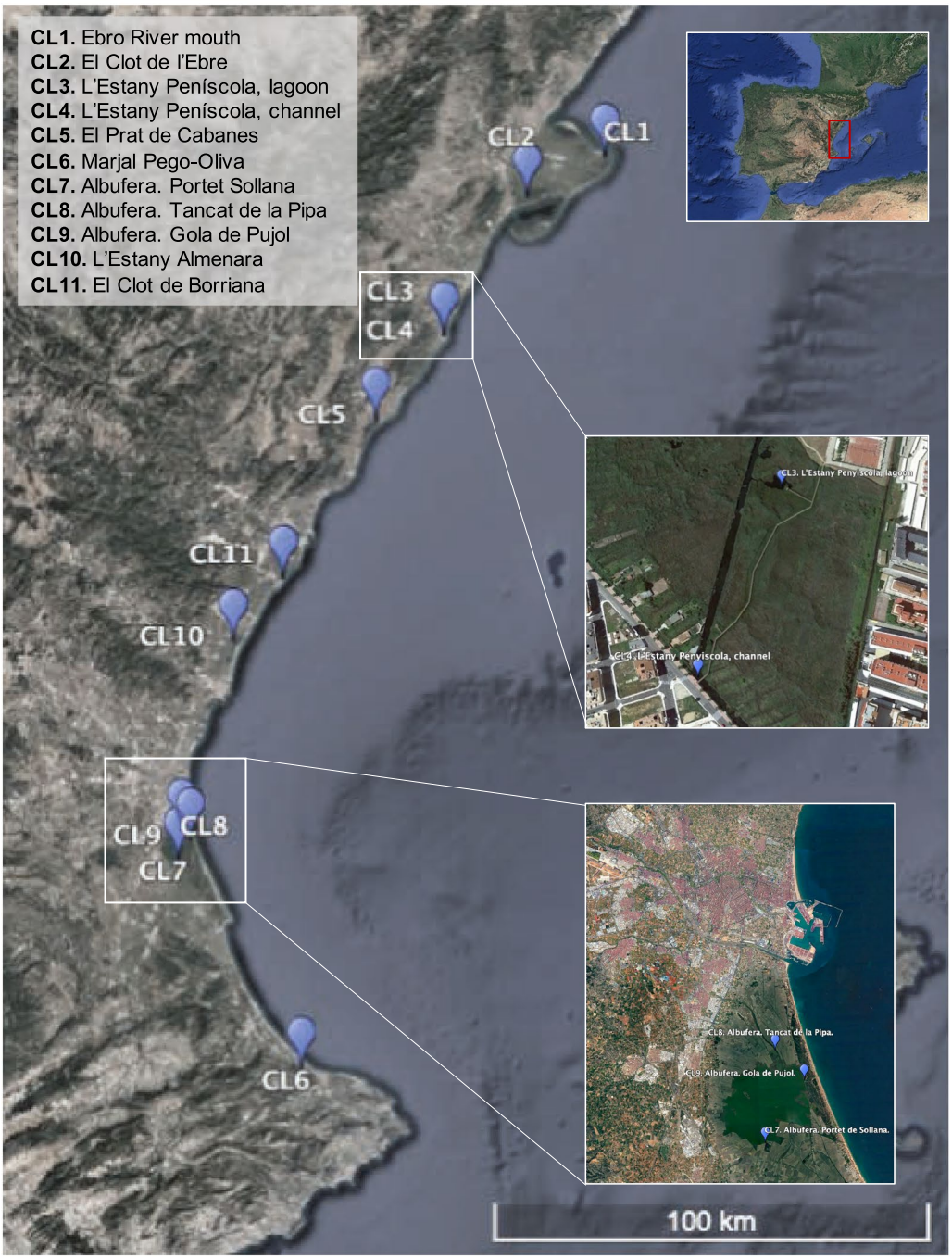

Fig. 1 Sampling points across Spanish Mediterranean coastline. Magnified areas show locations where sampling points were nearby. Upper-right map indicates the region within the Iberian peninsula under study (red square)

CL4 and CL9 were collected in points where human activity can be of importance (CL3 and CL4 close to urbanized areas, and CL9 within a highly touristic part of 'Albufera' natural park). Finally, sampling points for CL1 and CL8 might be affected by treated wastewater streams and urban runaways from large cities, although CL1 is a flowing river that might reduce its impact into water quality.

Grab samples (8 L) were collected in polyethylene terephthalate (PET) plastic bottles in February 2019. Prior to sample collection, bottles were rinsed three times with the water sample. After collection, water samples were

Table 1 Sample location details, relevance of sample and observations

ID	Sampling location	Coordinates	Relevance	Observations
CL1	Ebro River mouth	40° 43′ 10.20″ N 00° 51′ 20.88″ E	Part of 'Delta del Ebro' natural park. Included in list of Wetlands of International Importance [32]	Largest river in Spain. Instream of treated wastewater from large populations
CL2	El Clot de l'Ebre	40° 38′ 35.52″ N 00° 38′ 24.36″ E		Instream of irrigation water from rice agriculture
CL3	L'Estany Peníscola, lagoon	40° 21′ 59.04″ N 00° 24′ 01.80″ E	Flora micro reserve. Designated as Place of Community Interest by regional government	Potentially affected by human activity
CL4	L'Estany Peníscola, channel	40° 21′ 51.12″ N 00° 23′ 56.76″ E		
CL5	Prat de Cabanes-Torreblanca	40° 11′ 50.28″ N 00° 12′ 31.32″ E	Included in list of Wetlands of International Importance [32]	Area with potential impact of citrus-fruit agriculture
CL6	Marjal Pego-Oliva	38° 52′ 23.52″ N 00° 02′ 53.88″ W	Included in list of Wetlands of International Importance [32]	Area with potential impact of citrus-fruit agriculture
CL7	Albufera. Portet Sollana	39° 18′ 37.08″ N 00° 21′ 25.92″ W	Part of 'L'Albufera' natural park. Included in list of Wetlands of International Importance [32]	Area with potential impact of rice agriculture
CL8	Albufera. Tancat de la Pipa	39° 22′ 05.52″ N 00° 20′ 45.60″ W		Instream of treated wastewater and urban runaways from the city of Valencia (~ 2,500,000 inh., Spain)
CL9	Albufera. Gola de Pujol	39° 20′ 14.04″ N 00° 11′ 32.64″ W		Highly tourist area of the natural park. Impact of human activity
CL10	L'Estany Almenara	39° 45′ 14.04″ N 00° 11′ 32.64″ W	Included in list of Wetlands of International Importance [32]	Area with potential impact of citrus-fruit agriculture
CL11	El Clot de Borriana	39° 52′ 46.92″ N 00° 03′ 14.04″ W	Natural park supplied with water from a natural spring	Area with potential impact of citrus-fruit agriculture

stored at 4 °C until extraction performed within 24 h. Alongside wetland water samples, two procedural blank samples were also collected under the same conditions. Milli-Q water (8 L) were collected in PET bottles and stored at 4 °C until extraction to account for potential migration of chemicals from sampling containers into the aqueous phase.

Sample treatment

Water samples (2.5 L) were extracted by means of offline Solid Phase Extraction (SPE) using Oasis HLB cartridges (20 cc, 1 g) from Waters Corporation (Milford, MA, USA). Cartridges were previously conditioned with 3×10 mL of methanol (LC–MS grade, Scharlab, Barcelona, Spain) and 2×10 mL Milli-Q water (Millipore, Bedford, MA, USA). In order to avoid clogging, silanized glass wool was located inside the cartridge so bigger particulate matter could be retained and easily discarded. After sample loading, SPE cartridges were eluted with 3×10 mL of methanol. The three eluates were pooled and evaporated at 40 °C under N2 beam (0.7 bar) using a TurboVap II system (Caliper LifeSciences, Hopkinton, MS, USA) for 45 min reaching a final volume of 0.2 mL. Ethanol (absolute grade, Scharlab, Barcelona, Spain) was then added up to a final volume of 1 mL and extracts were frozen overnight at − 20 °C. Finally, extracts were centrifuged at 12,000 rpm for 10 min to remove fine particulate matter. A final concentration factor of 2500 was accomplished. Sample extracts were stored at − 20 °C until analysis.

Procedural blank samples, consisting of Milli-Q water, underwent the same sample treatment as wetland water sample to account for potential impact of sample treatment on the bioactivity of samples.

Bioanalysis of surface water samples

Wetland water samples, procedural blanks and positive controls were evaluated for AhR, agonistic AR (AR+) and antagonistic AR (AR−), agonistic ER (ER+) and antagonistic ER (ER−), Nrf2, and agonistic VDR (VDR+) and antagonistic VDR (VDR−) activities in reporter gene assays (Table 2). Cytotoxicity was evaluated in all cell lines using cell viability assays (MTS-based colorimetric assay and CellTiter-Glo® luminescent cell viability assay).

The activity of AhR was evaluated in transiently transfected human hepatocarcinoma cells (HepG2), which were transfected with a luciferase reporter plasmid under control of a DNA element responsive to ligand activated AhR (donated by Prof. Michael Denison, University of California, Davis, USA) [33]. AR+ and AR− activity was studied in the stably transfected Chinese Hamster Ovary cell line AR-EcoScreen™ (National Institutes of Biomedical Innovation, Health and Nutrition JCRB cell bank) with a human AR expression construct and a luciferase reporter construct under the control of the androgen response element [33]. ER+ and ER− activities were

Table 2 Panel of reporter gene assays for the assessment of toxicological fingerprint of wetland samples. Cell lines used, cytotoxicity assay method and positive control used for bioanalysis

Bioassay		Cell line	Cytotoxicity assay	Bioactivity positive control
Aryl hydrocarbon receptor (AhR)		Human hepatocellular carcinoma (HepG2)	MTS-based colorimetric assay	2,3,7,8-Tetrachlorodibenzodioxin (TCDD)
Androgen receptor (AR)	Agonistic	Chinese Hamster Ovary (AR-EcoScreen)	MTS-based colorimetric assay	Dihydrotestosterone (DHT)
	Antagonistic			Hydroxyflutamide (OHF)
Estrogen receptor (ER)	Agonistic	VM7Luc4E2	CellTiter-Glo® Luminescent Cell Viability Assay	17β-Estradiol (E2)
	Antagonistic			Raloxifene (Ral)
Nuclear factor erythroid 2-related factor 2 (Nrf2)		MCF7C32ARE	MTS-based colorimetric assay	tert-Butylhydroquinone (tBHQ)
Vitamin D receptor (VDR)	Agonistic	VDR-UAS-bla HEK 293T	MTS-based colorimetric assay	1α, 25-Dihydroxyvitamin D3 (1,25-D3)
	Antagonistic			*n.a*

n.a. not available

evaluated in a variant of human breast carcinoma MCF7 cell line, VM7Luc4E2 (donated by Prof. Michael Denison, University of California, Davis, USA), which contains a stably integrated ER-responsive luciferase reporter plasmid [33]. AR and ER activities were analyzed mainly according to OECD guidelines [33, 34]. The stably transfected human breast adenocarcinoma cell line MCF7 ARE c32 was used to measure oxidative stress corresponding to Nrf2 activity, and was kindly provided from R. Wolf (University of Dundee, Nethergate, Scotland) [35]. Finally, VDR+ and VDR− activity were evaluated in human embryonic kidney cell line HEK 293, containing a human vitamin D receptor ligand-binding domain and a Gal4 DNA binding domain as well as beta-lactamase reporter gene under the control of a UAS response element (VDR-UAS-bla HEK 293T) (ThermoFisher Scientific, Stockholm, Sweden). Further details and an expanded description of activity and cell viability assays are available in Additional file 1: Section S1.

Positive controls for each bioassay (Table 2) were analyzed alongside wetland water samples, procedural blanks and vehicle controls. For antagonistic effects, cells were co-treated with an agonistic stimulator as negative control test at a concentration corresponding to approximately EC80. 2,3,7,8-Tetrachlorodibenzodioxin (TCDD) and tert-butylhydroquinone (tBHQ) were used as positive control for AhR and Nrf2 reporter gene assays, respectively. In ER reporter gene assay, 17β-estradiol (E2) was used as control for agonistic activity and raloxifene (Ral) for antagonistic activity. For the AR bioassay, dihydrotestosterone (DHT) was used as a positive control for agonistic activity and hydroxyflutamide (OHF) for antagonistic. Finally, 1α, 25-dihydroxyvitamin D3 (1,25-D3) was used as a positive control for VDR+ activity. No appropriate positive control for VDR− activity is commercially available and, as a consequence, it could not be controlled. The positive controls in the reporter gene assay were analyzed in 6–12 concentration levels to obtain a standard calibration curve.

For incubation with cells, wetland water sample and procedural blank SPE extracts (2500 times enriched) were diluted 100 times with cell medium to get a final concentration of 1% ethanol. In consequence, the relative enrichment factor (REF) in the bioassays was 25. All water samples were tested for cell viability and bioactivity in concentration–response relationships (REF = 25, 12.5, 6.75 and 3.125) with 4 replicates for each concentration as previously proposed by Mehinto et al. [36]. In all experiments, vehicle controls were included, consisting of 1% ethanol, equivalent to water sample ethanol content. Vehicle controls were tested in 8 replicates.

Data processing

Bioactivities of wetland water samples, procedural blanks and positive controls we normalized to vehicle controls on each plate. Bioactivity was then expressed as fold-change compared to VHC (set as 1). Standard curves for AhR, AR and VDR (nuclear receptor bioassays) were drawn by fitting data to a four-parameter sigmoidal curve. For Nrf2, since no maximum effect can be reached, standard data were fitted to a liner regression. No appropriate standard is available for the antagonistic VDR reporter gene assay, and therefore, no standard curve could be obtained.

Cut-off values for cytotoxicity were set at 0.8 compared to VHC set at 1. Cut-off values for bioassays, i.e., for classification of samples as active, were based on the limit of detection (LOD) calculated as 1 plus 3 times the standard deviation of the VHC from all plates within the experiment for agonistic assays; and 1 minus 3 times the standard deviation of the VHC from all plates within the

experiment for antagonistic assays [37]. Cut-off values for AR antagonistic assays were established following OECD guideline [34]. Wetland samples were analyzed at different concentrations to enable the calculation of effect concentration (EC) values by means of statistical analysis. Given the observed activity for each sample at REF = 1, the bioanalytical equivalent concentration (BEQ) was interpolated from the dose–response curve for the positive control. For this purpose, concentration–response curve of each sample was adjusted to a linear regression [37]. Statistical analysis and graphical presentation were performed using GraphPad Prism 6.01.

Results and discussion

Cell viability

Cell viability was evaluated in all cell lines used in the study for the whole set of VHC, procedural blanks and wetland samples at different concentrations (Additional file 1: Figure S1). In general, no cytotoxicity was observed for any sample in any cell line except for sample CL8. The highest concentration tested (REF = 25) showed a slight cytotoxic effect on VM7luc4E2 cell line (ER assay, Additional file 1: Figure S1b), MCF7C32ARE cell line (Nrf2 assay, Additional file 1: Figure S1d) and AR-EcoScreen cell line (AR assay, Additional file 1: Figure S1e). Attending to these results, it was decided to use the 4 concentrations tested (REFs 25, 12.5, 6.3 and 3.1) to assess the bioactivity of wetland samples in all cell lines, paying special attention to results obtained for CL8 at the highest REF value tested. Procedural blanks and VHC did not show cytotoxicity in any cell line.

Evaluation of toxicity end-points

The bioactivities of the 11 wetland water samples were evaluated by a panel of 5 reporter gene assays for 8 different toxicity endpoints. Table 3 shows the bioactivity observed in the samples, the EC_{10} (or IC_{75}) values and the BEQ of the samples with determined EC_{10} (or IC_{75}) values. EC_{10} (or IC_{75}) values can be used for comparison purposes since they indicate the REF required to reach the 10% of the maximum activity observed for the positive control (or the 25% inhibition activity for antagonistic assays). A lower EC_{10} (or IC_{75}) value indicates a more potent activity. Accordingly, values equal to or lower than 1 indicate that activity is detected in the collected surface water sample prior to SPE, or at even lower concentrations. A different way to evaluate the bioactivity of the sample is the calculation of the BEQ; based on the activity observed in the sample, it gives an estimation of the concentration of the positive control needed to inflict the same activity in the water sample at REF 1. Thus, higher BEQ values indicate higher activity of the original water sample.

Table 3 Bioactivity observed in wetland water samples for the panel of bioassays used in the study

		Cut-off (fold-change)	EC/BEQ	Wetland samples										
				1	2	3	4	5	6	7	8	9	10	11
AhR		2.0	EC_{10}	24.7	> 25	> 25	> 25	19.5	10.6	13.3	7.9	21.6	–	–
			TCDD-eq (pM)	10.2	< 7.7	< 6.9	< 9.3	12.4	20.5	17.1	22.2	11.3	–	–
AR	AR+	1.5	EC_{50}	–	–	–	–	–	–	–	–	–	–	–
			DHT-eq (pM)	–	–	–	–	–	–	–	–	–	–	–
	AR−	0.7	IC_{75}	–	–	–	–	–	–	–	*d*	–	–	–
			OHF-eq (pM)	–	–	–	–	–	–	–	–	–	–	–
ER	ER+	1.5	EC_{50}	–	–	–	–	–	–	–	–	–	–	–
			E2–eq (pM)	–	–	–	–	–	–	–	–	–	–	–
	ER−	0.8	IC_{75}	–	–	–	*d*	*d*	*d*	–	3.21	3.84	*d*	*d*
			Ral-eq (nM)	–	–	–	–	–	–	–	441	23.1	–	–
Nrf2		1.5	$EC_{IR1.5}$	–	–	–	–	–	–	–	–	–	–	–
			tBHQ–eq (μM)	–	–	–	–	–	–	–	–	–	–	–
VDR	VDR+	1.5	EC_{50}	–	–	–	–	–	–	–	–	–	–	–
			1,25-D3-eq (pM)	–	–	–	–	–	–	–	–	–	–	–
	VDR−	0.7	IC_{75}	–	–	–	–	–	–	–	*d*	–	–	–

–: no activity detected above limit of detection at any REF, *d:* activity detected above limit of detection but no dose–response observed, EC_{10}: sample REF value to produce 10% of the maximum effect produced by positive control, EC_{50}: sample REF value to produce 50% of the maximum effect produced by positive control, IC_{75}: sample REF value to produce 25% of the maximum antagonistic effect produced by positive control, $EC_{IR1.5}$: sample REF value to induce a 1.5 fold-change activity versus vehicle control, *BEQ:* bioequivalent concentration, *TCDD:* 2,3,7,8-Tetrachlorodibenzodioxin, *DHT*: Dihydrotestosterone, *OHF:* Hydroxyflutamide, *E2:* 17β-estradiol, *Ral:* Raloxifene, *tBHQ:* Tert-butylhydroquinone, *1,25-D3:* 1α, 25-dihydroxyvitamin D3

Among the bioassays analyzed, AhR seems to be most responsive showing activity for all samples except for CL10 and CL11 (Table 3). From another perspective, sample CL8 seems to be the most polluted sample showing activity in every reporter gene assay except for Nrf2. This sample was collected in an area potentially affected by wastewater (either treated or untreated) and street runaways from a big city in the Spanish Mediterranean Coast (Fig. 1). Therefore, it was expected to be the most polluted sample and, as a consequence, the one showing more activity in the assays.

Procedural blanks were analyzed for all toxicity endpoints and no activity was detected for any of them (Additional file 1: Figure S2).

Aryl hydrocarbon receptor bioactivity

Figure 2 highlights the bioactivity observed for the AhR reporter gene assay for both the positive control and wetland samples. Linear regression was performed on the concentration–response data and used for calculation of the EC_{10} value. Neither of the REF values tested for samples CL10 and CL11 exceeded the cut-off value. Consequently, samples CL10 and CL11 were classified as inactive. However, bioactivity for samples CL1 to CL9 was detected above the cut-off value and the activity increased with increasing REF values. The REF values calculated to achieve the EC_{10} are shown in Table 3. As indicated, samples CL2, CL3 and CL4 were the least active samples with EC_{10} values > 25; and samples CL6, CL7 and CL8 were the most active samples for the AhR bioassay. The relatively high bioactivity observed in the surface water bodies studied indicated the presence of bioactive chemicals in the samples. Although this might not be an issue for environmental water samples [23, 24], it highlights that these samples can potentially activate the AhR, with multiple physiological functions involving energy metabolism, chemical and microbial defense, reproduction, development, immunity and inflammation [22]. At this moment, it is unknown whether this activity is being caused by anthropogenic chemicals or naturally occurring compounds, therefore the results observed cannot lead to the conclusion that water quality of the studied wetland is inadequate. In this respect, the highest activity observed for sample CL8 might indicate the elevated presence of small chemicals inducing AhR effect. Bioanalytical equivalents (BEQ) at REF 1 were calculated by

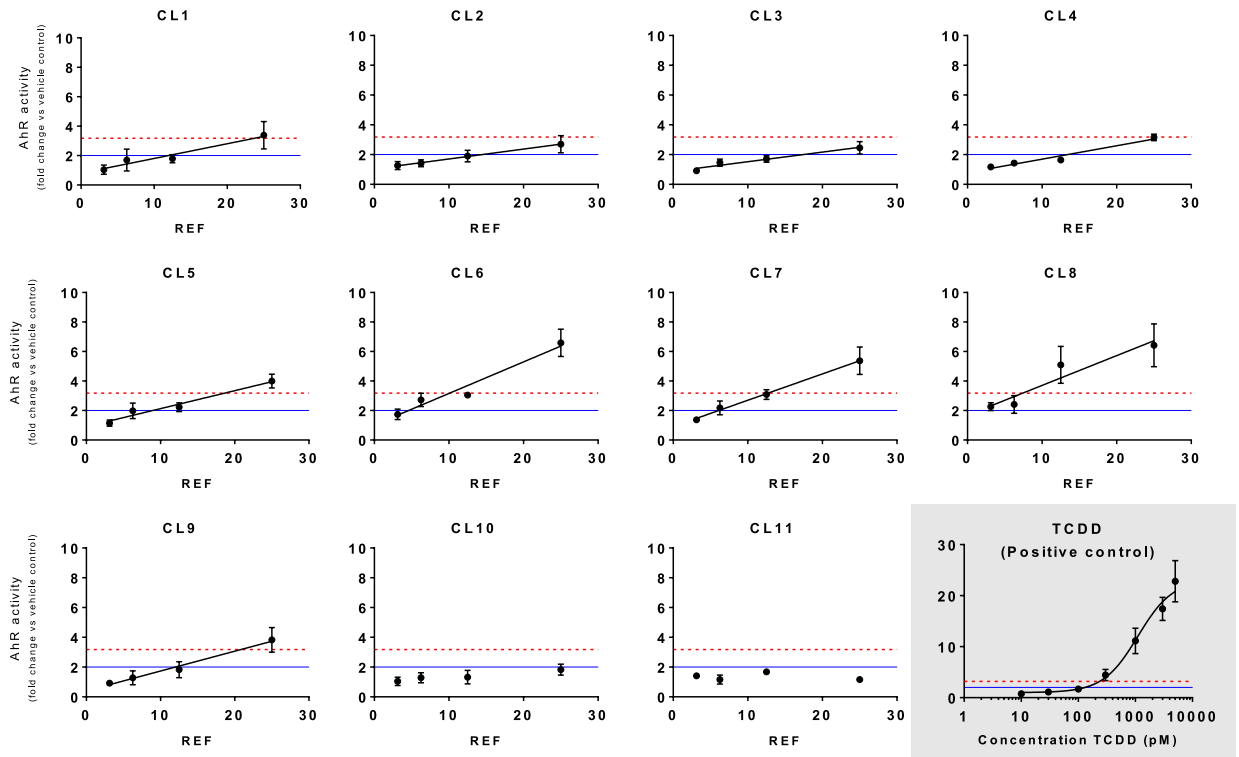

Fig. 2 AhR bioactivities for wetland water samples at REF 3.125, 6.25, 12.5 and 25 ($n = 4$ at each REF value) and positive control TCDD (grey-shadowed area). Straight black line indicates the trend curve fitting experimental data (linear regression for samples, sigmoidal for positive control), blue line indicates cut-off value for activity value and red dotted line indicates the 10% of assay maximum for comparison purposes

means of the linear range of the concentration–response curve in the samples as well as the dose–response curve for TCDD. The active samples had BEQ values in the range 7.34–22.24 pM of TCDD-Eq, with CL8 showing the highest BEQ value among samples (Table 3).

In general, AhR activity in environmental surface water samples has been reported repeatedly. Other studies involving natural water bodies found all surface water assessed bioactive for AhR [28, 38, 39]. In addition, Lundqvist et al. [40] identified a correlation between the activity observed and the total content of pesticides found in the sample denoting that samples with an increased exposure to agricultural activities may show a larger bioactivity for AhR reporter gene assay. Also, water bodies affected by industrial wastewater discharges results in an enhanced AhR activity [38] as it would be the case for sample CL8.

Androgen receptor (ant)agonistic bioactivity

In general, no bioactivity was observed with the AR reporter gene assay. Individual results for agonistic and antagonistic bioassays are shown in Additional file 1: Figure S3 and S4, respectively. In both analyses, appropriate dose–response activities were observed for the positive controls (DHT for agonistic and OHF for antagonistic).

Although no agonistic activity above the cut-off value was detected in any wetland samples, CL8 showed a decrease in the AR+ activity at higher concentrations. This diminution in the activity observed might respond to the slight cytotoxicity effect observed on the cell line used for the bioassay (Additional file 1: Figure S1) at high REF values. Apart from CL8, the other samples analyzed did not show any dose–response trend highlighting no remarkable presence of hormonal activity for the AR+ in the surface water bodies under study.

Similar results were obtained for the AR- reporter gene assay. Only sample CL8 presented activity below cut-off value at REF 25 with a concentration–response curve. However, the apparent increase of antagonistic activity might be due to the cytotoxic effect observed of CL8 at REF 25 in AR-EcoScreen cell line (Additional file 1: Figure S1) rather than being antagonistic active. None of the other samples analyzed showed detectable AR− activity. However, an increase over 1 in the activity value can be observed for the vast majority of them although data are normalized to the stimulated vehicle control activity. This behavior can be explained based on a potential cocktail effect between matrix-endogenous chemicals and the DHT added to stimulate the AR. Yet, none of them showed inhibition (except for CL8).

Detection of androgenicity or antiandrogenicity in surface water samples is common. Several studies have detected AR (ant)agonistic activities in wide sampling campaigns [19, 28, 33, 38] while others did not detect bioactivity for the AR reporter gene assays [41].

Estrogen receptor (ant)agonistic bioactivity

Estrogenicity and antiestrogenicity was evaluated by means of an ER-responsive luciferase reporter plasmid stably integrated in a cell line. ER+ activity is reported in Additional file 1: Figure S5. As illustrated, appropriate dose–response was observed for E2 (positive control), although no samples showed estrogenicity above the cut-off value. However, CL8 sample showed a clear decreasing trend in the activity fold-change (compared to vehicle control) that can be related to the cytotoxicity observed of such sample at REFs 12.5 and 25. In the case of ER-, the observations are more complex. Several samples showed activity below the cut-off value (CL4, CL5, CL6, CL8, CL9, CL10 and CL11) (Fig. 3); however, no dose–response was observed for CL4, CL5, CL6, CL10 and CL11 and, therefore, the activity could only be detected (Table 3) and no IC or BEQ value could be calculated. On the contrary, CL8 and CL9 showed a clear dose–response trend. Linear regression is usually used for the interpolation of IC_{75} and BEQ values when the activity observed is up to 30% of the positive control; however, the inhibition observed for those samples is around 60 or 70% of that from the positive control and, therefore, sigmoidal fitting is needed to be applied for a better adjust of the curve. Using this approach, CL8 and CL9 has BEQ in the range of 23–441 nM of Ral-eq. It is noteworthy that slight cytotoxicity was observed at high REF values for CL8 (Additional file 1: Figure S1) and, therefore, the elevated BEQ observed might be overestimated.

Estrogenic activity is triggered by the presence of natural and synthetic hormones, and to a lower extent by alkylphenols or phytoestrogens [18], whereas, anti-estrogenic activities are induced by dioxin-like substances, polychlorinated dibenzo-*p*-dioxins, polychlorinated dibenzofurans and polychlorinated biphenyls [42]. Several studies have detected ER activity at relatively low levels [19, 28, 33, 38, 40] with König et al. observing strong estrogenic effects in river water samples from Serbia [43].

Vitamin D receptor (ant)agonistic bioactivity

The vitamin D endocrine system regulates multiple important physiological functions and is conserved across many species. If this endocrine system is activated or blocked by environmental pollutants, it could be an endocrine disruptive effect with potentially serious consequences. For the VDR+ reporter gene assay, good dose–response was obtained for the positive control in

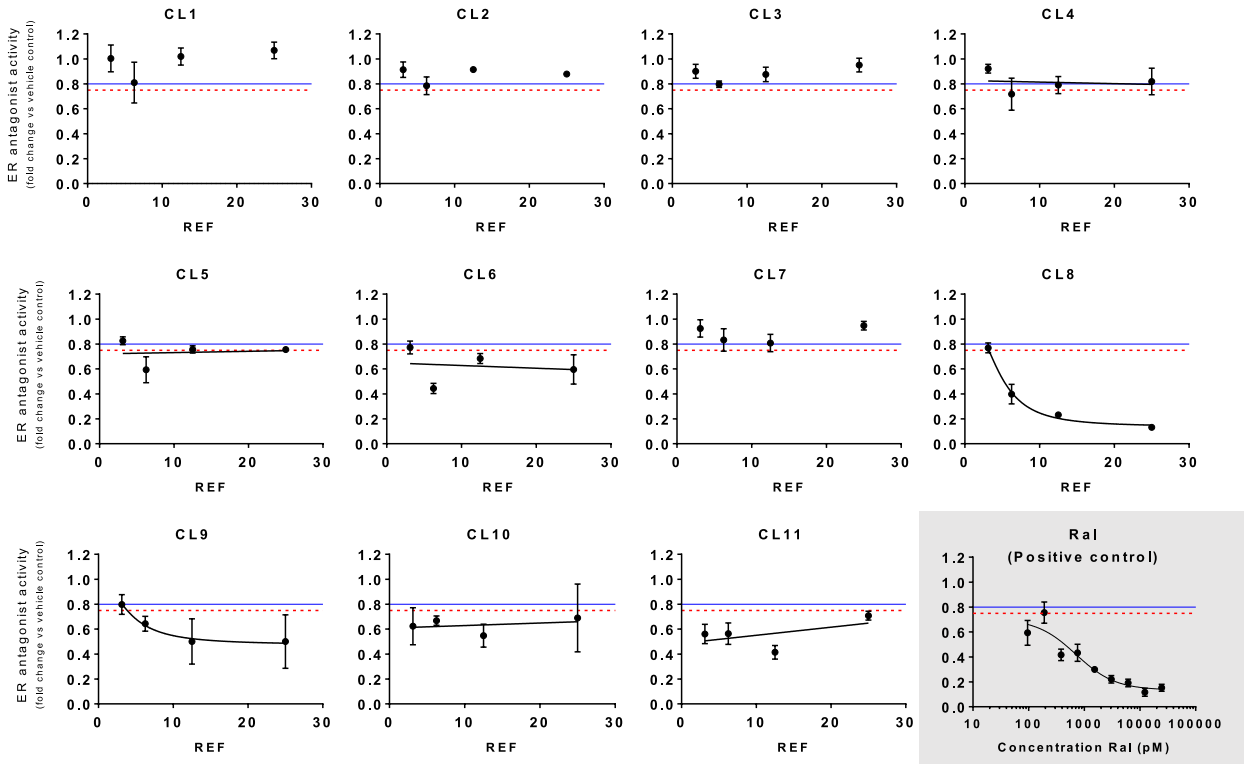

Fig. 3 Anti-estrogenicity observed in wetland water samples at REF 3.125, 6.25, 12.5 and 25 ($n = 4$ at each REF value) and positive control Ral (grey-shadowed area). Straight black line indicates the trend curve fitting experimental data, blue line indicates cut-off value and red dotted line indicates the 25% of assay maximum for comparison purposes

the agonistic mode (Additional file 1: Figure S6). However, there is no commercially available positive control for the VDR- behavior (Additional file 1: Figure S7). For the wetland water samples, no agonistic activity was observed above the cut-off value for any sample and only CL8 showed a slight antagonistic activity at REF 25. However, the VDR- activity of CL8 at REF 25 was only marginally below the cut-off value while all other REFs tested were above. As a consequence, the activity was classified only as detected and no IC value was calculated.

The application of VDR reporter gene assay for the assessment of (ant)agonistic activity in surface water samples has been scarcely explored in the literature. Inoue et al. [44] evaluated a set of 4 river water samples from Japan for the activity of VDR finding generally low response. Riegraf et al. [42], however, found no activity in a set of wastewater samples. The present work is one of the few studies incorporating the VDR bioassay to evaluate the potential activity of endocrine disrupting chemicals in environmental water bodies.

Nuclear factor erythroid 2-related factor 2 bioactivity

Oxidative stress response, evaluated by means of the Nrf2 reporter gene assay, is depicted in Additional file 1: Figure S8. Clearly, none of the samples assessed showed activity above the cut-off value at any REF assessed; and, consequently, no dose–response was observed for any sample. These results support the idea that no oxidative inducing compounds are present in the wetland water bodies studied at detectable and active levels. Previous studies also reported none or sporadic detection of oxidative stress response of environmental water samples [28, 40]. However, there are also studies depicting sustained oxidative response activity in surface water samples in Sweden [33], in the Danube River Basin [38] or even pronounced response in a sample collected in the same river basin [16]. The fact that previous studies have often reported oxidative stress response for surface water samples highlights the relevance of not having encountered activity over the Nrf2 reporter gene assay in this study.

Bioanalysis for the assessment of quality of natural water bodies in Spanish Mediterranean coastline

Assessment of water quality and pollution of natural water bodies from the Spanish Mediterranean coastline by means of a panel of 5 reporter gene assays was performed in the present study, where 8 toxicity endpoints were evaluated. Bioactivity was detected in only 20% of the analyses performed on 11 water samples. Among all of them, AhR activity was detected most frequently and found to be a sensitive indicator of the mixture effect of chemicals present in the water samples. The bioactivities observed in this study for AhR were in the range of 7.7–22.2 pM TCDD-eq (2.5–7.4 ng L^{-1}). It is remarkable that the TCDD-eq observed are similar to those previously measured in other studies. Rosenmai et al. [33] detected AhR activities in Swedish lakes at TCDD-eq of 4.2–7.8 ng L^{-1}, while Oskarsson et al. [28] detected 1.5 ng L^{-1} in river Göta Älv (Sweden). Lower activities (0.15–0.8 ng L^{-1}) were observed in Santa Cruz river (Arizona, US) by Daniels et al. [45]. Although the activity observed for AhR bioassay is in line with other studies about surface water quality assessment by bioanalysis, further research towards the analysis of which chemicals, either naturally occurring or anthropogenic, are causing this effect should be conducted.

The most active sample for the set of bioassays studied was CL8. As abovementioned, this sampling location was affected by wastewater effluents and street runaways from a big city (Valencia, 2.5 million inhabitants) and, therefore, bioactivity results could be expected. Most of the samples analyzed did not exhibit detectable activity, which per se denotes good water quality and low levels of chemical contamination. Considering that the water bodies used for the study are of special interest for the regional ecosystem, this finding can be considered of paramount importance.

Conclusions

A panel of 8 toxicity endpoints has been evaluated for a set of 11 surface water samples from environmentally relevant water bodies from the Spanish Mediterranean coastline. In general, only 20.5% of the bioassays applied showed detectable activity with the most relevant reporter gene assay being aryl hydrocarbon receptor. AhR activity was detected in water from 9 of the 11 sampling sites. The highest activity was found 'Albufera–Tancat de la Pipa' which may be influenced by contamination through wastewater effluents from Valencia with 2.5 million inhabitants although more research is needed to confirm this hypothesis. The AhR activities observed were in the same range as activities reported in surface water samples in Sweden and US. Antiestrogenic activity was detected in water from 7 of the 11 sampling sites. No estrogenic, androgenic, antiandrogenic, oxidative stress and VDR activities were detected in any of the samples, apart from sporadic, not dose-dependent activities in a few samples. Thus, the water samples only had minor impact on the studied bioactivities denoting the high water quality and low pollution of the water bodies studied.

Abbreviations

AhR: Aryl hydrocarbon receptor; AR: Androgen receptor; BEQ: Bioanalytical equivalent concentration; BS: Procedural blank sample; 1,25-D3: 1α,25-Dihydroxyvitamin D3; DHT: Dihydrotestosterone; E2: 17β-estradiol; EC: Effect concentration; ER: Estrogen receptor; HepG2: Human hepatocellular carcinoma; Nrf2: Nuclear factor erythroid 2-related factor 2; OECD: Organisation for economic cooperation and development; OHF: Hydroxyflutamide; PET: Polyethylene terephthalate; Ral: Raloxifene; REF: Relative enrichment factor; SPE: Solid phase extraction; tBHQ: Tert-butylhydroquinone; TCDD: 2,3,7,8-Tetrachlorodibenzodioxin; VDR: Vitamin D receptor; VHC: Vehicle control.

Supplementary Information

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Additional file 1: Section S1. Materials and methods for cell viability and activity assays. **Figure S1.** Cytotoxicity evaluation in all cell lines used in the study. **Figure S2.** Bioactivity responses for vehicle control (VHC), stimulated VHC, procedural blank samples (BS1 and BS2) and positive controls for AhR, Nrf2, VDR+, VDR−, AR+, AR-, ER+ and ER−. **Figure S3.** AR+ bioactivities for wetland water samples at REF 3.125, 6.25, 12.5 and 25. **Figure S4.** AR− bioactivities for wetland water samples at REF 3.125, 6.25, 12.5 and 25. **Figure S5.** ER+ bioactivities for wetland water samples at REF 3.125, 6.25, 12.5 and 25. **Figure S6.** VDR+ bioactivities for wetland water samples at REF 3.125, 6.25, 12.5 and 25. **Figure S7.** VDR- bioactivities for wetland water samples at REF 3.125, 6.25, 12.5 and 25. **Figure S8.** Nrf2 bioactivities for wetland water samples at REF 3.125, 6.25, 12.5 and 25.

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Authors' contributions

AC: study design, sample collection, formal analysis, writing first draft of manuscript, review and editing, funding. GM: study design, formal analysis, review and editing. AO: formal analysis, review and editing. JVS: sample collection, review and editing. LB: study design, review and editing, funding. JL: study design, formal analysis, review and editing, funding. All authors have read and approved the final manuscript.

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Availability of data and materials

Additional data are available in the supplementary information file and upon request to the corresponding author.

Declarations

Ethics approval and consent to participate
Not applicable.

Competing interest
The authors declare no competing interests.

Consent for publication
Not applicable.

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