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Application of the sea urchin embryo test in toxicity evaluation and effect directed analysis of wastewater treatment plant effluents

*Leire Mijangos,**[†,‡,§] *Martin Krauss,*[§] *Laura de Miguel,*[‡,∥] *Haizea Ziarrusta,*[†,‡] *Maitane Olivares,*[†,‡] *Olatz Zuloaga,*[†,‡] *Urtzi Izagirre,*[‡,∥] *Tobias Schulze,*[§] *Werner Brack*[§▲] *Ailette Prieto,*[†,‡] *Nestor Etxebarria* [†,‡]

[†]Department of Analytical Chemistry, Faculty of Science and Technology, University of the Basque Country (UPV/EHU), P.O. Box 644, 48080 Bilbao, (Basque Country), Spain

[‡]Research Centre for Experimental Marine Biology and Biotechnology (PIE), University of the Basque Country (UPV/EHU), Areatza z/g, 48620 Plentzia, (Basque Country), Spain

[§]UFZ - Helmholtz Centre for Environmental Research, Permoserstr. 15, 04318 Leipzig, Germany

▲ Institute for Environmental Research, RWTH Aachen University, 52074 Aachen, Germany

[∥]Department of Zoology and Animal Cell Biology, University of the Basque Country (UPV-EHU), E-48080 Bilbao, Basque Country, Spain

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ABSTRACT. Sea urchin embryo assay was used to assess general toxicity at four wastewater treatment plant effluents of Biscay (Gorliz, Mungia, Gernika and Galindo) and, within the tested range, all the extracts showed embryo growth inhibition and skeleton malformation activities with EC_{50} values, in relative enrichment factor units, between 1.1-16.8 and 1.1- 8.8, respectively. To identify the causative compounds, effect-directed analysis was successfully applied for the first time using sea urchin embryo test to the secondary treatment of the Galindo effluent. To this end, two subsequent fractionation steps were performed using a C18 (21 fractions) and an aminopropyl column (15 fractions). By this fractionation, the number of features detected by LC-HRMS in the raw sample was drastically reduced from 1500 to 9, and among them, two pesticides (mexacarbate, 17 ng/L, and fenpropidin, 23 ng/L), two antidepressants (amitriptyline, 304 ng/L, and paroxetine, 26 ng/L) and two anthelmintic agents (mebendazole, 65 ng/L, and albendazole, 48 ng/L) could be identified in the two toxic fractions. The artificial mixture of the identified 6 compounds could explain 79% of the observed effect with albendazole and paroxetine as the predominant contributors (49% and 49%, respectively) affecting the sea urchin embryogenesis activity.

1 INTRODUCTION

The presence of emerging contaminants (ECs) in the aquatic environment is an issue of growing concern due to the chronic exposure of many aquatic ecosystems and the risks for environmental and human health[1]. Considering that hundreds of known organic micropollutants can be detected in aquatic systems, and tens of thousands are likely to be present, a targeted analysis of all these compounds can hardly be achieved in regular monitoring[2]. At the same time, the lack of toxicological information for most of the chemicals present in surface waters hampers reliable risk assessment of these complex mixtures. Effect-based methods such as *in vitro* and *in vivo* bioassays have been recommended as a powerful tool for monitoring the toxic environmental mixture as a whole by directly measuring effects and diagnosing modes of action (MoAs) with specific cellular assays[3,4]. Screening typical water contaminants for existing knowledge on their MoAs revealed more than 30 MoA categories, of which only a minority can be diagnosed with existing in vitro assays[5]. Thus, for effect-based monitoring bioassay batteries have been recommended that combine specific *in vitro* assays with apical *in vivo* tests addressing major organism groups such as algae, invertebrates and fish [6,7]. Although both freshwater[8] and marine ecosystems[9] are known to suffer from toxic pollution, effect-based monitoring is still mostly based on freshwater organisms while there is still a gap in application of marine organisms[10,11]. The sea urchin (*Paracentrotrus lividus*) embryo test (SET)[12,13] is one of the few well established marine biotests that has been applied in a number of studies because these equinoderms are key benthic species for the study of coastal environments, they are affordable to use and to maintain in aquaria, and they are sensitive to many emerging contaminants[14–16].

Effect-based monitoring is a powerful tool to detect toxic mixtures in the environment though it does not provide much information on the drivers of detected effects. Information on these

drivers is often crucial for efficient abatement. Effect-directed analysis (EDA) has been generalized as a diagnostic approach for driver identification and the establishment of cause-effect relationships by combining bioassays with chromatographic fractionation in order to reduce the complexity of chemical mixtures and chemical analysis to identify and quantify the components of toxic fractions [17]. This approach has been particularly successful in the identification of drivers of specific *in vitro* effects such as endocrine disruption[18–20]. The identification of drivers of apical effects in whole organisms seems to be more challenging and success stories are limited to very few studies with algae, daphnids and fish embryos[1–3], being marine algae the only marine organisms used for EDA of estuaries and coastal waters[21].

The main aim of this work is to address the potential impact of urban wastewater effluent, after its conventional purification at four wastewater treatment plants (WWTP), on estuary and coastal organism[24,25], by means of the sea urchin embryo test (SET). In this context we have characterized the toxicity of several effluents entering the coastal region in the Basque Country and we have also identified drivers of toxicity in the main WWTP effluent entering the estuary of Bilbao. To this end, for the first time an EDA protocol is established and demonstrated implementing the SET as a test for adverse effects on a key organism in coastal environments. Since *P. Lividus* is widely found in Europe (Mediterranean and Atlantic coast) and there are equivalent echinoderms in American eastern (*Lytechinus variegates*) and western coasts (*Strongylocentrotus purpuratus*), and even in the Antartic (*Sterechinus neumayeri*), the bioassay has the potential of world wide application. Additionally, the use of sea urchin models has been included in the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM), though it still requires the standardization and validation to achieve the rank of zebrafish[23]. In addition to this, the work includes a deep insight of SET with mixtures of contaminants to provide the ecotoxicological assessment of the studied effluent.

2 EXPERIMENTAL SECTION

2.1. Reagents and materials

All chemicals and laboratory material are provided in **section 1** of the **supplementary material 1 (SM1).** Names, main use, CAS numbers and other relevant physicochemical properties for the organic compounds used are summarized in **S1 SM2**.

2.2.- Sampling and sample preparation

For the toxicity analysis of the WWTP effluents four treatment plants of Biscay were selected (i.e. Gorliz, Mungia, Gernika and Galindo) and, in the case of Galindo, the samples were taken from the secondary treatment (Ga2) and from a third treatment effluent (Ga3) that uses a chlorination process. Further details about the treatments, water discharge and sources of all those effluents are summarized in **Table S1**. Water flow and physicochemical parameters data of the collection day have been added in Table S2 of SM1 for Mungia, Gorliz and Galindo WWTPs (all of them with 2nd treatment). Unfortunately, we could not measure the psychochemical parameters of Gernika WWTPs, which only has a primary treatment. .

From each effluent a punctual sample of 5 L were taken in pre-cleaned plastic bottles and transported to the laboratory in cooled boxes and filtered within 48 h with a 1.2 µm glass microfiber filter (GE Whatman, Maidstone, UK) before extraction.

The filtered samples were extracted with 200 mg HLB-solid phase extraction (SPE) according to a previously validated method with slight modifications[26]. Each cartridge was sequentially conditioned with 5 mL of acetone, 5 mL of ethyl acetate (EtOAc), 5 mL of methanol (MeOH) and 5 mL of ultrapure water. In the case of Ga3, sodium thiosulfate (30 mg/L) was added to the raw sample, prior to perform the SPE, to neutralise the presence of chlorine [27]. A maximum of 500 mL of each effluent sample were passed through each cartridge (several

cartridges were used in parallel) assisted by a vacuum pump at ca. 5 mL/min. Subsequently, the cartridges were washed with 6 mL of ultrapure water, vacuum dried for 40 min and eluted with 6 mL of methanol. All the eluted extracts were pooled together and the final extract was concentrated to dryness under a gentle stream of nitrogen at 35°C, re-dissolved in pure MeOH, and submitted to the sea urchin bioassay (see **section 2.3**).

For EDA 225 L (punctual sample) of the effluent of the secondary treatment of Galindo (Ga2) were sampled and filtered in the lab. For the SPE extraction, the cartridges were prepared in-house by filling an empty PP column (20 mL) with 1.5 g Strata X-AW (bottom) and 3.5 g of Bond-Elut Plexa (top). Previous to the extraction, both bulk materials were individually cleaned with 400 mL of acetone followed by EtOAc, MeOH, MeOH with 2% ammonia (v/v) solution and Milli-Q (30 min for each solvent, 3 cycles) in an ultrasonic bath. The 225 L of the effluent sample were percolated through the cleaned cartridges assisted by a vacuum pump at ca. 5 mL/min (the ratio mass of sorbents/volume of effluent was scaled up from an amount of 0.2 g of total sorbent amount per 0.5 L of water). After the extraction, all cartridges were kept at -40°C for 24 h and freeze-dried (Cryodos-50 laboratory freeze-dryer from Telstar Instrument, Sant Cugat del Vallés, Barcelona, Spain). Elution was carried out with 90 mL of MeOH: EtOAc (50:50, v:v) solvent mixture followed by 60 mL of MeOH with 2% ammonia (v/v). All extracts were neutralized by adding formic acid and the pooled extracts were evaporated using a rotary evaporation (Büchi, Switzerland) and adjusted to a final volume of 225 mL (i.e. the raw sample with an extraction factor SPE (EF_{SPE}) of 1000).

2.3.-Sea Urchin Embryo Test (SET)

Adults of sea urchins (*P lividus*) were provided by the ECIMAT (Galicia, Spain) or collected from an intertidal area of Armintza (43.43347N, 2.89889W, Basque Country) and maintained

in aquaria at the Plentzia Marine Station (PiE). Seawater tanks were maintained at 15±1°C and natural photo period. Every two days sea urchins were fed with macroalgae and dregs were siphoned. The procedure followed to obtain the gametes and embryos is described in **section3** in **SM1.**

In order to perform the dose-response curve, two different dose ranges were used: relative enrichment factor REF[28,29] 0.05-75 (3 mL, n=3) for the analysis of toxicity in the effluents and REF 1-75 (3 mL, n=3) for the EDA approach. The methanolic solutions obtained from the extraction and fractionation (see **sections 2.2** and **2.4**) were concentrated to dryness under a gentle stream of nitrogen at 35°C and re-dissolved with 3 mL of FSW containing 0.1% of dimethyl sulfoxide (DMSO, (v/v)). Afterwards, fertilized sea urchin embryo eggs were added to test samples (40 egg/mL) and placed in an incubator at 20°C for 48 h in darkness until larvae reach the four arm-pluteus stage. After the incubation, larvae were preserved by adding one drop of 40% formalin per sample.

The quantitative assessment of the toxic effects was evaluated in two ways: by measuring the index of toxicity (IT) accounting for the skeleton malformations[12] and by measuring the growth inhibition of the larvae by calculating the size increase (SI) as proposed by Saco-Álvarez and co-workers[13].

For the calculation of the IT, 100 individual embryos were categorized for their level of malformation according to Carballeira et al[12]. Normal larvae or Level 0 correspond to larvae at four arm-pluteus stage with fully developed arms, complete skeletal rods and of similar size to control larvae. Level 1 toxicity (slightly toxic) was characterized by larvae presenting an incorrect arrangement of skeletal rods (crossed tip, separated tip, fused arms and incomplete skeletal rods). Level 2 (moderate toxicity) was featured by larvae with no skeleton or in which skeletal rods were absent or incomplete, or anomalous shape. Level 3 toxicity (highly toxic)

158 was characterized by the blockage of development at early stages and larvae that did not reach
159 the pluteus stage. Then, the general index of toxicity (IT) was calculated according to
160 **Equation 1**.

161 $$IT = \frac{(0 \text{ x } \% \text{ Level } 0)+(1 \text{ x } \% \text{ Level } 1)+(2 \text{ x } \% \text{ Level } 2)+(3 \text{ x } \% \text{ level } 3)}{100} \qquad \textbf{(Eq.1)}$$

162 where IT ranges from no toxicity (IT = 0) to highly toxic (IT = 3).

163 The growth inhibition was recorded according to Saco-Álvarez[13]. The maximum dimension of
164 35 embryos was measured and the SI was calculated by subtracting the fertilized egg diameter
165 at t=0 (fertilized eggs were fixed once the initial size was measured).

166 As quality control tests, four different control samples (n=3) were included: i) eggs (fertilized
167 eggs development was blocked just after fertilization), ii) FSW, iii) solvent control (FSW with
168 DMSO at 0.1% v/v) and iv) procedural blank control. Procedural blanks were processed in
169 parallel to the effluent samples and fractions. A test was acceptable when the mean size of the
170 control eggs exceeds 218 µm (in the case of SI) or the length of control larvae was >340 µm
171 (in the case of IT criterion) [13], and the fertilization success (indicated by the presence of a
172 fertilization membrane) was > 90 %. Water quality was also measured at the beginning and at
173 the end of the bioassay to ensure acceptance of incubation (temperature 20°C, salinity 3.3-3.4
174 (accetpanñe criteria > 3.2%), dissolved oxygen 7-8 mg/L (> 5 mg/L), pH 7.6-7.8 (> 7) and
175 ammonia 0 mg/L (< 40000 ng/L, NOEC 40000 ng/L))[13]. Additionally, to assure the accuracy
176 of the test, copper (Cu) standard solutions (0-10^6 ng/L) were used as positive controls[13]

177 Full dose-response curves were recorded, modelled with the probit model using SPSS
178 Statistics 23 package (v17, IBM SPSS) software. Effect concentrations of extracts and
179 fractions thereof were calculated and given as REF as the product of the EF_{SPE} and the

dilution factor in the bioassay reflecting the volume of the extract added to the bioassay divided by the total volume of the assay.

All statistical analyses were performed with the SPSS Statistics 23 package (v17, IBM SPSS), using data corrected by the control response. To test the normal distribution of the data, a normality analysis was conducted using the Shapiro–Wilk test and non-normal data were modified with an angular transformation ($P' = \arcsin P^{0.5}$). The median effective concentrations (EC_{50} and EC_{10}) with 95% confidence limits were calculated by the probit model. Sizes measuring (for growth inhibition) and images were taken with NIS-Elements Imaging Software v4.30 (Nikon Instruments BV, Europe).

2.4.- EDA workflow and fractionation

As illustrated in **Figure S1,** the raw sample obtained previously was subjected to a two-fold fractionation step and the SET bioassay was applied to all the fractions obtained at each fractionation (see **section 2.3**), but non-targeted chemical analysis was restricted to biologically active and non-active neighboring fractions and the parent extract (see **section 2.5**). In the same way, a procedural blank was also submitted to fractionation and analysis.

At each fractionation step a recombined mixture of all the fractions was prepared and tested in the bioassay to assure that no major losses of bioactivity occurred during fractionation. Finally, SET dose-effect curves of identified candidate drivers were recorded in those cases where standards were available in order to confirm the toxicity of these compounds and to assess their contribution to the entire bioactivity of the active fractions (see **section 2.6**). Concentrations of effluents along the whole procedure are given in relative enrichment factors (REF) as defined in **section 2.3**.

The extracts were fractionated by semi-preparative reverse phase liquid chromatography. The HPLC was operated under the control of Chromeleon 6.7 (Dionex) software and was comprised of a Rheodyne manual valve, a Varian Prostar 210 Pump and a Foxy 2000 fraction collector (Teledyne Isco Inc.Lincon USA). A Dionex UVD 340U UV/VIS detector was used for recording of chromatograms at wavelengths of 210 nm and 254 nm.

The sequential fractionation was performed combining two different columns with an orthogonal selectivity[19]: a reverse phase C_{18} column (Macherey-Nagel Nucleodur C_{18} column, 250 x 10 mm, 5 µm particle size) and an aminopropyl column (AP, Imtakt, 150 x 10 mm , 3 µm particle size) using a gradient elution with water and MeOH, both containing 0.1% of formic acid, at a flow rate of 2.36 mL/min. In the first fractionation the gradient started at 30% of MeOH, held for 5 min, linearly increasing to 95% of MeOH within 30 min and maintained for the next 15 min before returning back to the initial conditions for 15 min re-equilibration. In total, 18 fractions (F1-F18) of two minute intervals were collected followed by two fractions of three minutes (F19-F20) and a last fraction (F21) of 8 minutes (see **Table S3** in **SM1**). In the second fractionation the gradient started at 5% of MeOH, held for 2 min, linearly increased to 95% MeOH within 32 min and maintained for the next 10 min before returning back to the initial conditions for 20 min re-equilibration. Fifteen fractions (F13-1-F13-15) of three minute intervals were collected.

Fractionation blanks (FB_{C18} and FB_{AP}) were obtained and processed prior to the sample fractionation. The recombined samples (R_{C18} and R_{AP}) were constituted from equal volumes of all 21 and 15 fractions collected, respectively, and processed in the same way as the fractions.

After further concentrating the raw sample to an EF_{SPE} of 10,000, aliquots of 500 µL enriched extract were injected per run and the corresponding fractions from each of the 12 injections

were combined. For both biotesting and chemical analysis, the fractions, the blanks (FB_{C18} and FB_{AP}) and the recombined samples (R_{C18} and R_{AP}) were re-extracted with SPE on Plexa:Strata-X-AW (70:30, m:m, conditioned with 12.5 mL of LC-MS grade acetone, ethyl acetate, MeOH and 25 mL of LC-MS grade water) after dilution with LC-MS grade water to less than 5% of MeOH[18] (see **Table S3** in **SM1**),. The loaded cartridges were dried and eluted with 9 mL of MeOH:EtOAc (1:1, v:v) and 6 mL of MeOH containing 2% (v/v) 7N ammonia in MeOH (Supelco). The extracts were neutralized with formic acid and aliquots were evaporated to dryness under a gentle stream of nitrogen at 35°C and dissolved in MeOH for chemical analysis and filtered sea water (0.1 µm, FSW) with 0.1 % of DMSO for biotesting.

The recovery of the whole proc dure (extraction with SPE and fractionation) was assessed with a synthetic mixture containing 215 micropollutants (see **S2** in **SM2**) including several classes of environmentally relevant compounds. The set of compounds (each at 500 ng/mL) was submitted to each fractionation procedure using the same elution program explained above and the resulting fractions were analysed by LC-HRMS (see **section 2.5**).

2.5.-LC-HRMS analysis

The raw effluent extract from Ga2, the fractions showing a significant toxicity in the SET and the non-toxic neighboring fractions were analyzed by LC-Q-Exactive HRMS operated in full scan mode with data dependent MS^2 data acquisition mode, as detailed in **section 4** of the SM.

Data were analyzed using Compound Discoverer 2.1 (CD; Thermo-Fisher Scientific). The workflow (see **Figure S2** in **SM1**) and settings (see **Table S4** in **SM1**) used for the data evaluation are summarized in the **section S4** in **SM1**. Briefly, peak picking and peak alignment were performed with a retention time deviation of 0.5 min, a mass tolerance of 5 ppm and a signal higher than $5 \cdot 10^5$. The m/z values of the predicted compounds were searched in the peak list considering the criteria of 5 ppm for mass tolerance and 30% for the

intensity tolerance for the isotope search. The peaks that fulfilled both criteria were manually checked and only those with available MS^2 spectra, a maximum of 10 background contamination to sample ratio and resembling Lorentzian or Gaussian peak shape, were further considered. Structural assignments were carried out based on $ddMS^2$ fragments annotated by Compound Discoverer. Afterwards, we compared the exact mass, isotope pattern, MS^2 fragmentation and abundances of the selected features with those available in the mzCloud (best match > 70%) library. When the substance was not available in the mzCloud library, the experimental fragmentation pattern was compared against *in silico* fragmentation obtained in MetFrag (https://msbi.ipb-halle.de/MetFragBeta/)[30]. Plausible candidates were selected based on the number of references in *ChemSpider* as an indicator of human use and commercial importance.

Only the peaks with an intensity 4 times higher in the active (toxic) fractions than in the neighboring inactive ones were considered. Since the C_{18} column is expected to separate complex mixtures according to hydrophobicity [17], retention time and log D (at pH=3, calculated with JChem for Office provided by ChemAxon) values were used as criteria for candidate selection based on the log D calibration of the C_{18} chromatographic system with the synthetic mixture of 215 micropollutants (see **S2** in **SM2**). Tentatively identified mixture components were confirmed with neat standards using retention times and MS/MS spectra.

2.6.- Chemical and effect confirmation

The quantitative contribution of the identified chemicals to the mixture effect was confirmed on the basis of the model of concentration addition using toxic units (TU) in agreement with previously published EDA studies. [17]

Individual concentration of mixture components (C_i) normalized to individual 50% effect concentrations ($EC_{50(i)}$) were summed up to achieve TU_{chem} based on chemical analysis (eq. 2)

for comparison with biologically derived TUs for the sample respective fraction (TU_{bio}) (eq. 3) and for an artificial mixture containing the identified chemicals at the same concentrations as in the sample or fraction ($TU_{artificial\ mixture}$).

$$TU_{chem} = \sum_{i=1}^{n} \frac{C_i}{EC_{50(i)}} \qquad \textbf{eq. 2}$$

$$TU_{bio} = \frac{1}{EC_{50(sample)}} \quad \textbf{eq. 3}$$

$$TU_{artificial\ mixture} = \frac{\sum C_i}{EC_{50(mixture)}} \textbf{ eq. 4}$$

The effect concentrations for the mixtures $EC_{50(sample)}$ and $EC_{50mixture}$ are expressed as dimensionless relative enrichment factor (REF).

Concentrations were quantified with the TraceFinder 4.1 software (Thermo). EC_{50} values were calculated by recording and modeling dose-response curves. Stock solutions were made up by dissolving standards in FSW approximately 2 hours before the beginning of the experiment. Dose range (1-10^8 ng/L) of the identified single compounds were chosen on the basis of their measured concentrations in the extracts and their water solubility.

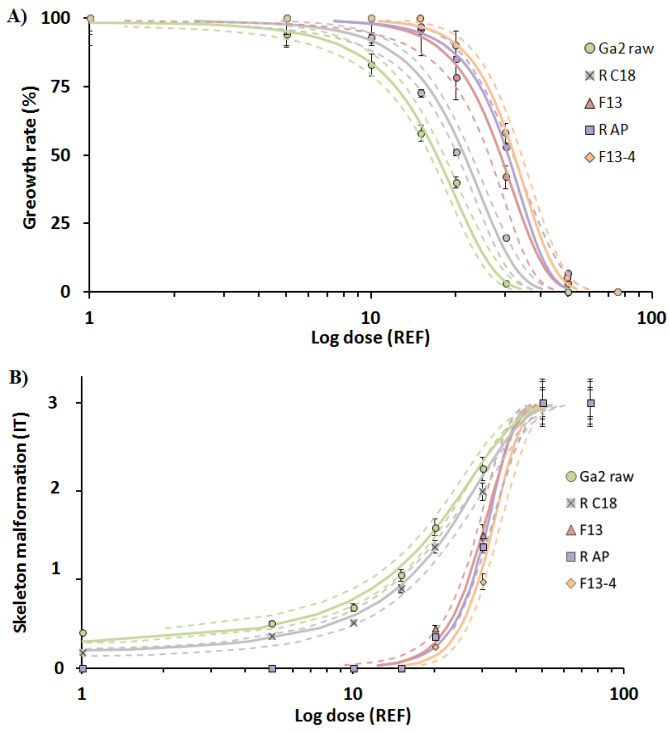
3 RESULTS AND DISCUSSION

3.1.- Effluent toxicity evaluation

Through the experimental period the positive control Cu exhibited a mean EC_{50} of 37 µg/L with all EC_{50} values in the range of 24 to 55 µg/L (within average ± 2 x standard deviation). These values are in concordance with results reported previously[31].

The procedural blanks did not induce any effect with the tested endpoints below the

maximum dose level (REF75) and all the extracts showed embryo growth inhibition and skeleton malformation activity within the dose range tested. These facts allowed us to model the dose-response curve of the tested effluents samples as shown in **Figure 1** and to calculate the EC_{10} and EC_{50} values as summarized in **Table 1**. In addition to this, **Figure S3** in **SM1** shows representative malformations observed for the tested effluents in this work. The effluent of Gernika WWTP was identified as the most toxic one followed by Ga3 (EC_{50}-SI= 1.1 REF and 4.3, respectively) and the effluents with the secondary treatment (EC_{50}-SI = 7.0, 17.4 and 23.9 for Mungia, Ga2 and Gorliz, respectively). These investigations revealed a 6-23 times higher bioactivity of the effluent of the Gernika WWTP effluent compared to the other two effluents after secondary treatments. EC_{10} values of 0.4 (SI) and 0.3 (IT) REF indicates significant effects in Gernika WWTP even in diluted samples and thus this effect may be of concern for the Biosphere Reserve (see **Table S1** in **SM1**), even considering the tidal dilution of the discharge into the estuary [32].



310 **Figure 1**. The log dose-response curves of the tested effluents samples (Gernika, Mungia, Ga2, Ga3 and Gorliz)
311 obtained with A) size increase end-point and B) skeleton malformation end-point. Straight lines show the EC fit
312 values obtained with probit and dashed line the confidence level (95%).

313

Table 1. Effect concentrations EC_{10} and EC_{50} obtained with both end-points (IT: larvae malformations and SI: size increase) and their confidence level (95%) obtained for each sample expressed as relative enrichment factor (EC REF).

Sample	EC REF (confidence level 95%)			
	Skeleton malformation		Size increase	
	$EC_{10\text{-}IT}$	$EC_{50\text{-}IT}$	$EC_{10\text{-}SI}$	$EC_{50\text{-}SI}$
Gernika	0.3 (0.1-0.4)	1.1 (1.0-1.4)	0.36 (0.26-0.44)	1.1 (1.0-1.2)
Mungia	2.9 (1.0-4.1)	5.7 (4.6-7.5)	3.3 (2.5-4.0)	7.0 (6.2-7.8)
Gorliz	8.8 (7.3-10.0)	16.8 (15.5-18.2)	10.6 (7.5-13.5)	23.9 (20.8-28.0)
Ga2	<0.05	12.2 (10.8-13.9)	7.9 (6.6-9.1)	17.4 (16.1-18.9)
Ga3	1.6 (1.1-2.1)	2.9 (2.4-3.6)	2.1 (1.2-2.7)	4.33 (4.29-5.24)

314

315 Effluents from Galindo (Ga2 and Ga3) exhibited two different patterns regarding the selected
316 endpoints. Ga2 showed a lower $EC_{10\text{-}IT}$ value for larvae development compared to Ga3 (<
317 0.05 vs 1.6, see **Table 1**), while growth was inhibited at lower dose by Ga3 ($EC_{50\text{-}SI}$ 2.1 vs
318 7.9). Even though larvae treated with Ga2 reached the 4 arm pluteous stage at any dose lower
319 than REF 50, a high number of crossed tip malformations (level 1) were observed even at low
320 REF (see **Figure S3** in **SM1**). This fact would suggest a slightly different susceptibility of
321 both endpoints to complex mixtures with malformations being more sensitive than the growth
322 inhibition at low doses.

323 Enhanced growth inhibition at Ga3 might be driven by by-products formed during the
324 advanced treatment[27]. Comparable results were obtained by Rueda-Marquez et al.[33], who

applied the SET bioassay to study the viability of H_2O_2/UV photolysis. In fact, they also found a higher toxicity on embryos in AOP treated effluents than the non-treated ones.

3.2.- Identification of active fractions

The effluent extract from the secondary treatment of Galindo (Ga2) was selected to demonstrate the power of SET-based EDA to identify drivers of sea urchin toxicity. SET of the tested extract exhibited monotonic dose–response curves with REF 75 causing full inhibition (100%) and 0.052 TU_{Bio} and 0.058 TU_{Bio} for $EC_{50\text{-}IT}$ and $EC_{50\text{-}sI}$, respectively, indicating no significant difference in sensitivity between skeleton malformation and growth. Procedural blanks (FB_{C18} and FB_{AP}) did not induce any effect on the tested endpoints up to REF 75.

After the first fractionation step only fraction 13 (F13) showed significant toxicity with TU_{Bio}=0.034 skeleton malformation and TU_{Bio}=0.035 for SI (**Figure 2)**. The biological activities of the recombined sample (R_{C18}) and of the raw sample were identical in a window of +/- 20% confirming the excellent recovery of the fractionation procedure (**Table 2**). The latter has been confirmed chemically with the mixture of 215 standard compounds (acceptable recoveries from 53% to 89% were obtained for most of the tested compounds, for details see **S2** in **SM2**). Since about 75% of the activity of the raw extract was recovered in F13, the rest is probably distributed over the other fractions without getting significant in any of them. Interestingly, on the basis of $EC_{10\text{-}IT}$ much higher values are observed for the raw extract (TU_{Bio} >1) and R_{C18} (TU_{Bio} = 0.24) than for F13 (TU_{Bio} = 0.049) indicating that slightly increased skeleton malformations might be already induced at very low doses of the complex mixture even if this effect cannot be recovered in the fractions. This is also evident from the reduced slope of the dose-response curves for raw extract and R_{C18} and skeleton malformation (see **Figure S4** in **SM1**).

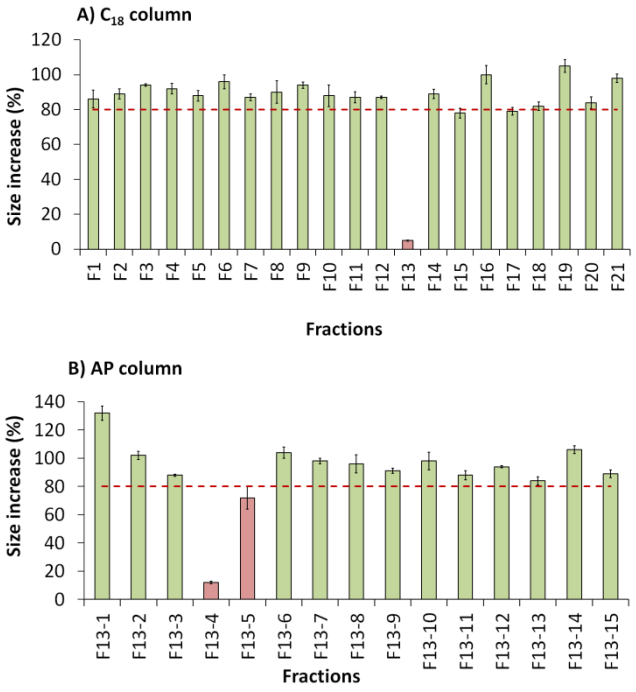


Figure 2. Size increase (%) response of the single fractions obtained with a) C_{18} column, $F1_1$-$F1_{21}$ and b) aminopropyl (AP) column, fraction F13-1 to F13-15. Red bars represent the identified active fractions. Fractions with the mean value under the dashed line at 80 % are defined as active. All the fractions are at REF 75.

Table 2. Toxic units (TU_{Bio}) for effective concentrations (EC_{10} and EC_{50}) obtained with both end-points (larvae malformation and size increase) and their confidence level (95%) obtained for each sample.

Sample	Toxic units			
	Skeleton malformation (IT)		Size increase (SI)	
	$TU_{10\text{-}IT}$	$TU_{50\text{-}IT}$	$TU_{10\text{-}SI}$	$TU_{50\text{-}SI}$
RAW	<1	0.052 (0.048-0.052)	0.13 (0.11-0.16)	0.058 (0.055-0.062)
R_{C18}	0.24 (0.16-0.67)	0.046 (0.042-0.049)	0.09 (0.08-0.11)	0.047 (0.044-0.049)
F13	0.049 (0.054-0.046)	0.034 (0.032-0.035)	0.06 (0.05-0.07)	0.035 (0.031-0.035)

R_{AP}	0.041 (0.039-0.045)	0.032 (0.031-0.035)	0.046 (0.040-0.049)	0.038 (0.034-0.043)
F13-4	0.043 (0.041-0.047(	0.031 (0.029-0.033)	0.049 (0.045-0.054)	0.031 (0.029-0.033)

In order to further reduce complexity, the active primary fraction F13 was separated into 15 secondary fractions using the AP column. Embryo growth inhibition was observed in the secondary fractions F13-4 and F13-5 with TU_{Bio} in the range of 0.031-0.049 for F13-4 for both endpoints. No full dose-response relationship was recorded for the much less toxic F13-5 and thus, no exact EC_{50} can be given. However, more than 90% of the EC_{50} of F13 for skeleton malformation and 86% for growth inhibition could be recovered in F13-4. This indicates only minor contributions of F13-5 and other secondary fractions to the activity of F13.

3.3.-Nontargeted analysis of toxic fractions

The toxic fractions (F13 and F13-4), the neighboring nontoxic fractions, the recombined fractions and the raw and blank samples were analyzed in order to identify the most likely toxic candidates.

More than 15,000 features (in both positive and negative ionization modes) were detected in the raw sample. Among them, 49 could be identified (Level 1), 67 tentatively identified as probable structures (Level 2a) and 59 as tentative candidates (Level 3), according to classification by Schymanski et al.[34] (see **S3** in **SM2**).

The list of feasible features present in the raw sample was drastically reduced if only those features were considered which could be found in the two toxic fractions with peak intensity

at least 4 times higher in the active fractions (F13 and F13-4) than in the neighboring non-active fractions and in the retention time's windows from 4.5 to 7.5 min (see **S2** in **SM2**). Lastly, the pre-calibrated $C_{18\ step}$ (C_{18} vs log D $_{(pH=3)}$, r^2=0.89) indicated that the log D $_{(pH=3)}$ of the components of F13 should bein the range of 1.27-2.49 (see **S2** in **SM2**).

Using these filtering steps, the number of candidate features was limited to nine peaks (see **Table 3**). Six of these features could be confirmed with standards, as can be seen in the MS^2 spectra shown in **Figure S5** in **SM1**. The determination of the unequivocal molecular formula was not possible in the case of the remaining three features due to poor MS^2 spectra. The nine identified compounds include two pesticides, mexacarbate and fenpropidin, and four pharmaceuticals, the antidepressants drugs amitriptyline and paroxetine, and the anthelmintic agents mebendazole and albendazole. All these compounds were detected in the active fractions (F13, F13-4), and the raw sample except fenpropidin, which was not detectable in the raw sample. We attribute this to the complex matrix of the raw sample compared to that of the individual fractions (F13, F13-4).

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Table 3. Overview of the 9 non-target peaks detected commonly in all the active samples (raw, F13 and F13-4) .

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#	Ionization	Molecular weight	RT [min]	Formula	Compound name	Level	Log D (pH = 3)[a]	Mode of action	Use	Concentration in raw sample (ng/L)	$EC_{50\text{-}SI}$ ng/L (confidence level 95%)
1	$[M+H]^+$	222.1367	4.2	$C_{12}H_{18}N_2O_2$	Mexacarbate	2a	1.8	Acetylcholinesterase inhibition	Pesticide	17	9000 (2400-49.6)
2	$[M+H]^+$	204.1513	4.4	$C_{14}H_{20}O$	Unknown	4	-				-
3	$[M-H]^-$	265.0885	5.0	$C_{12}H_{15}N_3O_2S$	Albendazole	1	2.2	Mitosis, cell cycle	Anthelminthic	48	1700 (1300-2300)
4	$[M-H]^+$	295.0958	5.3	$C_{16}H1_3N_3O_3$	Mebendazole	1	2.4	Mitosis, cell cycle	Anthelminthic	65	4460 (2400-7600)
5	$[M+H]^+$	329.1423	5.8	$C_{19}H_{20}FNO_3$	Paroxetine	2a	1.9	Serotonin reuptake inhibitors	Antidepressant	26	910 (600-1300)
6	$[M+H]^+$	277.1827	6.2	$C_{20}H_{23}N$	Amitriptyline	1	1.3	Serotonin reuptake inhibitors	Antidepressant	304	60200 (26200-1252)
7	$[M+H]^+$	273.2452	6.9	$C_{19}H_{31}N$	Fenpropidin	1	1.9	Sterol biosynthesis inhibition	Fungicide	23	560000 (400000-766000)
8	$[M-H]^-$	317.0343	7.5	$C_6H_{11}N_3O_{12}$	Unknown	4	-				-
9	$[M-H]^-$	307.0056	7.5	$C_{10}H_6ClN_7OS$	Unknown	4	-				-

a) Log D (pH = 3) were calculated using the Calculator Plugins in JChem for Excel (version 18.11.0.301).

As an example, the identification of mexacarbate (m/z 233.1440, RT 4.2 min) is explained in detail. Only one plausible molecular formula ($C_{12}H_{18}N_2O_2$) remained after the mass accuracy (< 5 ppm) and isotopic fit criteria and only two structures showed an mzCloud score above 70%: mexacarbate (a pesticide, ChemSpider ID 9043, log $D_{(pH=3)}$ = 1.8) and neostigmine (a parasympathomimetic pharmaceutical, ChemSpider ID4301, log D $D_{(pH=3)}$ = -1.6). The main differences between their structures arise in the position of two methyl groups. Metfrag explained the fragments found in the MS^2 spectra of both candidates: Mexacarbate explained nine out of the ten most intense fragments and neostigmine explained eight. Neostigmine could not explain the peak m/z 178.12175 (see **Figure S5** in **SM1**) present in the spectra, and in the case of mexacarbate it was feasible by the loss of N-methylamine [$C_9H_{11}NO + H^+$], m/z 178.1227. The presence of mexacarbate in F13 was also in agreement with the log D $D_{(pH=3)}$ range of 1.27-2.49 characterizing this fraction, and thus, neostigmine (log $D_{(pH=3)}$=-1.6) was discarded as a possible candidate.

3.4.-Assessment of toxicity

The current knowledge of SET in response to individual organic chemicals is still very limited. In fact, we could not narrow down or compare the risk of the identified list of candidate toxicity drivers with the information available in the EPA Dashboard web application (https://comptox.epa.gov/dashboard) or ecotoxicology knowledgebase (ECOTOX Knowledgebase, https://cfpub.epa.gov/ecotox/). Therefore, in order to quantify their individual contribution all identified compounds in the toxic fraction were tested individually obtaining EC_{50} values in the range of 900-560000 ng/L (see **Table 3** and**Figure S6** in **SM1**).

By recording a full dose-response curve with the artificial-mixture of the 6 identified compounds at the same proportions as they have been detected in the toxic fraction (see **Figure S7)** a $EC_{50(mixture)}$ value of 22 REF was obtained, which could explained 79% of sea urchin embryogenesis activity observed in the biological active F13-4 fraction (EC_{50} =28 REF). When TU_{chem} and $TU_{artificial\text{-}mixture}$ were compared, the artificial mixture a lower effect (almost 60% of the arithmetic sum), which may suggesting the presence of an antagonistic effect among the tested targeted compounds (see **Figure 3**).

Regarding the contribution of each identifies compound, antihelmitics (albendazole TU_{Chem}= 0.03 and mebendazole TU_{Chem}= 0.02) were the predominant contributors (74%) followed in a less extend by antidepressants (58%, paroxetine TU_{Chem}= 0.03 and amitriptyline TU_{Chem}= 0.005), whereas mexacarbate (TU_{Chem}= 0.002) and fenpropidin (TU_{Chem}= 0.00004) could only explain the 3% and 0.1% of the sea urchin embryogenesis, respectively.

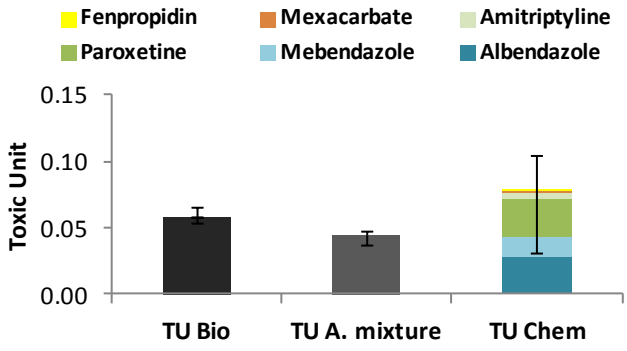

Figure 3. The biological toxic unit (TU_{Bio}) determined for raw sample obtained with size increase, the toxic unit ($TU_{A.\ mixture}$) determined for the artificial mixture of the 6 targeted compounds at the same concentration as they have been detected in the toxic fraction (see Table 3) and the arithmetic sum toxic units (TU_{Chem}) of the chemicals tested individually (albenzadole, paroxetine, mebendazole, amitriptyline, mexacarbate and fenpropidin).

The high contribution of albendazole (49%) and mebendazole (25%) is in agreement with their specific MoA. They are benzimidazoles extensively used as an anthelmintic agent to

treat parasitic infections of humans and animals [35]. Adults and larvae are affected by depolymerisation of microtubules[36], a process that plays an essential role in sea urchin embryos since this process is involved in many cellular processes such as cell division during early embryogenesis, intracellular transport and four arm-pluteus stage shape maintenance[37–39]. For instance, Stepanov et al.[40] evaluated the microtubule-destabilizing properties of a series of benzimidazole drugs and reported alterations in swimming pattern of blastulae treated after hatching indicating the possible underestimation of risk for sea urchins whichs are not usually considered in biomonitoring campaigns.

The antidepressant, paroxetine showed a high biological activity (TU_{Chem}= 0.03), being one of the main contributor (49%) of the observed sea urchin embryogenesis. The contribution (9%) of amitriptyline, with a TU_{Chem}=0.005, can be interpreted by its high effluent concentration (304 ng/L), an order of magnitude higher than the other compounds (17-65 ng/L). These neuroactive antidepressants have been reported to be toxic for crustaceans[5,41] and, for zebrafish[42]. Among other alterations, it was demonstrated to alter the swimming behavior and body length of *Danio rerio* embryos[43]. However, this is the first time that the potential toxicity of amitriptyline to the observed toxicity on sea urchin embryos has been evaluated.

The present study confirms sea urchin embryos bioassay as a powerful and novel bio-diagnostic tool for the detection and identification of drivers of toxicity in complex emissions using effect-directed analysis. This approach revealed so far hardly monitored and investigated drugs driving risks on marine invertebrates such as anthelmintic agents. Although designed for a better assessment of risks to marine communities, the results with sea urchin embryos presented here gives a first indication that anthelmintic agents into monitoring and assessment should be also considered in the monitoring of freshwater environments.

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ASSOCIATED CONTENT

SUPPORTING INFORMATION

The following files are available free of charge

- SM1: Reagent and materials, sampling points details, Table S1, Table S2, SET procedure, EDA and fractionation, Figure S1, Table S3, LC-q-Orbitrap analysis details, Figure S2, Table S4, EDA-SET, Figure S3, Figure S4, Figure S5, Figure S6, Table S5, Figure S7 and references (PDF).

- SM2: Reagents information (S1); Information of the set compounds used to calibrate the fractionation (S2), compounds identified in the raw sample as level 1, 2a and 3 according to the criteria of Schymanski[34] (S3) (MS Excel)

AUTHOR INFORMATION

Corresponding Author

*Phone: + 34 94 601 55 51; fax: +34 94 601 35 00; e-mail: leire.mijangos@ehu.eus; ORCID:

Leire Mijangos: 0000-0003-1872-4624

Notes The authors declare no competing financial interest

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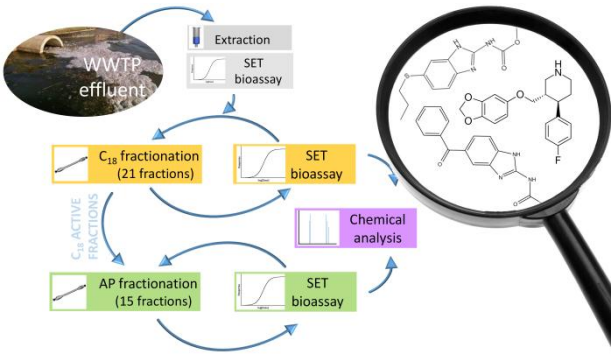

Graphical abstract. For Table of Contents Only

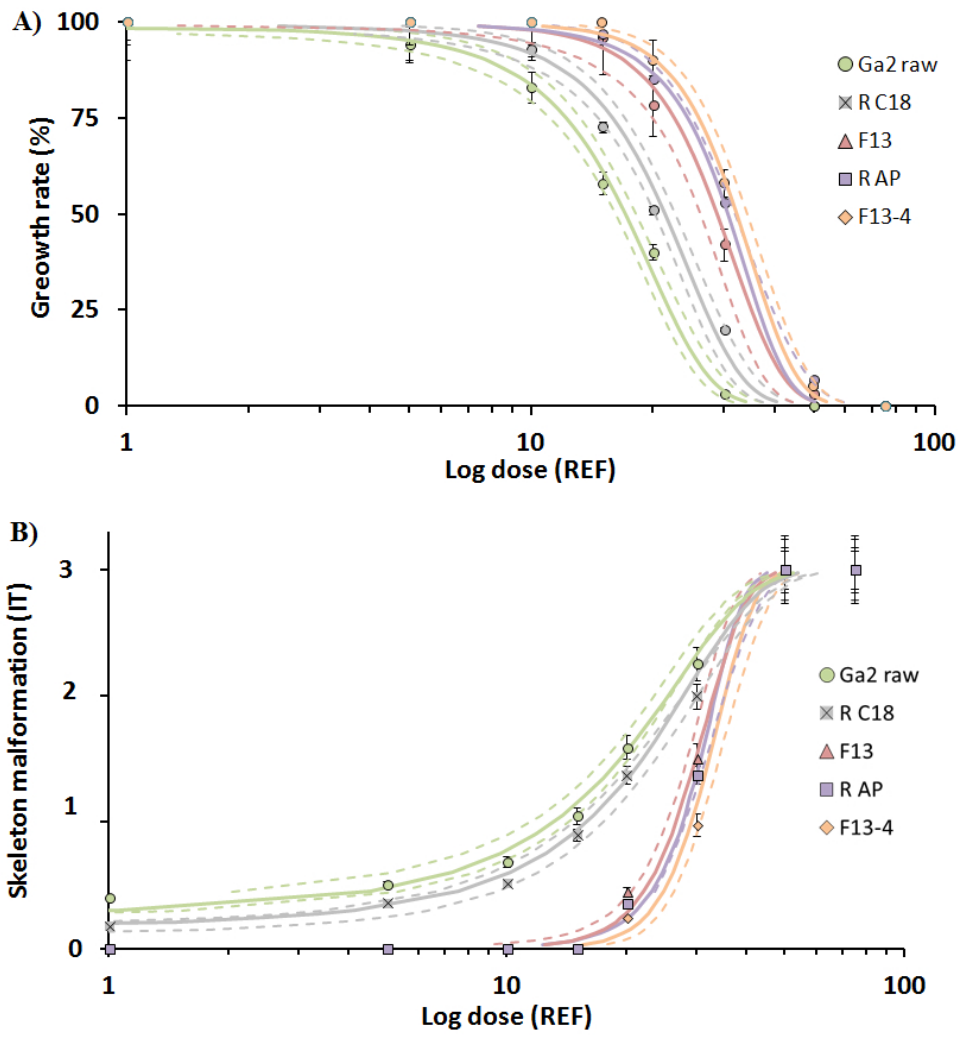

Figure 1. The log dose-response curves of the tested effluents samples (Gernika, Mungia, Ga2, Ga3 and Gorliz) obtained with A) size increase end-point and B) skeleton malformation end-point. Straight lines show the EC fit values obtained with probit and dashed line the confidence level (95%).

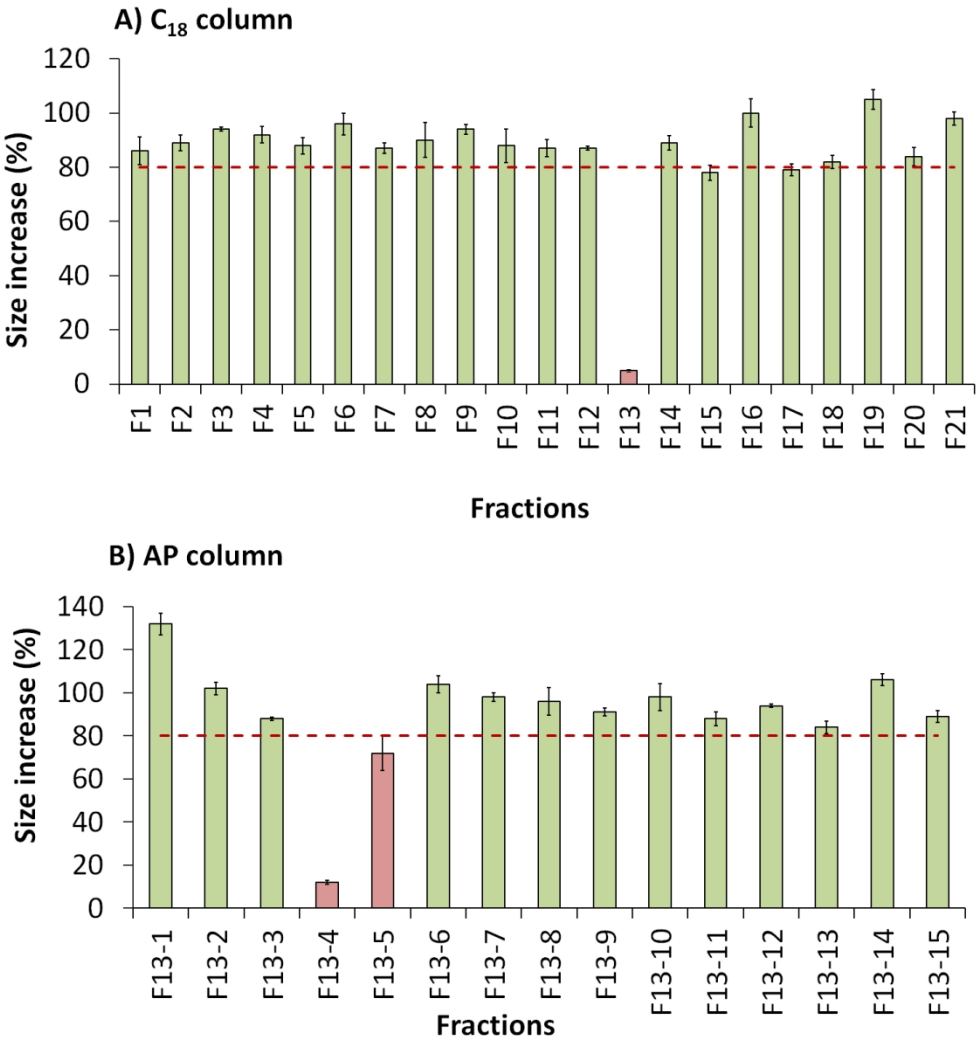

Figure 2. Size increase (%) response of the single fractions obtained with a) C18 column, F11-F121 and b) aminopropyl (AP) column, fraction F13-1 to F13-15. Red bars represent the identified active fractions. Fractions with the mean value under the dashed line at 80 % are defined as active. All the fractions are at REF 75.

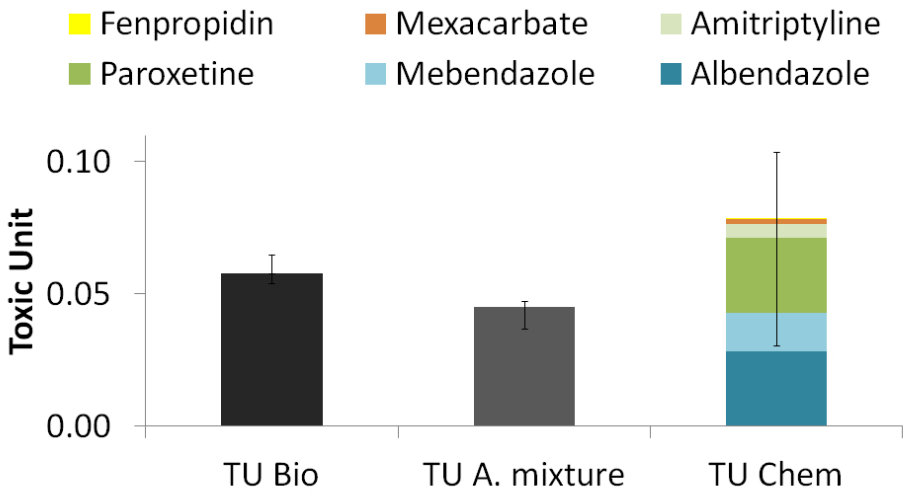

Figure 3. The biological toxic unit (TUBio) determined for raw sample obtained with size increase, the toxic unit (TUA. mixture) determined for the artificial mixture of the 6 targeted compounds at the same concentration as they have been detected in the toxic fraction (see Table 3) and the arithmetic sum toxic units (TUChem) of the chemicals tested individually (albenzadole, paroxetine, mebendazole, amitriptyline, mexacarbate and fenpropidin).

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