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

Supplementary material

Leire Mijangos[a, b]*, Martin Krauss[d,e], Laura de Miguel[b, c], Haizea Ziarrusta[a, b], Maitane Olivares[a, b], Olatz Zuloaga[a, b], Urtzi Izaguirre[b, c], Tobias Schulze[d,e], Werner Brack[d,e], Ailette Prieto[a, b], Nestor Etxebarria[a, b]

[a]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

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

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

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

[e] Institute for Environmental Research, RWTH Aachen University, 52074 Aachen, Germany

Supplementary Material 1 is formed by 22 pages which include the following information:

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FIGURES

Figure S5. MS^2 spectra (HCD 10, 35 and 75) of a) albendazole, b) mebendazole, c) amitriptyline, d) fenpropidin e) paroxetine and d) fragments explanation of two potential candidates (mexacarbate and neostigmine) which match with the precursor ion. Only the mayor fragments have been included (rounded).

Figure S6. The log dose-response curves of the identified chemicals tested individually: a) paroxetine, b) albendazole, c) mebendazole, d) mexacarbate, e) amitriptyline and f) fenpropidin. Straight lines show the EC fit values obtained with Probit and dashed line the confidence level (95%).

Figure S7. The log dose-response curves of the artificial mixture of the identified 6 chemicals tested all together. Straight lines show the EC fit values obtained with Probit and dashed line the confidence level (95%).

S1. Reagents and materials

Names, use, CAS numbers, molecular formulas and brand target compounds are summarized in **S1 supplementary material (SM) 2**. All the reference standards used in this study have a purity of at least 97%.

Oasis hydrophilic-lipophilic balanced (HLB) 200 mg-SPE cartridges were purchased from Waters (Milford, USA). Bond-Elut Plexa and Strata X-AW bulk sorbents used in the effect-directed analysis approach were purchased to Agilent (Santa Clara, CA, USA) and Phenomenex (Torrance, CA, USA), respectively. Empty solid phase extraction (SPE) tubes (6 mL and 20 mL) and polypropylene (PP) frits were purchased from Supelco (Bellefonte, PA, USA).

Formic acid (> 98%), ethylenediaminetetraacetic sodium salt (EDTA≥99.9%) and sodium thiosulfate (≥ 98) were supplied by Panreac (Barcelona, Spain). Methanol (MeOH, HPLC grade, 99.9%), ethyl acetate (EtOAC, HPLC grade, 99.9%), acetone (HPLC grade, 99.9%) and ammonium solution (25% as NH_3) used in the SPE extraction were obtained from Sigma Aldrich (St. Louis, MO, USA). Ultra-pure water was obtained using a Milli-Q water purification system (<0.05 S/cm, Milli-Q model 185, Millipore, Bedford, MA, USA). Dimethyl sulfoxide (DMSO, cell culture grade) used in the bioassays was supplied by Panreac.

LC-MS grade MeOH, water and formic acid (Optima grade) purchased from Sigma Aldrich were used as mobile phase in the fractionation, whereas Optima grade water, acetonitrile, isopropanol and formic acid provided by Fischer Scientific (Geel, Belgium) were used as mobile phase in the LC-HRMS.

S2. Sampling.

Table S1. Name, location, treatment, effluents discharge estuaries, water flow and influents sources of the WWTPs studied in this work.

WWTP	Coordinates	Treatment	Effluents discharge estuaries	Median water flow (m^3/day)	Influent sources
Galindo	-2.97103 W, 43.28796 N	2nd (Ga2)	Bilbao estuary	1.0e9	Industrial 3.2%, Hospital 0.5%, Domestic 96.3% (> 1000000 inhabitant)
		3rd chlorination (Ga3)	-[a]		
Gorliz	-2.94244W, 43.41229N	2nd	Plentzia estuary It releases the effluent into the estuary mouth through a submarine pipe located to 1000 m from the coast with an 18 m depth.	1.4e6	Industrial 0%, Hospital 1.3%, Domestic 98.7% (10600 inhabitants)
Mungia		2nd	Plentzia estuary It releases the effluent into the upper part (22 km with to respect the mouth) of Plentzia estuary	5.4e3	Industrial 3.1%, Hospital 0%, Domestic 96.9% (17000 inhabitants)
Gernika	-2.6739 W, 43.3239 N	1st	Urdaibai estuary. It discharges directly to the estuary of Urdaibai, which is declared Reserve of The Biosphere by Unesco since 1984.	-[b]	Industrial 25.33%, Hospital 0.2%, Domestic 74.46% (70000 inhabitants)

a) Currently, it is only for private use in the WWTP

b) Unknown (but <10% of the total flow)

Table S2. Water flow and effluent physicochemical parameters from Galindo, Gorliz and Mungia WWTPs

WWTP	Flow m^3/day	pH	TSS mg/L	VSS mL/L	COD mg/L	BOD mg/L	NH_3 mg/L	NO_3^- mg/L	PO_4^{3-} mg/L	Conductivity (µS/CM)
Galindo	305572	7.10	<6	4.00	37.00	<4	<0,50	7.42	3.89	3,670.00
Gorliz	3404	7.40	16.00	<3	51.00	4.80	7.51	12.70	2.88	1,270.00
Mungia	6161	7.50	6.00	<3	43.00	<4	0.58	0.64	0.53	531.00

TSS: total suspended solid; VSS: volatile suspended solids, COD: chemical oxygen demand; BOD: Biological oxygen demand after 5 days;.

S3. 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 photoperiod. Every two days sea urchins were fed with macroalgae and dregs were siphoned.

Gametes were obtained by osmotic-shock-induced spawning injecting 1 mL of potassium chloride (KCl, 0.5 mol/L) through the peri-oral membrane into coelom[1]. Afterwards, gametes were observed under a microscope to check their viability (eggs roundness and sperm mobility) and the viable ones were pooled.

The fertilisation procedure was carried out as described by Fernández and Beiras [2]. A dense suspension of oocytes in control FSW was fertilised with a few µL of non-diluted sperm. 20 µL-aliquots (n=4) were taken to record fertilisation success (assessed by the percentage of eggs showing a fertilisation membrane) and egg density through an inverted microscope (Nikon eclipse Ti-S). Eggs were counted using a Sedgewick-rafter counting cell (Pyser Optics, Edenbridge, United Kingdom). Within 30 minutes, the fertilised egg suspension was distributed in glass vials (20 mL) containing a known volume of test sample (3 mL), assuring a final concentration of 40 eggs/mL. Afterwards, fertilized sea urchin embryo egg were added to test samples 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 a one drop per sample of 40% formalin.

S4. EDA and fractionation

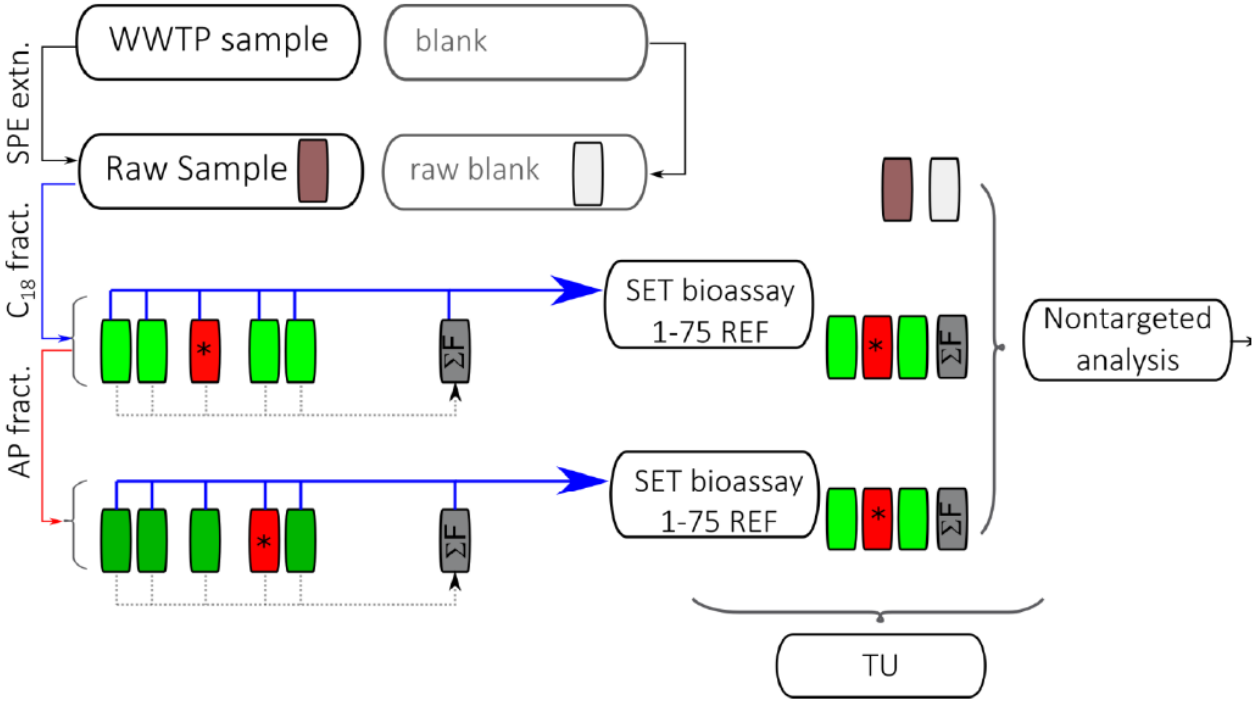


Figure S1. Schematic representation of the experimental design of the effect-directed analysis approach. ΣF, recombined fractions; AP fract., fractionation with aminopropyl column; C_{18} fract., fractionation with C_{18} column; REF, relative enrichment factor; SET, sea urchin embryo toxicity; SPE, solid phase extraction; TU, toxic unit.

Table S3. Fraction names, elution time windows and water content of the resulting fractions after the consecutive fractionation performed with two columns (Nucleodur C_{18} gravity and Imtakt aminopropyl).

Fraction	Fractionation approaches					
	1st fractionation step Nucleodur C_{18} Gravity column			2nd fractionation step Imtakt aminopropylcolumn		
	Name	Fraction RT[a] (min)	Water content (%)	Name	Fraction RT[a] (min)	Water content (%)
1	F1	0-2	70	F13-1	0-3	92
2	F2	2-4	70	F13-2	3-6	84
3	F3	4-6	68	F13-3	6-9	77
4	F4	6-8	64	F13-4	9-12	69
5	F5	8-10	60	F13-5	12-15	61
6	F6	10-12	56	F13-6	15-18	53
7	F7	12-14	52	F13-7	18-21	45
8	F8	14-16	48	F13-8	21-24	37
9	F9	16-18	44	F13-9	24-27	29
10	F10	18-20	40	F13-10	27-30	21
11	F11	20-22	36	F13-11	30-33	13
12	F12	22-24	32	F13-12	33-36	6
13	F13	24-26	28	F13-13	36-39	5
14	F14	26-28	24	F13-14	39-42	5
15	F15	28-30	20	F13-15	42-45	5
16	F16	30-32	16			
17	F17	32-34	12			
18	F18	34-36	10			
19	F19	36-39	5			
20	F20	39-42	5			
21	F21	42-50	5			

[a] The fraction collector was started with a delay of 4 min.

S5. Non-targeted analysis

The analysis were performed in a Thermo Scientific Dionex UltiMate 3000 UHPLC coupled to a Thermo Scientific Q Exactive quadrupole-Orbitrap mass spectrometer equipped with a heated ESI source (HESI, Thermo, CA, USA).

The separation was carried out at 0.3 mL/min and 35 °C of flow rate and temperature, respectively, on an ACE UltraCore 2.5 SuperPhenylhexyl (2.1 mmx 100 mm, 2.5 µm) column coupled to a pre filter (Vivi Jour, Schenkon, Suitzlerdan) from Waters (Milford, Massachusetts, United States). Milli-Q water was used as mobile phase A and acetonitrile as mobile phase B, both containing 0.1% formic acid. The injection volume was set to 5 µL. The eluent gradient profile was as follows: linear change of 85% A to 70% up to 4 min, another linear change to 50% A up to 4 min (hold 12 min), another linear change to 10% A up to 10 min (hold 15 min) and a final linear change to 85% A up to 3 min. Lastly, 5 min to regain initial conditions.

The Orbitrap was operated in the corresponding ionization mode in full scan – data dependant MS^2 (Full MS-ddMS^2) discovery acquisition mode. One full scan at a resolution of 70,000 full width at half maximum (FWHM) at m/z 200 over a scan range of m/z 70-1000 was followed by three ddMS^2 scans at a resolution of 17,500 FWHM at m/z 200, with an isolation window of 0.8 Da. The stepped normalized collision energy (NCE) in the higher-energy collisional dissociation (HCD) cell was set to 10, 35 and 75 eV. Negative and positive voltages were measured in different injections runs. The HESI source parameters in positive mode were set to 3.2 kV spray voltage, 300 °C capillary temperature, 35 arbitrary units (au) sheath gas (nitrogen), 10 au auxiliary gas, 1 au sweep gas, 280 °C auxiliary gas heater and S-lens RF level 55.0. The HESI source parameters in negative mode were set to 3.2 kV spray voltage, 330 °C capillary temperature, 48 au sheath gas, 11 au auxiliary gas, 2 au sweep gas, 310 °C auxiliary gas heater and S-lens RF level 55.0. External calibration of the instrument was conducted immediately prior to analysis using Pierce LTQ ESI Calibration Solutions (Thermo Scientific, Waltham, Massachusetts, United States). The instrument was controlled by Xcalibur 4.0 software (Thermo).

Data analysis was done using Compound Discoverer 2.1 (CD; Thermo-Fisher Scientific). The workflow and settings used for the data evaluation are summarized in **Figure S2** and **Table S4** in **SM1**

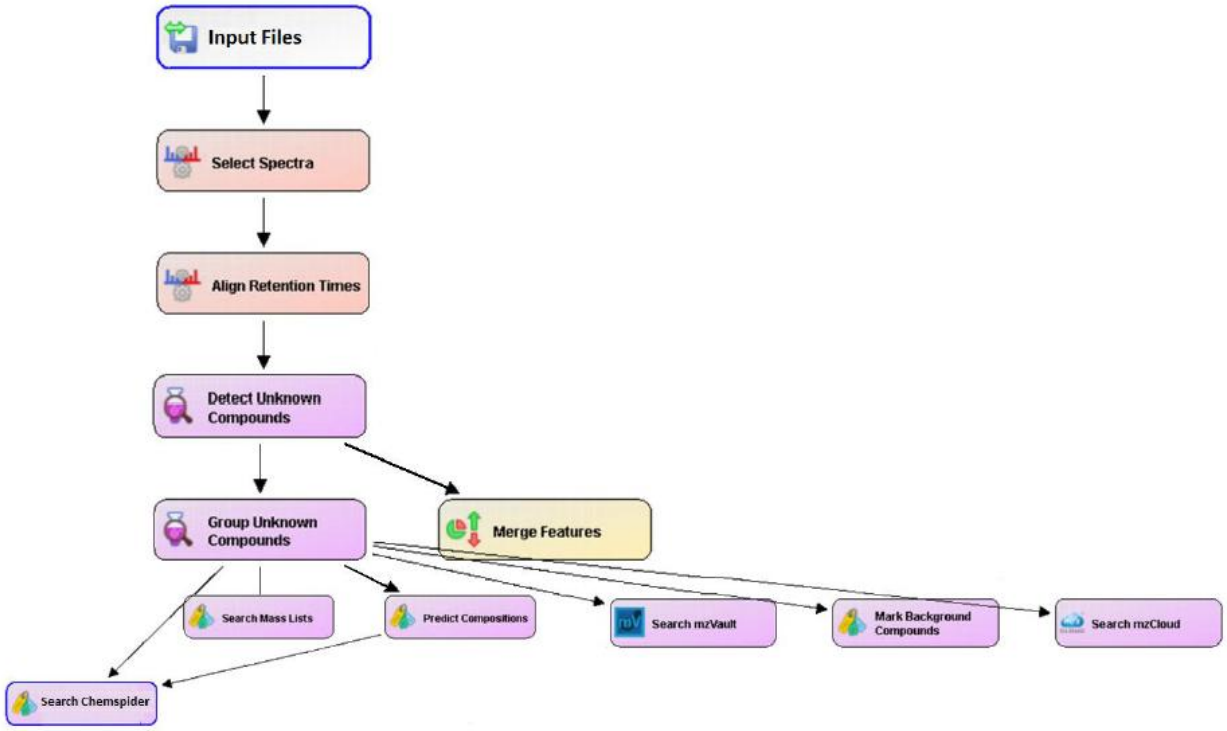


Figure S2 Compound Discoverer (2.1) workflow

Table S4. Compound Discoverer (2.1) workflow settings and parameters

1. Select Spectra	1.1 General settings	- Precursor Selection: Use MS (N - 1) Precursor - Use New Precursor Reevaluation: True - Use Isotope Pattern in Precursor Reevaluation: True - Store Chromatograms: False
	1.2 Spectrum properties Filter	- Lower RT Limit: 0 - Upper RT Limit: 0 - First Scan: 0 - Last Scan: 0 - Ignore Specified Scans: (not Specified) - Lowest Charge State: 0 - Highest Charge State: 0 - Min. Precursor Mass: 100 Da - Max. Precursor Mass: 5000 Da - Total Intensity Threshold: 0 - Minimum Peak Count: 1

Table S4. Compound Discoverer (2.1) workflow settings and parameters

	1.3 Scan event Filters	- Mass Analyzer: (not Specified) - MS Order: Any - Activation Type: (not Specified) - Min. Collision Energy: 0 - Max. Collision Energy: 1000 - Scan Type: Any - Polarity Mode: (not Specified)
1. Select Spectra	1.4 peak filters	- S/N Threshold (FT-only): 1.5
	1.5. Replacements for Unrecognized Properties	- Unrecognized Charge Replacements: 1 - Unrecognized Mass Analyser Replacements: ITMS - Unrecognized MS Order Replacements: MS^2 - Unrecognized Activation Type Replacements: CID - Unrecognized Polarity Replacements: + - Unrecognized MS Resolution@200 Replacements: 60000 - Unrecognized MSn Resolution@200 Replacements: 30000
2. Align Retention times	*2.1. General Settings*	- Alignment Model: Adaptive curve - Alignment Fallback: Use Linear Model - Maximum Shift [min]: 2 - Shift Reference File: True - Mass Tolerance: 5 ppm - Remove Outlier: True
3. Detect Unknown Compounds	*3.1. General Settings*	- Mass Tolerance [ppm]: 5 ppm - Intensity Tolerance [%]: 30 - S/N Threshold: 3 - Min. Peak Intensity: 500000 - Ions: [M+Cl]-1; [M+FA-H]-1; [M+H]+1; [M+H+MeOH]+1; [M+K]+1; [M+Na]+1; [M-H]-1; [M-H-H2O]-1 - Base Ions: [M+H]+1; [M-H]-1 - Min. Element Counts: C H - Max. Element Counts: C90 H190 Br3 Cl4 F20 K2 N10 Na2 O18 P3 S5
	3.2. Peak Detection	- Filter Peaks: True - Max. Peak Width [min]: 0.8 - Remove Singlets: True - Min. # Scans per Peak: 3 - Min. # Isotopes: 1
4. Merge Features	*4.1 Peak consolidation*	-mass tolerance: 5 ppm - RT Tolerance 0.1 min
5. Group Unknown Compounds	*5.1. Compound Consolidation*	- Mass Tolerance: 5 ppm - RT Tolerance [min]: 0.5
	5.2. Fragment Data Selection	- Preferred Ions: [M+H]+1; [M-H]-1
6 Search ChemSpider	*6.1. Search Settings*	Database(s): ACToR: Aggregated Computational Toxicology Resource; DrugBank; EAWAG BIOcatalysis/Biodegradation Databse; EPA DSSTox; EPA Toxcast; FDA UNII-NLMBioCyc; KEGG; Mass Bank - Mass Tolerance: 5 ppm - Max. # of results per compound: 100 - Max. # of Predicted Compositions to be searched per Compound: 3 - Result Order (for Max. # of results per compound): Order By Reference Count (DESC)
	6.2. Predict Composition	- Check All Predicted Compositions: True

Table S4. Compound Discoverer (2.1) workflow settings and parameters

7. Search Mass Lists	*7.1. Search Settings*	- Input file(s): \EFS HRAM Compound Database_OZZ.csv - Show extra Fields as Columns: False - Consider Retention Time: True - RT Tolerance : 0.5 - Mass Tolerance: 5 ppm
8.Predict Composition	*8.1. Prediction Settings*	Mass Tolerance: 5 ppm - Min. Element Counts: C H - Max. Element Counts: C90 H190 Br3 Cl4 F20 K2 N10 Na2 O18 P3 S5 - Min. RDBE: 0 - Max. RDBE: 40 - Min. H/C: 0.1 - Max. H/C: 3.5 - Max. # Candidates: 10 - Max. # Internal Candidates: 200
	8.2. Pattern Matching	Intensity Tolerance [%]: 30 - Intensity Threshold [%]: 0.1 - S/N Threshold: 3 - Min. Spectral Fit [%]: 30 - Min. Pattern Cov. [%]: 80 - Use Dynamic Recalibration: True
	8.3. Fragments Matching	- Use Fragments Matching: True - Mass Tolerance: 5 ppm - S/N Threshold: 3
9. Seach mzVault	*9.1 Seach settings*	- mzVault Library: \mzVault February 2017.db - Compound Classes: All - Match Ion Activation Type: True - Match Ion Activation Energy: Match with Tolerance - Ion Activation Energy tolerance: 20 - Match Ionization Method: True - Apply Intensity Method: true - Remove precursor Ion: true - Precursor Mass Tolerance: 10 ppm - FT Fragment Mass Tolerance: 10 ppm - IT Fragment mass tolerance: 0.4 Da - Match Analyzer Type: True - Search Algorithm: HighChem HighRes - Match factor Threshold: 50 - Max. # results: 10
10. Mark BackGround compounds	*10.1 Seach settings*	- Max. Sample/Blank: 5 - Max Max. Blank/Sample: 0 - Hide Background: True

Table S4. Compound Discoverer (2.1) workflow settings and parameters

11. Search mzCloud	*11.1. Search Settings*	- Compound Classes: All - Match Ion Activation Type: True - Match Ion Activation Energy: Match with Tolerance - Ion Activation Energy Tolerance: 20 - Apply intensity threshold: True - Precursor Mass Tolerance: 10 ppm - FT Fragment Mass Tolerance: 10 ppm - IT Fragment Mass Tolerance: 0.4 Da - Search Algorithm: Cosine - Similarity Search: Similarity Forward --Library: Reference - Post Processing: Recalibrated - Match factor threshold: 50 - Max. # results per compound and spectrum: 20

S6. EDA-SET

Figure S3a-l in SM1 show representative malformations observed for the tested effluents in this work and the **Figure S4** the modelled dose-response curves for the identified toxic samples (Raw, R_{C18}, F13, R_{AP} and F13-4) in EDA approach. **Figure S5a-e** shows the MS^2 spectra of albendazole, mebendazoleamitriptyline, fenpropidin and paroxetine, respectively.

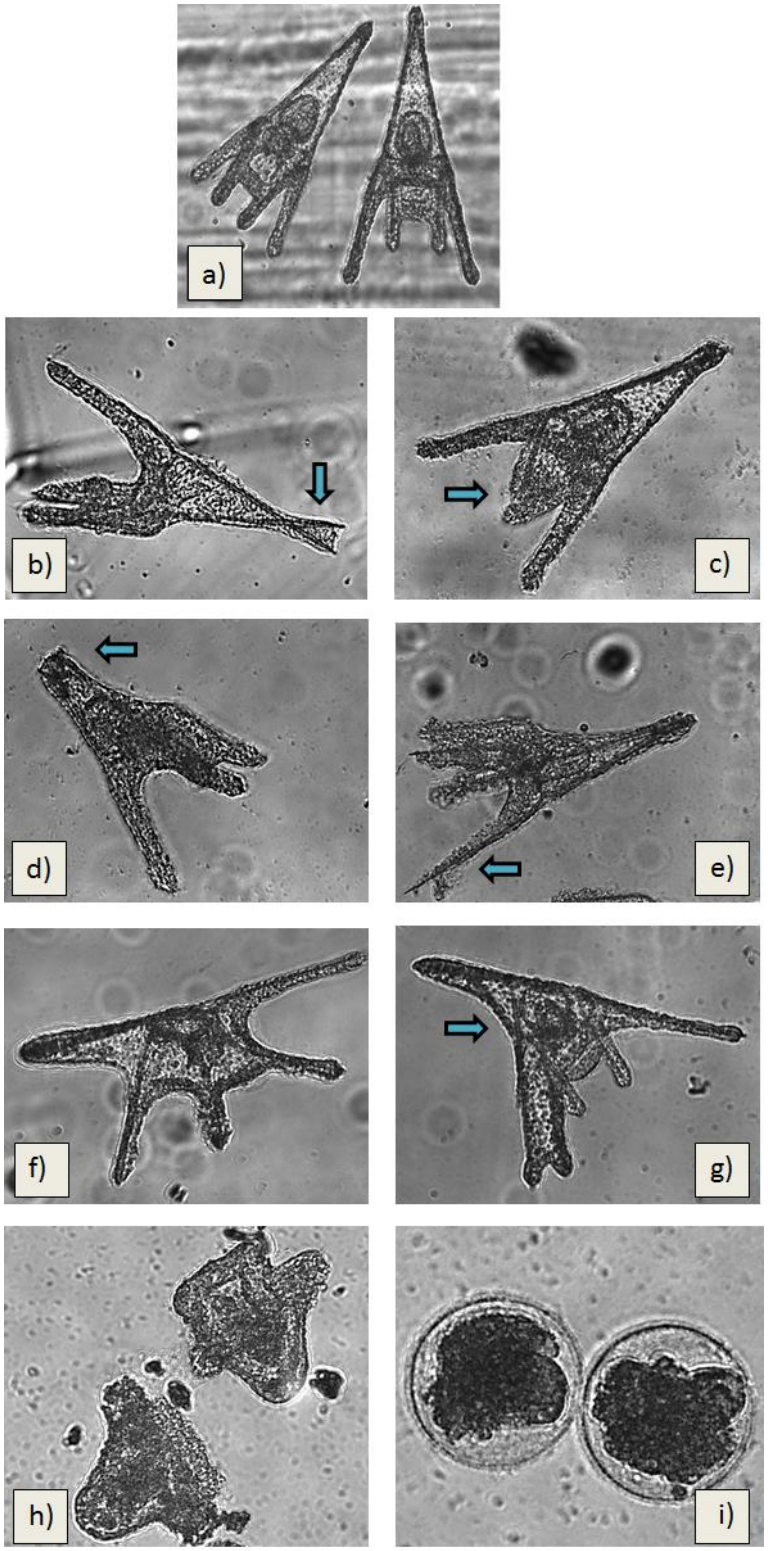


Figure S3 *Types of embryonic stages and developmental abnormalities of sea urchin Paracentrotus lividus observed in this study after 48 h incubation period. a) normal 4 arm pluteus stage (level 0); b) crossed tip (level 1); c) fused arms (level 1); d)separated tip (level 1); e) incomplete skeletal rods (level 2); f) absence of skeletal rods (level 2); g) folded tip (level 2); h) pre-pluteus stage; i) Undeveloped stage.*

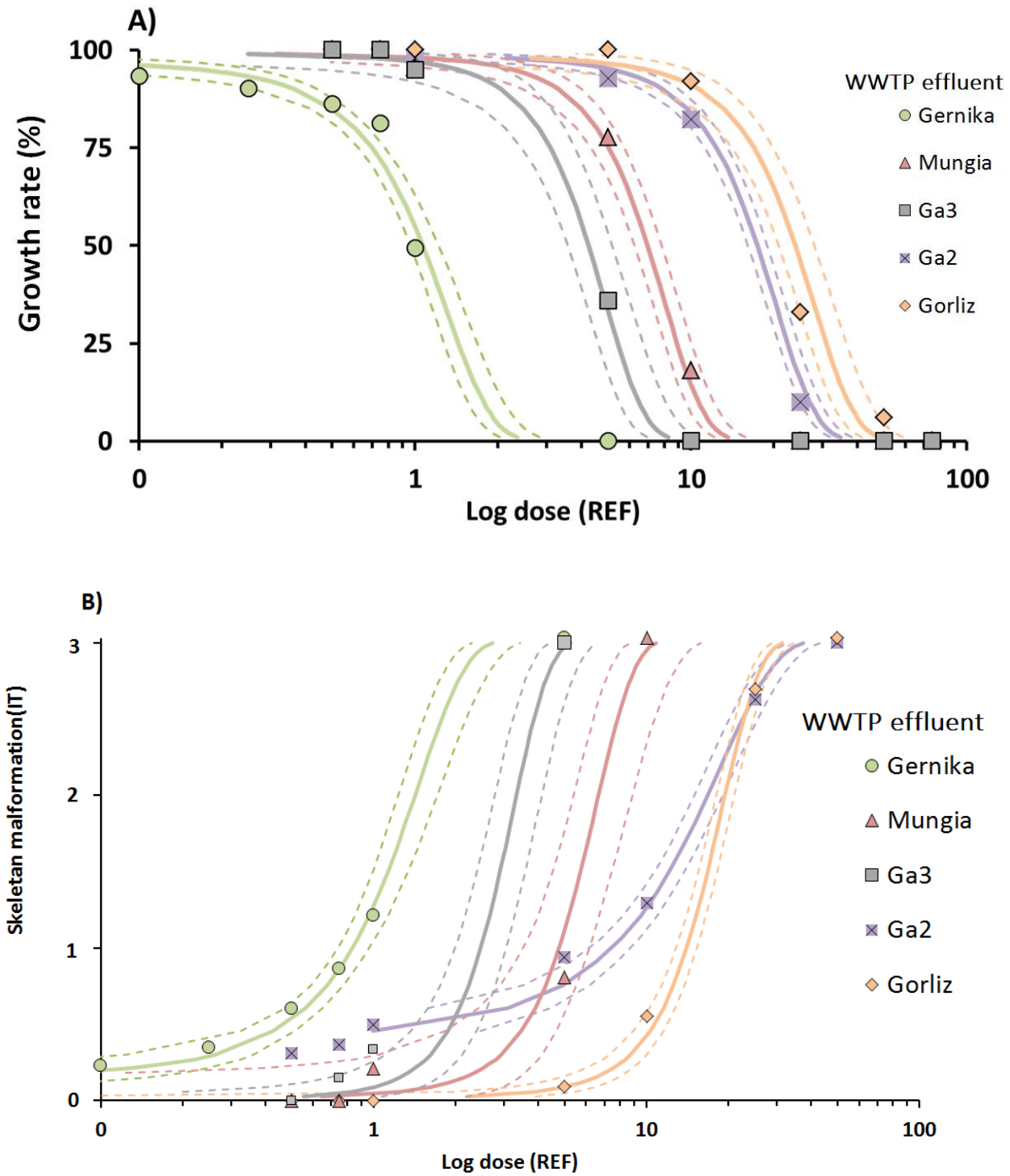
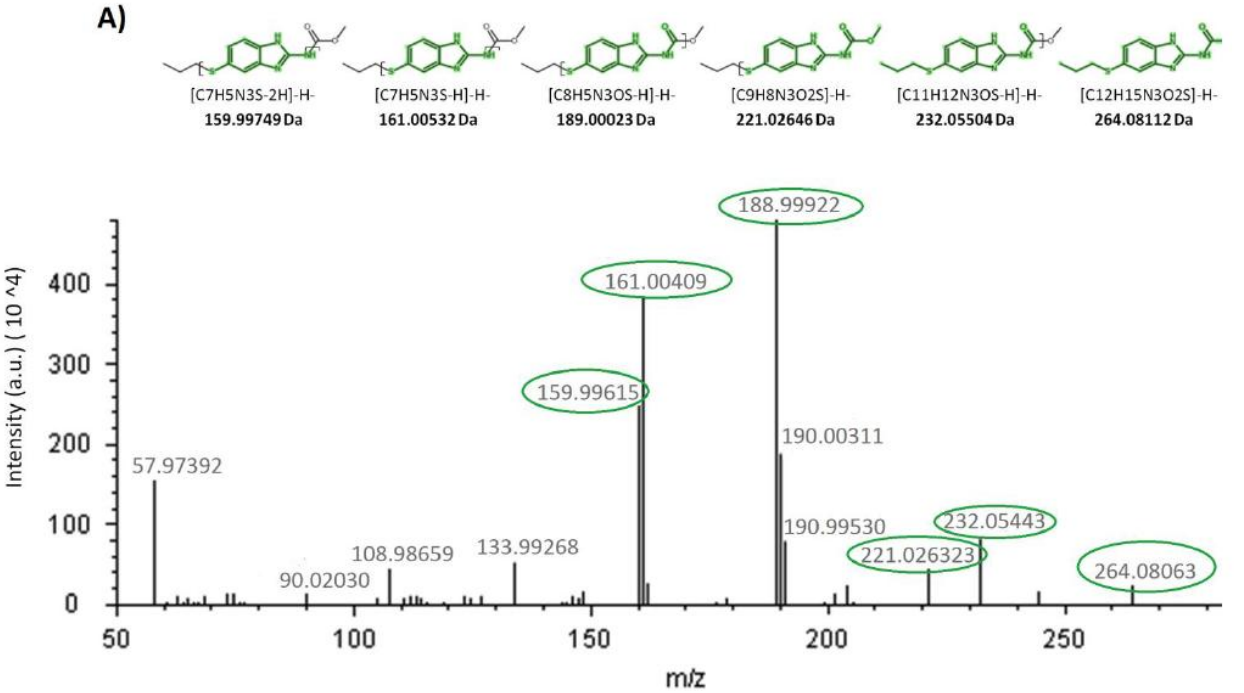
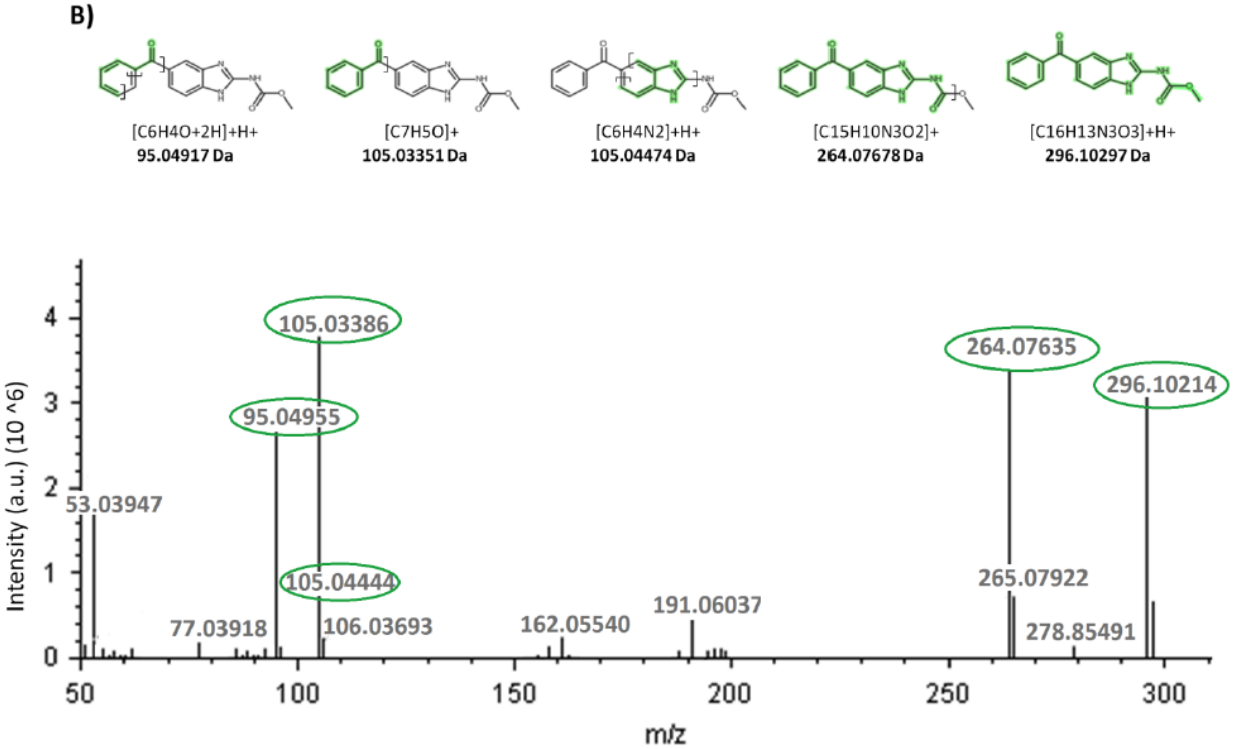
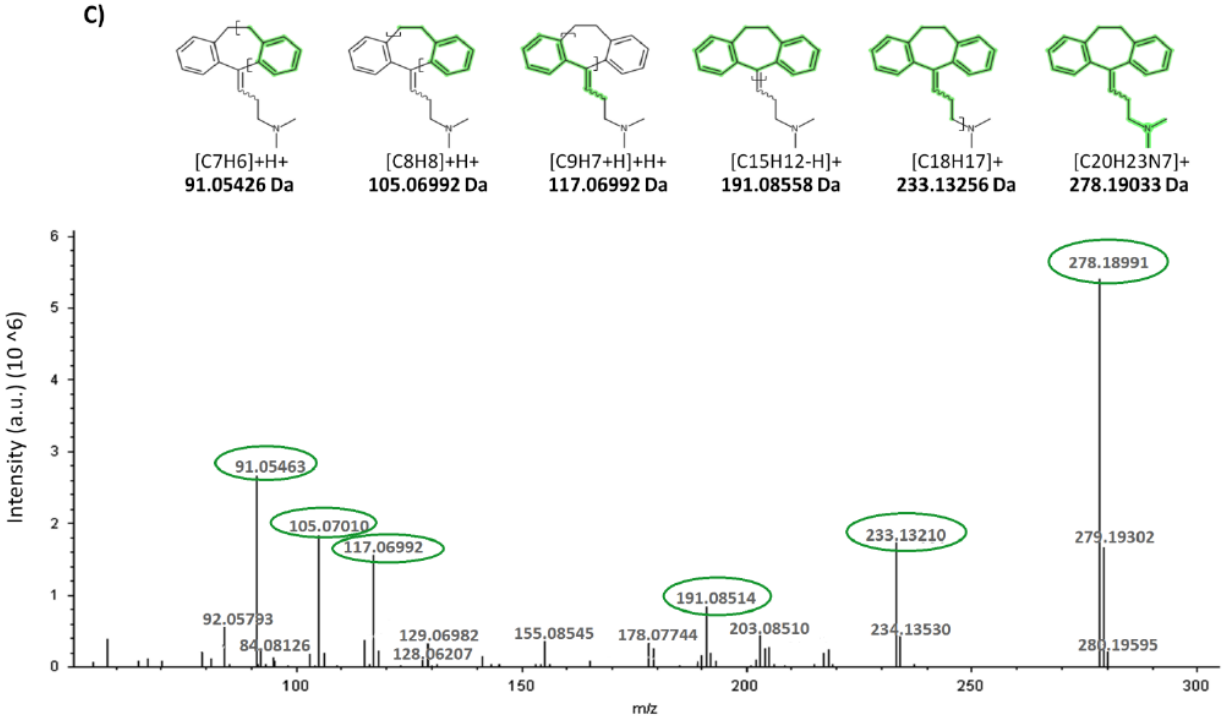


Figure S4. The log dose-response curves of the active samples (Ga2 raw, R_{C18}, $F1_{13}$, R_{AP} and $F2_4$) obtained with a) size increase end-point and b) skeleton malformation end-point. Straight lines show the EC fit values obtained with Probit and dashed line the confidence level (95%). For interpretation of colored legend in these figures, the reader is referred to the web version of this article.

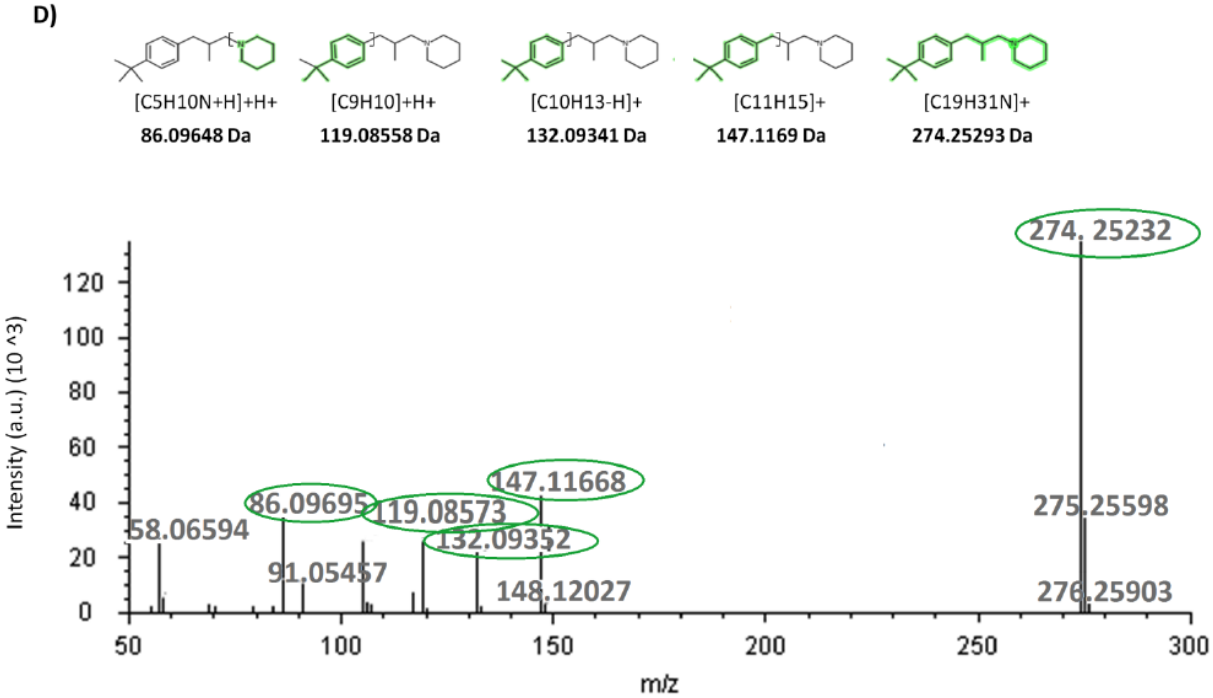

A)
[C7H5N3S-2H]-H-
159.99749 Da
[C7H5N3S-H]-H-
161.00532 Da
[C8H5N3OS-H]-H-
189.00023 Da
[C9H8N3O2S]-H-
221.02646 Da
[C11H12N3OS-H]-H-
232.05504 Da
[C12H15N3O2S]-H-
264.08112 Da
Intensity (a.u.) (10 ^4)
m/z
188.99922
161.00409
159.99615
190.00311
57.97392
190.99530
232.05443
221.026323
264.08063
108.98659
133.99268
90.02030


B)
[C6H4O+2H]+H+
95.04917 Da
[C7H5O]+
105.03351 Da
[C6H4N2]+H+
105.04474 Da
[C15H10N3O2]+
264.07678 Da
[C16H13N3O3]+H+
296.10297 Da
Intensity (a.u.) (10 ^6)
m/z
105.03386
264.07635
296.10214
95.04955
53.03947
105.04444
265.07922
191.06037
77.03918
106.03693
162.05540
278.85491

C)


[C7H6]+H+
91.05426 Da
[C8H8]+H+
105.06992 Da
[C9H7+H]+H+
117.06992 Da
[C15H12-H]+
191.08558 Da
[C18H17]+
233.13256 Da
[C20H23N7]+
278.19033 Da
Intensity (a.u.) (10 ^6)
278.18991
91.05463
105.07010
117.06992
233.13210
279.19302
191.08514
92.05793
84.08126
129.06982
128.06207
155.08545
178.07744
203.08510
234.13530
280.19595
m/z

D)


[C5H10N+H]+H+
86.09648 Da
[C9H10]+H+
119.08558 Da
[C10H13-H]+
132.09341 Da
[C11H15]+
147.1169 Da
[C19H31N]+
274.25293 Da
Intensity (a.u.) (10 ^3)
274. 25232
147.11668
86.09695
119.08573
58.06594
132.09352
91.05457
148.12027
275.25598
276.25903
m/z

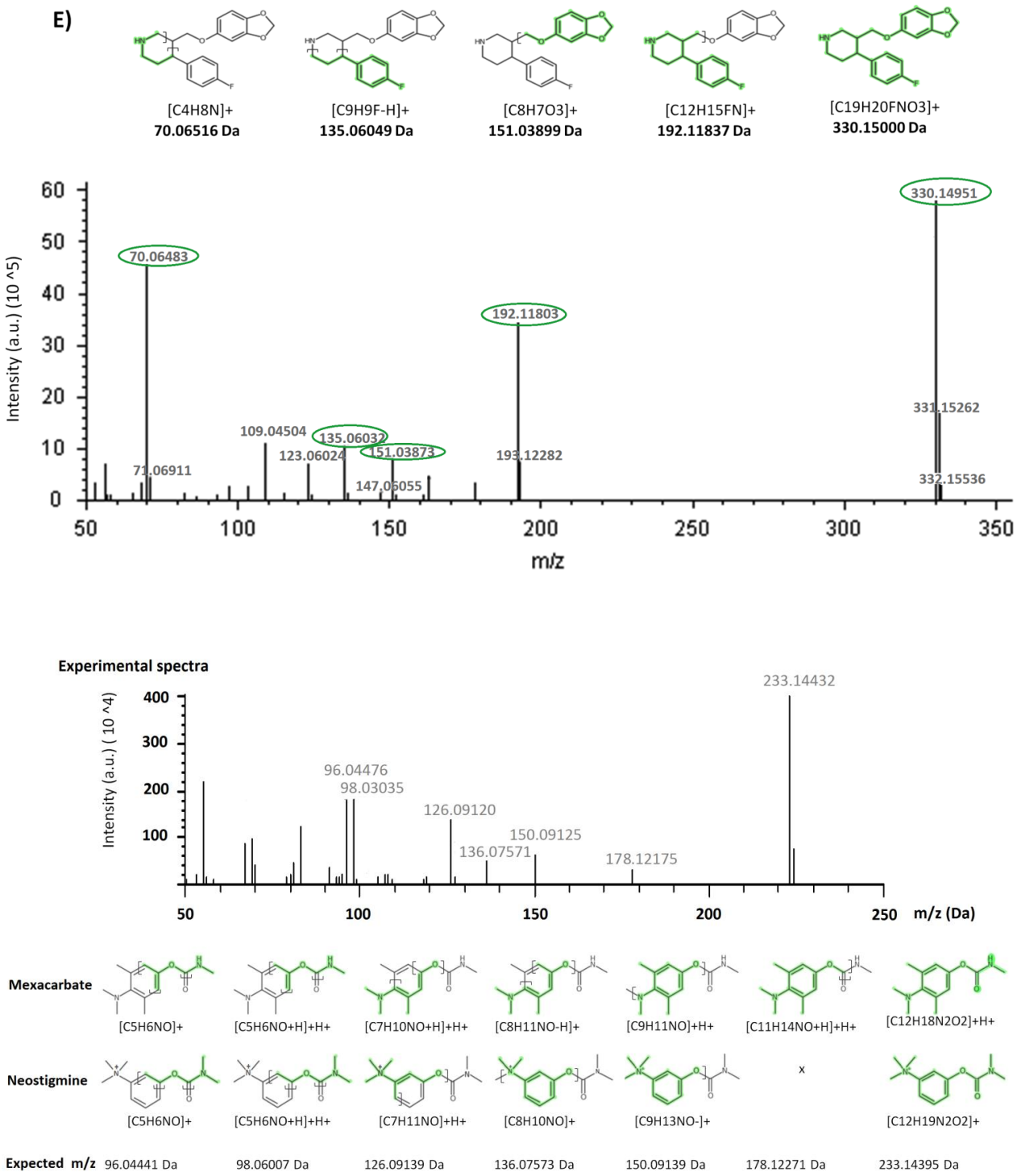


Figure S5 MS^2 spectra (HCD 10, 35 and 75) of a) albendazole, b) mebendazole, c) amitriptyline, d) fenpropidin e) paroxetine and d) fragments explanation of two potential candidates (mexacarbate and neostigmine) which match with the precursor ion. Only the mayor fragments have been included (rounded).

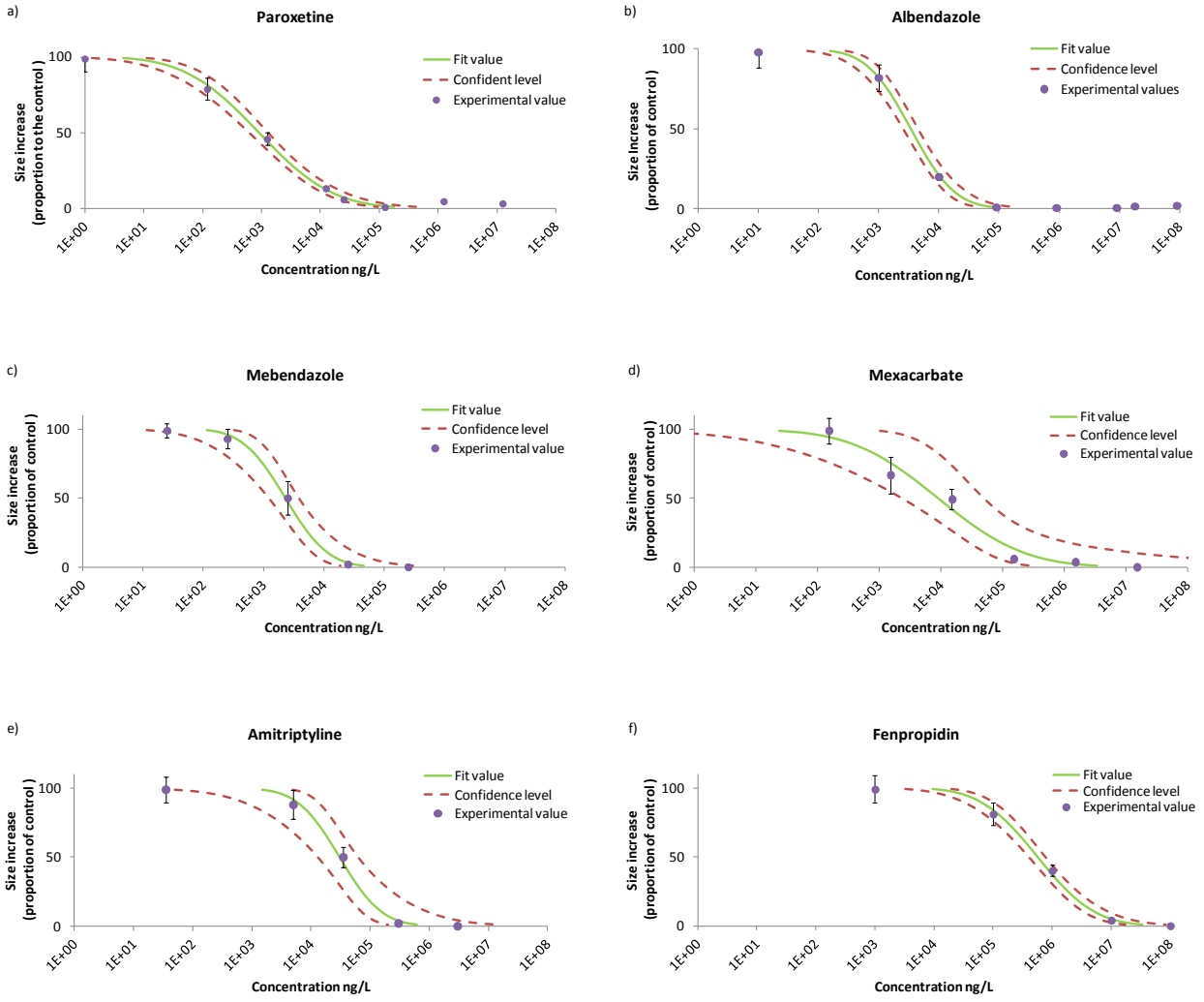


Figure S6 The log dose-response curves of the identified chemicals tested individually: a) paroxetine, b) albendazole, c) mebendazole, d) mexacarbate e) amitriptyline and f) fenpropidin. Straight lines show the EC fit values obtained with Probit and dashed line the confidence level (95%).

Table S5. Concentrations of each of the 6 targeted compounds (mexacarbate, albendazole, mebendazole, paroxetine, amitriptyline and fenpropidin) of the artificial mixture at each of the tested relative enrichment factor (REF) dose. Concentrations of REF1 is based on the concentrations presented in the raw sample at equal REF

	Concentration (ng/L)					
	REF 1	REF 5	REF 10	REF 50	REF100	REF1000
Mexacarbate	17	85	170	850	1700	17000
Albendazole	48	240	480	2400	4800	48000
Mebendazole	65	325	650	3250	6500	65000
Paroxetine	26	13	260	1300	2600	26000
Amitriptyline	304	1520	3040	15200	30400	304000
Fenpropidin	23	115	230	11500	2300	23000

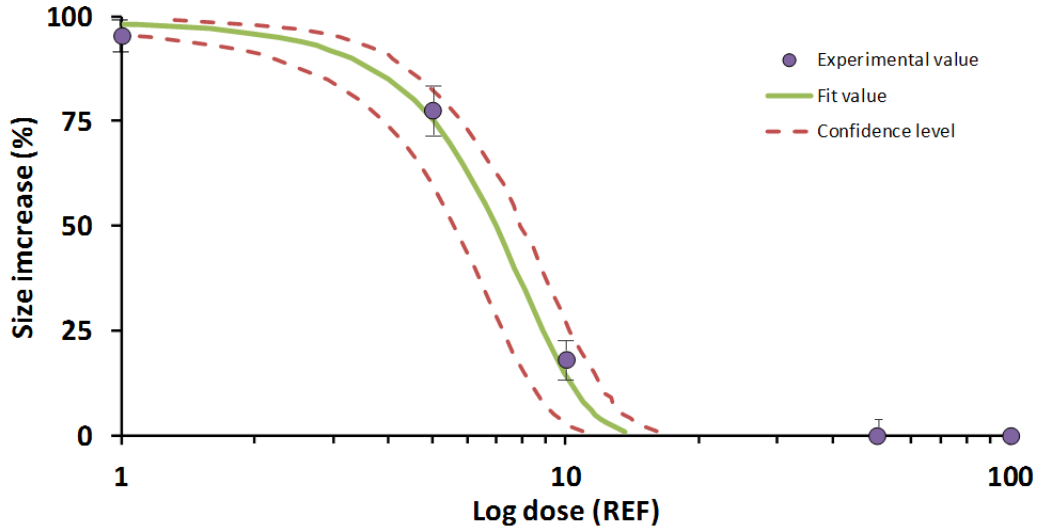


Figure S7. The log dose-response curves of the artificial mixture of the identified 6 chemicals tested all together. Straight lines show the EC fit values obtained with Probit and dashed line the confidence level (95%).

S7. References

(1) Carballeira, C.; Ramos-Gómez, J.; Martín-Díaz, L.; DelValls, T. A. Identification of Specific Malformations of Sea Urchin Larvae for Toxicity Assessment: Application to Marine Pisciculture Effluents. *Mar. Environ. Res.* 2012, *77*, 12–22.

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