

## Contaminants in Aquatic and Terrestrial Environments

# TRACING URBAN WASTEWATER CONTAMINANTS INTO THE ATLANTIC OCEAN BY NONTARGET SCREENING

Pablo Antonio Lara-Martín, Aurea C. Chiaia-Hernández, Miriam Biel-Maeso, Rosa María Baena-Nogueras, and Juliane Hollender

*Environ. Sci. Technol.*, **Just Accepted Manuscript** • DOI: 10.1021/acs.est.9b06114 • Publication Date (Web): 02 Mar 2020

Downloaded from pubs.acs.org on March 4, 2020

### Just Accepted

“Just Accepted” manuscripts have been peer-reviewed and accepted for publication. They are posted online prior to technical editing, formatting for publication and author proofing. The American Chemical Society provides “Just Accepted” as a service to the research community to expedite the dissemination of scientific material as soon as possible after acceptance. “Just Accepted” manuscripts appear in full in PDF format accompanied by an HTML abstract. “Just Accepted” manuscripts have been fully peer reviewed, but should not be considered the official version of record. They are citable by the Digital Object Identifier (DOI®). “Just Accepted” is an optional service offered to authors. Therefore, the “Just Accepted” Web site may not include all articles that will be published in the journal. After a manuscript is technically edited and formatted, it will be removed from the “Just Accepted” Web site and published as an ASAP article. Note that technical editing may introduce minor changes to the manuscript text and/or graphics which could affect content, and all legal disclaimers and ethical guidelines that apply to the journal pertain. ACS cannot be held responsible for errors or consequences arising from the use of information contained in these “Just Accepted” manuscripts.

1 TRACING URBAN WASTEWATER CONTAMINANTS INTO THE ATLANTIC  
2 OCEAN BY NONTARGET SCREENING

3  
4 Pablo A. Lara-Martín<sup>1\*</sup>, Aurea C. Chiaia-Hernández<sup>2,3</sup>, Miriam Biel-Maeso<sup>1</sup>, Rosa M. Baena-  
5 Nogueras<sup>1</sup>, Juliane Hollender<sup>2,4</sup>

6  
7 <sup>1</sup>Physical Chemistry Department, Faculty of Marine and Environmental Sciences, University of  
8 Cadiz, Campus of International Excellence of the Sea (CEI-MAR), 11510 Puerto Real, Spain

9 <sup>2</sup>Eawag, Swiss Federal Institute of Aquatic Science and Technology (Eawag), 8600 Dübendorf,  
10 Switzerland

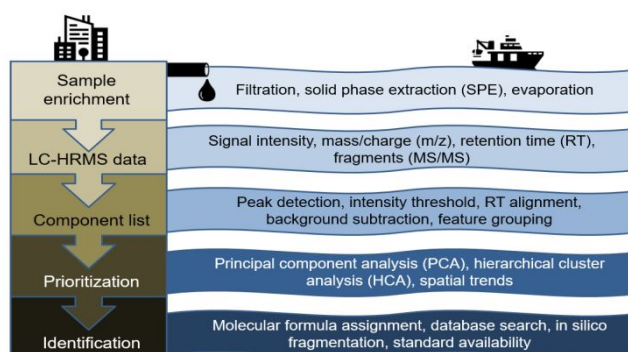
11 <sup>3</sup>Institute of Geography and Oeschger Centre for Climate Change Research, University of Bern, Bern,  
12 3012 Switzerland

13 <sup>4</sup>Institute of Biogeochemistry and Pollutant Dynamics, IBP, ETH Zurich, 8092 Zurich, Switzerland

## Abstract

Oceans are the ultimate sink for many of the over 100 million man-made substances. Until now, monitoring was limited to a reduced number of targeted persistent organic pollutants, reaching open waters mainly via atmospheric deposition. However, the composition and fate of the thousands of pollutants reaching the marine environment through wastewater discharges from coastal sources remain largely unexplored. By combining a newly developed non-target screening (NTS) workflow and high resolution mass spectrometry (HRMS), we have identified over 500 sewage-derived contaminants occurring in the ocean. Samples from the NE Atlantic contained this anthropogenic imprint at distances over 50 km from the coastline and > 500 m depth, beyond the continental margin. The range of identified compounds spans from pharmaceuticals and personal care products to food additives and industrial chemicals, including several that have never been reported in the environment, as they escaped conventional targeted analytical methods. Predicting the effects of the continuous input of this chemical “cocktail” on marine ecosystems is a formidable challenge, since 40% of the detected compounds lack information regarding their use and ecotoxicity.

## TOC



## 49    **1. Introduction**

50

51    Oceans are the vastest ecosystems on Earth, as well as sources of a wide number of resources such as  
52    fisheries, minerals, and energy. Coastal zones are the interface between land and the ocean, and have  
53    historically attracted human settlements because of these resources, as well as for logistical purposes  
54    (e.g., marine trade and transport). The development and utilization of coastal zones have greatly  
55    increased during the recent decades, undergoing tremendous socio-economic and environmental  
56    changes. The coastal population is projected to be nearly 1 billion by 2030, and most of the world's  
57    megacities are located by the coastline (1). These rapid changes in the coastal zones have had  
58    detrimental impacts towards marine species and habitats. Anthropogenic stressors on marine  
59    ecosystems, such as overfishing and nutrient input, are increasing globally (2). Among them,  
60    pollution by man-made chemicals has recently caught the attention of both environmental scientists  
61    and public interest, especially after becoming evident to the naked eye in the form of floating plastic  
62    debris in vast areas of the ocean (3).

63

64    Plastics are not the only artificial compounds mainly derived from land-based sources that can be  
65    detected in seawater. As of 2018, there are over 130 million registered chemical substances in the  
66    Chemical Abstracts Service (CAS) Registry File. Approximately 400,000 of them are somehow  
67    regulated in the international markets (e.g., U.S. Toxic Substances Control Act, or TSCA) and much  
68    less regularly monitored in aquatic systems (4). The vast number of chemical contaminants that  
69    consequently may be potentially present, but have not been detected, in the marine environment poses  
70    a great threat to the marine diversity and a challenge for both regulators and researchers. For  
71    regulators, one of the most common approaches has been to develop lists of substances to focus on  
72    their potential hazard to the ecosystems and/or to human health. In this context, there are several  
73    national and international legislations such as the US Clean Water Act (CWA), the European Water  
74    Framework Directive (WFD, 2000/60/EC1) and the Marine Strategy Framework Directive (MSFD,

2008/56/EC2) that established a limited number of substances of wide concern (named priority substances) to be regularly monitored in the coastline. Most of the target substances included in these legislations are either metals (As, Cd, Hg, etc.) or persistent organic pollutants (POPs) such as organochlorine compounds, pesticides, brominated flame retardants, perfluorinated chemicals, and recognized endocrine disruptors (e.g., nonylphenol and phthalates). The most comprehensive list of potential chemical contaminants in the marine environment, based on a compilation of substances from relevant aforementioned legislations, global conventions (e.g., Stockholm Convention), and other international organizations (e.g., US EPA) contains approximately 2700 substances (5). Many of these proposed compounds are considered as contaminants of emerging concern and have not been implemented yet in routine monitoring programs.

From an analytical point of view, the challenge is even bigger due to the wide range of chemical structures and properties considered and the very low concentrations (< parts per billion, or ppb) occurring in the ocean. To date, most of the effort has been focused on a targeted analysis of selected POPs, susceptible to long-range atmospheric transport and bioaccumulation in marine organisms (6). Analysis of non-regulated substances such as specific sewage-markers (e.g., coprostanol) and, most recently, selected pharmaceuticals has often been limited to enclosed seas such as the Baltic Sea and the Mediterranean Sea (7, 8). The identification of new contaminants is not only a challenge but also one of the most commonly-mentioned bottlenecks in the advance of disciplines such as environmental chemistry and ecotoxicology (9). Recent advances in high resolution mass spectrometry (HRMS), capable of discerning individual chemicals in complex matrices by accurate mass measurement, coupled with chromatographic separation are seen as key tools to address this challenge. By enabling higher throughput analysis of unknown substances at trace levels, the new so-called non-target screening (NTS) strategies aim to capture the universe of anthropogenic organic chemicals occurring in the environment. As we aim to explore the whole chemical composition of samples instead of specific target compounds, complexity in the interpretation of the results increases exponentially. The

101 resulting big data are managed by crafting analytical workflows capable of analyzing HRMS data  
102 with openly accessible online chemical compound databases, structure prediction algorithms, and  
103 statistical tools (10). To date, much effort has been dedicated to the identification of relevant  
104 contaminants and transformation products in wastewater treatment plants (WWTPs) (11, 12) and to  
105 characterize components in hydraulic fluids used for fracking (13).

106

107 Here, by using state-of-the-art NTS tools, we present a comprehensive characterization of the oceanic  
108 contamination by organic pollutants. As the release of synthetic chemicals into the marine  
109 environment may occur through a variety of pathways, including river and atmospheric transport, as  
110 well as directly at sea via aquaculture, shipping and fishing activities (5), we have focused our  
111 attention on sewage-derived contaminants by performing a directed NTS on these substances.  
112 Wastewater is the largest contributing source of emerging contaminants to aquatic environments (14)  
113 and a rising issue in coastal areas due to the aforementioned increase of urban settlements and  
114 population. The study area selected was the Gulf of Cadiz (NE Atlantic), as well as its coastline  
115 (Cadiz Bay, SW Spain) and the main WWTP (Fig. 1). Comparative analyses of wastewater, coastal  
116 waters, and offshore waters collected were performed to unravel the occurrence of 537 wastewater  
117 contaminants that could be detected from source to the open ocean, at distances further than 50 km  
118 from the coast and > 500 m depth.

119

## 120 2. Materials and Methods

121

122 **Water sampling.** All samples were collected in summer 2015 from the inlet and outlet of the WWTP  
123 serving Jerez de la Frontera, the main population in the area, and along 6 different transects: 3 within  
124 the Cadiz Bay area (Guadalete River estuary: G1-G10, Sancti Petri creek: S1-9, Rio San Pedro creek:  
125 R1-8) and 3 offshore (Cadiz Bay coast: CC1-4, Guadalquivir River mouth: GD1-4. and Trafalgar

126 Cape: TF1-5) (Fig. 1). Description of the sampling area and further details on how the sampling was  
127 conducted are in ref. 15 and Supporting Information (SI), respectively.

128

129 **Sample extraction and mass spectrometry determination.** Briefly, all water samples were filtered  
130 with glass fiber filters and extracted using Oasis HLB cartridges. Extracts were spiked with a mix of  
131 internal standards (Table S1) and analyzed by high performance liquid chromatography coupled to a  
132 Q Exactive hybrid quadrupole-Orbitrap mass spectrometer equipped with an electrospray ionization  
133 source (HPLC-ESI-HRMS) and operating in full-scan mode at a mass resolution of 140,000 FWHM  
134 referenced to  $m/z$  400 according to ref. 16. For structural elucidation of prioritized compounds (see  
135 next section), targeted MS/MS fragmentation was performed at 17,5000 FWHM. Specific  
136 information on SPE conditions and HPLC-HRMS instrumental settings can be found in SI.

137

138 **Selection, prioritization, and identification sewage-derived compounds.** A flowchart of the  
139 workflow used and the different nodes involved is presented in Fig. 2. The workflow consisted of a  
140 combination of nodes implemented with statistical software R (version 3.4.3) and the vendor software  
141 Compound Discoverer 2.1. The resulting dataset was subjected to statistical analyses using principal  
142 component analysis (PCA) and agglomerative hierarchical cluster analysis (HCA) to prioritize  
143 compounds of interest that were present both in wastewater and surface waters samples (Table S2).  
144 Identification of the molecular structure of these substances was carried out through a combination  
145 of different nodes (Fig. 2), which were complementary and provided identification confidence levels  
146 (17, 18) between 1 and 4. This process involved using reference standards (EAWAG standards, Table  
147 S1), library spectrum data search (mzCloud and MassBank), in-silico fragmentation (MetFrag and  
148 FISH), online compound databases (ChemSpider and PubChem) and suspect lists (NORMAN  
149 network and Environment and Food Safety, or EFS, lists) (Table S3). All the relevant information  
150 regarding the identification of sewage-derived compounds found in both coastal and oceanic waters

(n = 537) is provided in Table S4. Further details on the different workflow nodes, their optimization, and how the statistical analysis was performed can be found in SI.

### 3. Results and Discussion

**Prioritization of sewage-derived compounds.** HRMS analysis of wastewater samples at the influent and effluent from the WWTP serving Jerez, the biggest coastal city (215,000 inhabitants) in the study area (Fig. 1), revealed over 250,000 different compounds occurring in sewage. By compounds, we mean the integration of all the different MS features such as molecular ions, salt adducts, and isotopes. They cover a chemical space containing dissolved polar to semi-polar substances within a mass-to-charge ( $m/z$ ) window of 100 to 800 Da that are susceptible to electrospray ionization. Key aspects of the methodology developed here for such integration are provided in SI. To isolate and prioritize signals of interest (sewage-derived contaminants) from the background (naturally occurring components making up the dissolved organic matter), the NTS workflow was first calibrated by using a specific subset of samples. More specifically, we conducted a comparative analysis between the aforementioned wastewater samples and sewage-impacted surface waters from Guadalete Estuary (G1-10) (Fig. 1) to screen for common features. This estuary, highly polluted due to continuous sewage inputs from Jerez WWTP and other pollution sources, has been the subject of a series of studies on targeted contaminants, mainly surfactants (active ingredients in detergent formulations) (15, 19, 20).

We found an average number of approximately 47,000 compounds in the samples analyzed along Guadalete Estuary (G1-10). Principal component analysis (PCA) (Fig. S1A in the Supporting Information Appendix) revealed that locations adjacent to the discharge outlet of the WWTP (G1 and G2) differed substantially from the rest. They featured the highest numbers of compounds in the estuary (> 50,000), and most of these compounds had the highest signal intensities. Therefore, we



177 used the samples from these locations, together with the influent and effluent wastewater samples, to  
178 build a preliminary filter for prioritization of sewage-derived substances. The four samples (G1, G2,  
179 effluent, and influent) shared 11,256 common compounds. Many of these substances might be  
180 organic contaminants in wastewater persistent enough to partially escape sewage treatment and,  
181 consequently, be detected in the receiving surface waters. Although the full characterization of the  
182 contaminants entering the WWTP is out of the scope of this study, known substances frequently  
183 detected in urban wastewater such as caffeine and cocaine (21) were identified using certified  
184 standards (Table S1). For a considerable fraction of the detected chemicals (35%), relative signal  
185 intensities (previously normalized by considering sample volume and ion suppression using internal  
186 standards) were higher in the wastewater effluent than in the influent, suggesting either de-  
187 conjugation of parent compounds or transformation into different products at the WWTP. Examples  
188 are ranitidine S-oxide and dihydroxycarbamazepine, two pharmaceutical biodegradation products  
189 also confirmed with available standards.

190

191 In a second step, the number of possible sewage-derived contaminants was reduced to 6892 by  
192 hierarchical cluster analysis (HCA) of the 11,256 pre-selected compounds using all the samples taken  
193 along Guadalete Estuary (Fig. 3A). The main criterion was to select those compounds whose  
194 concentrations decreased continuously from G1 (adjacent to the WWTP outlet) towards G10 (at the  
195 mouth of the estuary), an indication that they are anthropogenically sourced through wastewater  
196 discharges and undergo dilution and/or degradation/sorption during their transport (Fig. 3B). The rest  
197 of the substances that did not follow this trend were assigned to either natural dissolved organic matter  
198 (34%) (Fig. 3C) or different contamination sources (5%) (Fig. 3D). More specifically, the latter group  
199 showed a maximum around sample site G8, where a port serving El Puerto de Santa Maria (90,000  
200 inhabitants) is located, so its occurrence may be associated with the maritime traffic and naval  
201 activities occurring in the estuary (15). The resulting lists of prioritized sewage-derived components  
202 (Table S2) were then included into the NTS workflow to limit the number of substances to detect and

203 identify in the rest of samples. These include coastal waters from Cadiz Bay (R1-8 and P1-9, at two  
204 tidal creeks) and surface and bottom samples taken offshore at the Gulf of Cadiz (CC1-4, GD1-4, and  
205 TF1-5, three transects of approximately 50 km length away from the coastline, covering the  
206 continental shelf and the shelf break) (Fig. 1).

207

208 **Identification of wastewater contaminants in the ocean.** The average number of possible organic  
209 compounds detected in the coastal ( $n = 26$ ) and oceanic samples ( $n = 27$ ) analyzed was approximately  
210 36,500 and 12,000, respectively (Fig. 4). The percentage of those compounds included in the list of  
211 sewage-derived contaminants was between 1 and 11%, depending on the sample. Specific examples  
212 are plotted in Fig. S2, where red dots are wastewater contaminants and the rest (grey dots) are either  
213 contaminants from other sources or naturally occurring organic matter. There were two coastal  
214 sampling sites, Guadalete Estuary (G1-10) and Rio San Pedro tidal creek (R1-R8) where more than  
215 50,000 substances were measured, indicative of higher concentrations of dissolved organic matter.  
216 As discussed in the previous section, this number is partly justified by the continuous input of  
217 wastewater from Jerez WWTP into the estuary, which resulted in 6% of total number of compounds,  
218 on average, being attributed to sewage-derived chemicals. The tidal creek, however, is not subjected  
219 to direct wastewater discharges, as confirmed by the considerably lower percentage ( $< 2\%$ ), number  
220 (450-1400), and signal intensities of sewage-derived chemicals detected here. PCA plots (Fig. S1A)  
221 also grouped samples from this area differently from the rest. The major contributors to the dissolved  
222 organic carbon (DOC) are attributed to effluents from fish farms (22), located in the vicinity of  
223 sampling site R1. The characterization of the contaminants derived from aquaculture activities is out  
224 of the scope of this work.

225

226 The spatial distribution of sewage-derived compounds showed a general trend, where the numbers  
227 and concentrations of contaminants (expressed as normalized signal intensities by considering sample  
228 volume and ion suppression using internal standards) progressively declined when moving from the

229 main source of wastewater discharge towards the open ocean (Fig. 4). This was expected due to  
230 dilution and co-occurrence of different environmental processes such as biodegradation, photolysis  
231 or sorption, aimed to reduce the concentrations of chemicals in aquatic environments (23). The  
232 decreasing trend was not only visible when comparing different coastal and ocean transects, but also  
233 to a lesser degree, within each transect as the distance increased from the coastline (Fig. S3). From  
234 the almost 7000 compounds included in this analysis (Table S2), the number of sewage-derived  
235 substances detected offshore was reduced to between 220 and 600. Some sampling sites (e.g.,  
236 Trafalgar Cape) were considerably more impacted than the rest (Fig. S1B) possibly due to direct  
237 discharges of untreated wastewater from popular touristic spots (e.g., Caños de Meca) not connected  
238 to the main sewage network, in agreement to local maximum values for DOC measured there (24).  
239 Additionally, there were also differences in the vertical distribution of sewage components along the  
240 water column. Higher relative signal intensities and numbers were always detected at the surface (Fig.  
241 S3), on average  $22.8 \pm 1.5\%$  higher. These differences have been previously reported for target  
242 contaminants such as pharmaceuticals and surfactants in urbanized estuaries (25) and can be  
243 attributed to positive buoyancy of less dense wastewater plumes entering the ocean via estuaries and  
244 marine outfalls.

245

246 As the main goal of this study was to characterize substances entering and occurring in the Atlantic  
247 Ocean, identification of compounds by assigning specific compound formulas, structures, and names  
248 was performed uniquely for those detected in offshore samples. This resulted in 537 different sewage-  
249 related chemicals (Table S4). Confidence identification levels from 1 (the highest confidence,  
250 confirmed with reference standards) to 4 (the lowest, tentative chemical compound from a chemical  
251 database) were assigned by using a combination of available chemical pure standards, mass spectra  
252 libraries, online chemical database searches, and in-silico mass spectral fragmentation algorithms (see  
253 Materials and Methods section). Some examples to illustrate different aspects used for identification  
254 are shown in Fig. S4. Here, the artificial sweetener sucralose, the food additive sulfurol, and the

255 industrial chemical 17-amino-3,6,9,12,15-pentaoxaheptadecan-1-ol, were identified at levels 1, 2, and  
256 3, respectively. Identification confidence levels and elemental composition of all the sewage-derived  
257 contaminants detected in oceanic waters from the Gulf of Cadiz is summarized in Fig. 5A, where  
258 information on their inclusion in chemical databases and known applications is presented in Fig. 5B.  
259 The vast majority of contaminants (85%) were exclusively formed of C, H, N, O, and S atoms,  
260 covering a wide range of functional groups including alcohols, carboxylates, alkylamines, sulfonated  
261 and sulfated compounds. The rest were either halogenated chemicals (mainly chlorinated substances,  
262 see blue bar in Fig. 5A) or had P and/or Si atoms in their composition (see red bar in Fig. 5A). Other  
263 chemicals such as hydrocarbons (composed of C and H only) were not detected due to the limitations  
264 of the analytical approach used, based on HPLC-ESI-HRMS.

265

266 Only about 20% of the prioritized sewage contaminants could be identified with high degree of  
267 confidence (levels 1 and 2, see light green and blue sectors, respectively, in Fig. 5A) due to the limited  
268 number of standards available in our laboratory (Table S1) and the still relatively low (although  
269 growing) number of HRMS spectra recorded from commercially available standards. For instance,  
270 as of 2018, there were approximately 16 thousand substances in one of the mass spectral libraries  
271 used here (MassBank); this number represents a tiny fraction of the more than 130 million registered  
272 chemical substances in the Chemical Abstracts Service (CAS). Our identification effort still resulted  
273 in over 100 chemicals positively identified in the ocean, many of them never reported before. The  
274 bulk of the prioritized list (60% of the substances), however, was composed by chemicals identified  
275 at level 3 by combining database searches (suspect lists in Table S3, ChemSpider, and PubChem) and  
276 in-silico fragmentation tools (MetFrag and FISH). Higher scores in Table S4 (columns P, R, and S)  
277 are indicative of higher probabilities of achieving successful identification. For the remaining  
278 chemicals (17%), HRMS information was not enough to elucidate their chemical structures. Tentative  
279 names were assigned from top-ranked compounds in ChemSpider and PubChem databases (in terms  
280 of number of references) that matched their molecular formula.

281

282 **Environmental relevance of the chemicals identified.** We could find 95% of the different  
283 substances identified in the ocean in online databases (ChemSpider and PubChem, light blue and light  
284 green sectors combined in Fig. 5B), and almost 50% of the possible candidates were already compiled  
285 in lists of suspected environmental contaminants (EFS and NORMAN network lists, see Table S3)  
286 (light green sector in Fig. 5B). Relevant environmental information such as their uses and applications  
287 was found for over 60% of them. This allowed their classification into six different groups of  
288 chemicals: pharmaceuticals and related bioactive substances, pesticides, personal care products,  
289 surfactants, industrial chemicals, and food additives. The top-ten compounds of each group, in terms  
290 of signal intensity and frequency of detection, are presented in Fig. 6. Their relative abundance is also  
291 depicted as maximum signal intensity ranges in coastal (G1-10, R1-8, and P1-9, red bars) and oceanic  
292 (CC1-4, GD1-4, and TF1-5, blue bars) samples, the latter often being several orders of magnitude  
293 lower. The rest of the contaminants were not considered for the analysis shown in Fig. 6 as no data  
294 on their possible uses and/or ecotoxicological relevance could be retrieved (red bar in Fig. 5B).

295

296 Pharmaceutically active compounds were, in terms of number of identified chemicals ( $n = 144$ ), the  
297 most relevant group of contaminants, comprising up to 25% of the prioritized wastewater components  
298 detected in the NE Atlantic. Those compounds showing highest intensities were analgesic (e.g.,  
299 antipyrine, also known as phenazone) and psychiatric drugs such as venlafaxine (an antidepressant),  
300 carbamazepine, and olanzapine. The former group were reported worldwide at the highest  
301 concentrations in coastal WWTPs due to their higher consumption (non-prescription drugs in many  
302 cases), whereas the latter are very recalcitrant when undergoing conventional sewage treatment (25).  
303 The presence of metabolites (4-formylaminoantipyrine, 4-acetamidoantipyrine, and valsartan acid,  
304 among others) and lidocaine (a local anesthetic) was also confirmed (level 1), whereas antivirals such  
305 as telvibudine and nevirapine were identified at level 3. Possible effects on marine biota derived from  
306 the occurrence of these bioactive substances have been recently reviewed by Mezzelani and co-

workers (26), including alterations in the enzymatic and immunological systems of filter feeders. As NTS workflows are not aimed for accurate quantification of the concentrations, we proceeded to re-analyze our samples using specific methodology for target determination of pharmaceuticals. Total concentrations of these contaminants were between 50 and 200 ng L<sup>-1</sup>, not currently known to be high enough to pose any foreseen risk towards marine biota (20). This preliminary environmental risk assessment, however, was not without limitations due to poor ecotoxicological data regarding the toxicity of these substances towards marine species, as well as unknown additive or synergistic effects with other chemicals co-existing in the water column. Among these chemicals, pesticides are also bioactive substances on their own, designed to kill specific organisms. Fungicides, such as metalaxyl and carbendazim, and herbicides (terbutryn, 2,4-D, and fluometuron) were identified at different confidence levels. Toxicity towards primary producers such as marine microalgae can be expected even at sub-ppb concentrations (27).

Surfactants were second in number of identified sewage-derived components, but first in terms of signal intensities. Although their removal in conventional WWTPs is very efficient (25) (> 95%), their current worldwide production is the highest of all synthetic chemicals (after plastics), with over 15 million tonnes per year (28). As a consequence, these compounds are reported at the highest concentrations in treated effluents and streams in comparison with other organic contaminants not so efficiently eliminated during wastewater treatment but used in lower amounts (e.g., psychiatric drugs) (29). Linear alkylbenzene sulfonates (LAS), polyethylene glycols (PEG), and polypropylene glycols (PPG) were the main components detected in seawater, together with their respective biodegradation products (e.g., sulfophenyl carboxylic acids, or SPCs, and oxidized PEG) and manufacturing impurities (dialkyltetralin sulfonates, or DATS). Rather than individual compounds, they are complex mixtures comprising different homologous and ethoxymer series (Fig. S5) with changing environmental and ecotoxicological properties (30). Although readily biodegradable according to screening tests, their elimination was shown to be considerably slower under marine conditions (31)

333 in comparison with freshwater environments. This is explained by a combination of marine microbial  
334 communities being less active than their freshwater counterparts toward xenobiotic chemicals and  
335 complexation with ions in seawater that reduces bioavailability, thereby inhibiting biodegradation  
336 (30). Surfactants are used in a myriad of applications including cleaning detergents (LAS), personal  
337 care products (PEG and PPG), and various industrial uses (e.g., oil, textiles, polymers, agriculture,  
338 and paints). The global surfactant market is forecast to grow at a compound annual rate of 6% over  
339 the next years, with rising demand for personal care products (PCPs) being the market driver (30).  
340 Other chemicals included in the formulation of PCPs that were detected in our samples were  
341 fragrances (galaxolidone, a degradation product from galaxolide), UV filters (sulisobenzone), and  
342 insect repellents (DEET and picaridin). Some of these compounds, reported in freshwater resources  
343 from all continents, are bioaccumulative and suspected endocrine disruptors, leading to potential  
344 alterations on fecundity, growth and development of exposed species (14).

346 Lastly, industrial chemicals were the third most relevant group identified by our NTS workflow. Their  
347 inclusion in the prioritized list of sewage-derived contaminants can be explained by the presence of  
348 industrial activities (e.g., wineries, laundry services, food processing, auto repair shops, etc.) within  
349 the city and its surroundings, also served by Jerez WWTP. This is a very diverse group comprising  
350 substances used in a wide range of applications, including processing aids (siloxanes), buffering  
351 agents (CAPSO), reaction intermediates (benzenesulfonamide), complexing agents (21-Crown-7),  
352 and corrosion inhibitors (benzotriazoles). The vast majority of the chemicals included in this group  
353 ( $n = 54$ ) has never been reported in the marine environment and there is no literature on their risks  
354 towards marine species. This includes food additives such as the flavoring agents sulfurol, identified  
355 at level 2, geranyl acetate, and eugenol benzoate (level 3). Some of them are not only synthetically  
356 produced, but also natural products. Such is the case of panthenol, a vitamin (B5) but also a  
357 moisturizer widely used in PCPs that was recently identified in WWTP using a NTS approach (12)  
358 and also detected in our seawater samples (Table S4). To discern between natural and anthropogenic

sources and the relative contributions of these sources is challenging and certainly would not be possible without using additional analytical techniques (e.g., compound-specific stable isotope analysis). Nevertheless, the highest concentrations observed in urban wastewater ensure that cities are one of the main sources of this type of chemicals towards the marine environment. Within this category, only non-volatile cyclosiloxanes (D6 and D7), also used in PCPs and with potential carcinogenicity, and benzotriazoles were previously reported in WWTP effluents (32, 33). Both are resistant to biodegradation and very persistent in aquatic environments, and the latter have been proposed as anthropogenic indicators in groundwater and rivers (34). Similarly, the artificial sweetener sucralose, the food additive detected at the highest intensity in oceanic samples, is considered as an ideal marker of sewage contamination in freshwater (35) and, more recently, coastal systems (36) due to its recalcitrance, source specificity, and high solubility. Concentrations up to 70 ng L<sup>-1</sup> were reported in the Gulf Stream (37), the only available data reported from offshore samples. In the present study, we determined an average 350-fold decrease in the relative abundance of these well-known sewage markers (Fig. S6A) from the most contaminated station (G1) to offshore samples. Due to the low reactivity of these markers, it was mostly attributed to dilution of wastewater in the ocean. Other chemicals more prone to undergo degradation (e.g., antipyrine and its main metabolites) showed a decrease in their concentration of more than 1000-fold from the estuary to the ocean. The transformation of the parent compound into different degradation products during their transport was evident as the relative abundances increased for the latter as we moved away from the contamination source (Fig. S6B).

**Future perspectives.** This work presents a list with over 500 different contaminants detected in coastal and oceanic samples from NE Atlantic. Their occurrence not only along the coastline but also in the whole continental shelf and beyond in a very dynamic environment, as opposed to previous targeted studies in enclosed seas, confirms their ubiquity. The detection of these chemicals in the open sea can only be explained by a combination of persistence in seawater and continuous input of



385 significant amounts from land-based sources such as WWTPs. Immediate efforts should be focused  
386 towards development of specific target methodologies aimed at the accurate determination of the  
387 concentrations of those contaminants detected by NTS, as well as ecotoxicity assays and/or modeling  
388 to discern whether they may pose risks towards marine biota. This is also a long-term task as the  
389 number of synthetic chemicals is continuously increasing (e.g., in 2015, 56 new drugs were launched  
390 and more of 7000 compounds were in trial) (38). Their global trends in terms of worldwide production  
391 and diversification has already outpaced the rates of change of other agents of global change such as  
392 rising CO<sub>2</sub> atmospheric concentrations and habitat destruction (39), but pollution by synthetic  
393 chemicals has not yet been included in most analyses of global change. In addition, and in spite of  
394 nowadays stricter environmental policies, concentrations of many sewage-derived chemicals in the  
395 marine environment are expected to keep rising within the next decades as the human population  
396 increases while conventional WWTPs are incapable of efficient removal of many contaminants. This  
397 growing trend has already been confirmed for pharmaceuticals from recent targeted analyses of dated  
398 marine sediment cores (40). Mitigation measures include improving wastewater treatment through  
399 the use of additional technologies (e.g., advanced oxidation processes) in current and planned coastal  
400 WWTPs, which have proved to increase water quality in sewage-impacted freshwater settings (41).

401

402 This study also explores the boundaries of the current existing methodologies for environmental high-  
403 throughput identification of compounds by HRMS. Although targeted analyses using reference  
404 standards remains the most reliable way to determine contaminants, it is not feasible to extend this  
405 approach over more than a few hundreds of substances of interest (more than 600 were used here) for  
406 routine monitoring. As mass spectral libraries are growing and computational mass spectrometry is  
407 advancing at a fast pace, the confidence in the identification of unknown substances using NTS  
408 workflows will increase in the near future. Already acquired HRMS data such as ours (available in  
409 File S1) will also benefit from upcoming achievements in the field (e.g., more robust in-silico  
410 fragmentation algorithms and identification of new contaminants) through the use of retrospective

411 screening, as has been recently tested with aqueous environmental samples from different European  
412 countries (42). Use of complementary techniques such as GC-HRMS for screening of hydrophobic  
413 and semi-volatile chemicals will allow to expand the analytical horizon towards an even more  
414 complete picture of the chemical universe in our environment (43). Lastly, by applying NTS  
415 workflows similar to the one described here, different potential contamination sources towards the  
416 marine environment such as aquaculture and maritime traffic could be investigated to unravel  
417 additional contaminants.

418

## 419 **Associated content**

420 **Supporting Information.** Materials and methods, Figures S1-S7, Tables S1-S4, and File S1 (MS/MS  
421 spectra). This material is available free of charge via the Internet at <http://pubs.acs.org>.

422

## 423 **Author information**

424 **\*Corresponding Author.** Address: Campus Río San Pedro s/n, Campus de Excelencia Internacional  
425 del Mar (CEI-MAR), 11510 Puerto Real, Cádiz, Spain. Phone: +34 956 016159. E-mail:  
426 [pablo.lara@uca.es](mailto:pablo.lara@uca.es)

427 **Notes.** The authors declare no competing financial interest.

428

## 429 **Acknowledgments**

430

431 Thanks to J.E Schollé and K. Kiefer for their HPLC-HRMS and MetFragR support, respectively, and  
432 to S. Salzmann during the revision process. This research was supported by the Spanish Ministry of  
433 Economy, Industry and Competitiveness (MINECO) (CTM2015-70731-R) and by the University of  
434 Bern through the Interfaculty Research Cooperation One Health.

435

## References

1. Neumann, B.; Vafeidis, A.T.; Zimmermann, J.; Nicholls, R.J. Future coastal population growth and exposure to sea-level rise and coastal flooding – A global assessment. *PLoS ONE* **2015**, 10, 3.
2. Halpern, B.S.; Frazier, M.; Potapenko, J.; Casey, K.S.; Koenig, K.; Longo, C.; Lowndes, J.S.; Rockwood, R.C.; Selig, E.R.; Selkoe, K.A.; Walbridge, S. Spatial and temporal changes in cumulative human impacts on the world's ocean. *Nat. Commun.* **2015**, 6, 7615.
3. Cózar, A.; Echevarría, F.; González-Gordillo, J.I.; Irigoien, X.; Úbeda, B.; Hernández-León, S.; Palma, A.T.; Navarro, S.; García-de-Lomas, J.; Ruiz, A.; Fernández-de-Puelles, M.L.; Duarte, C.M. Plastic debris in the open ocean. *PNAS* **2014**, 111, 10239-10244.
4. CAS, Database Counter, 2018, <http://www.cas.org/content/counter> (accessed 10.10.18).
5. Tornero, V.; Hanke, G. Potential chemical contaminants in the marine environment: An overview of main contaminant lists. ISBN 978-92-79-77045-6, EUR 28925, **2017**.
6. Luek, J.L.; Dickhut, R.M.; Cochran, M.A.; Falconer, R.L.; Kylin, H. Persistent organic pollutants in the Atlantic and southern oceans and oceanic atmosphere. *Sci. Total Environ.* **2017**, 583, 64-71.
7. Maldonado, C.; Dachs, J.; Bayona, J.M. Trialkylamines and Coprostanol as Tracers of Urban Pollution in Waters from Enclosed Seas: The Mediterranean and Black Sea. *Environ. Sci. Technol.* **1999**, 33, 3290-3296.
8. Björlenius, B.; Ripszám, M.; Haglund, P.; Lindberg, R.H.; Tysklind, M.; Fick, J. Pharmaceutical residues are widespread in Baltic Sea coastal and offshore waters - Screening for pharmaceuticals and modelling of environmental concentrations of carbamazepine. *Sci. Total Environ.* **2018**, 633, 1496-1509.
9. Schwarzenbach, R.P.; Escher, B.I.; Fenner, K.; Hofstetter, T.B.; Johnson, C.A.; von Gunten, U.; Wehrli, B. The challenge of micropollutants in aquatic systems. *Science* **2006**, 313, 1072-1077.

- 460 10. Hollender, J.; Schymanski, E.L.; Singer, H.; Ferguson, P.L. Non-target screening with high  
461 resolution mass spectrometry in the environment: Ready to go?. *Environ. Sci. Technol.* **2017**, 51,  
462 11505-11512.
- 463 11. Schollée, J.E.; Schymanski, E.L.; Avak, S.E.; Loos, M.; Hollender, J. Prioritizing Unknown  
464 Transformation Products from Biologically-Treated Wastewater Using High-Resolution Mass  
465 Spectrometry, Multivariate Statistics, and Metabolic Logic. *Anal. Chem.* **2015**, 87, 12121–12129.
- 466 12. Gago-Ferrero, P.; Schymanski, E.L.; Bletsou, A.A.; Aalizadeh, R.; Hollender, J.; Thomaidis, N.S.  
467 Extended Suspect and Non-Target Strategies to Characterize Emerging Polar Organic Contaminants  
468 in Raw Wastewater with LC-HRMS/MS. *Environ. Sci. Technol.* **2015**, 49, 12333-12341.
- 469 13. Hoelzer, K.; Summer, A.J.; Karatum, O.; Nelson, R.K.; Drollette, B.D.; O'Connor, M.P.;  
470 D'Ambro, E.L.; Getzinger, G.J.; Lee Ferguson, P.; Reddy, C.M.; Elsner, M.; Plata, D.L. Indications  
471 of Transformation Products from Hydraulic Fracturing Additives in Shale-Gas Wastewater. *Environ.*  
472 *Sci. Technol.* **2016**, 50, 8036-8048.
- 473 14. Montes-Grajales, D.; Fennix-Agudelo, M.; Miranda-Castro, W. Occurrence of personal care  
474 products as emerging chemicals of concern in water resources: A review. *Sci. Total Environ.* **2017**,  
475 595, 601-604.
- 476 15. Lara-Martín, P.A.; Gómez-Parra, A.; González-Mazo, E. Sources, transport and reactivity of  
477 anionic and non-ionic surfactants in several aquatic ecosystems in SW Spain: A comparative study.  
478 *Environ. Poll.* **2008**, 156, 36-45.
- 479 16. Chiaia-Hernández, A.; Günthardt, B.F.; Frey, M.P.; Hollender, J. Unravelling Contaminants in  
480 the Anthropocene Using Statistical Analysis of Liquid Chromatography–High-Resolution Mass  
481 Spectrometry Nontarget Screening Data Recorded in Lake Sediments. *Environ. Sci. Technol.* **2017**,  
482 51, 12547-12556.
- 483 17. Schymanski, E.L.; Jeon, J.; Gulde, R.; Fenner, K.; Ruff, M.; Singer, H.P.; Hollender, J. Identifying  
484 Small Molecules via High Resolution Mass Spectrometry: Communicating Confidence. *Environ. Sci.*  
485 *Technol.* **2014**, 48, 2097–2098.

- 486 18. Ruttkies, C.; Schymanski, E.L.; Wolf, S.; Hollender, J.; Neumann, S. MetFrag relaunched:  
487 incorporating strategies beyond in silico fragmentation. *J. Cheminform.* **2016**, 8, 3.
- 488 19. Corada-Fernández, C.; Lara-Martín, P.A.; Candela, L.; González-Mazo, E. Tracking sewage  
489 derived contamination in riverine settings by analysis of synthetic surfactants. *J. Environ. Monitor.*  
490 **2011**, 13, 2010-2017.
- 491 20. Biel-Maeso, M.; Baena-Nogueras, R.M.; Corada-Fernández, C.; Lara-Martín, P.A. Occurrence,  
492 distribution and environmental risk of pharmaceutically active compounds (PhACs) in coastal and  
493 ocean waters from the Gulf of Cadiz (SW Spain). *Sci. Total Environ.* **2018**, 612, 649-659.
- 494 21. Choi, P.M.; Tschärke, B.J.; Donner, E.; O'Brien, J.W.; Grant, S.C.; Kaserzon, S.L.; Mackie, R.;  
495 O'Malley, E.; Crosbie, N.D.; Thomas, K.V.; Mueller, J.F. Wastewater-based epidemiology  
496 biomarkers: Past, present and future. *TrAC* **2018**, 105, 453-469.
- 497 22. Burgos, M.; Ortega, T.; Bohórquez, J.; Corzo, A.; Rabouille, C.; Forja, J.M. Seasonal variation  
498 of early diagenesis and greenhouse gas production in coastal sediments of Cadiz Bay: Influence of  
499 anthropogenic activities. *Estuar. Coast. Shelf. Sci.* **2018**, 200, 99-115.
- 500 23. Jaffé, R. Fate of hydrophobic organic pollutants in the aquatic environment: A review. *Environ.*  
501 *Pollut.* **1991**, 69, 237-257.
- 502 24. González-Ortegón, E.; Amaral, V.; Sánchez-Leal, R.F.; Bellanco, M.J.; Jiménez, M.P.; Forja, J.;  
503 Vilas, C.; Tovar-Sanchez, A. Sources and coastal distribution of dissolved organic matter in the Gulf  
504 of Cadiz. *Sci. Total Environ.* **2018**, 630, 1583-1595.
- 505 25. Lara-Martín, P.A.; González-Mazo, E.; Petrovic, M.; Barceló, D.; Brownawell, B.J. Occurrence,  
506 distribution and partitioning of nonionic surfactants and pharmaceuticals in the urbanized Long Island  
507 Sound Estuary (NY). *Mar. Pollut. Bull.* **2014**, 85, 710-719.
- 508 26. Mezzelani, M.; Gorbi, S.; Regoli, F. Pharmaceuticals in the aquatic environments: Evidence of  
509 emerged threat and future challenges for marine organisms. *Mar. Environ. Res.* **2018**, 140, 41-60.

- 510 27. Moro, L.; Pezzotti, G.; Turemis, M.; Sanchis, J.; Farré, M.; Denaro, R.; Giacobbe, M.G.; Crisafi,  
511 F.; Giardi, M.T. Fast pesticide pre-screening in marine environment using a green microalgae-based  
512 optical bioassay. *Mar. Pollut. Bull.* **2018**, 129, 212-221.
- 513 28. Acmite Market Intelligence, 2016. Market Report: Global Surfactant Market, fourth ed., **2016**.
- 514 29. Kolpin, D.W.; Furlong, E.T.; Meyer, M.T.; Thurman, E.M.; Zaugg, S.D.; Barber, L.B.; Buxton,  
515 H.T. Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams,  
516 1999–2000: A national reconnaissance. *Environ. Sci. Technol.* **2002**, 36, 1202–11.
- 517 30. Jackson, M.; Eadsforth, C.; Schowanek, D.; Delfosse, T.; Riddle, A.; Budgen, N. Comprehensive  
518 review of several surfactants in marine environments: Fate and ecotoxicity. *Environ. Toxicol. Chem.*  
519 **2016**, 35, 1077-1086.
- 520 31. Perales, J.A.; Manzano, M.A.; Garrido, M.C.; Sales, D.; Quiroga, J.M. Biodegradation kinetics  
521 of linear alkylbenzene sulphonates in sea water. *Biodegradation* **2007**, 18, 63-70.
- 522 32. Giger, W.; Schaffner, C.; Kohler, H.P. Benzotriazole and tolyltriazole as aquatic contaminants.  
523 1. Input and occurrence in rivers and lakes. *Environ. Sci. Technol.* **2006**, 40, 7186-7192.
- 524 33. Capela, D.; Ratola, N.; Alves, A.; Homem, V. Volatile methylsiloxanes through wastewater  
525 treatment plants – A review of levels and implications. *Environ. Int.* **2017**, 102, 9-29.
- 526 34. Seit, W.; Winzenbacher, R. A survey on trace organic chemicals in a German water protection  
527 area and the proposal of relevant indicators for anthropogenic influences. *Environ. Monit. Assess*  
528 **2017**, 189, 244.
- 529 35. Lange, F.T.; Scheurer, M.; Brauch, H.J. Artificial sweeteners--a recently recognized class of  
530 emerging environmental contaminants: a review. *Anal. Bioanal. Chem.* **2012**, 403, 2503-2518.
- 531 36. Baena-Nogueras, R.M.; Traverso-Soto, J.M.; Biel-Maeso, M.; Villar-Navarro, E.; Lara-Martín,  
532 P.A. Sources and trends of artificial sweeteners in coastal waters in the bay of Cadiz (NE Atlantic).  
533 *Mar. Pollut. Bull.* **2018**, 13, 607-616.

- 534 37. Mead, R.N.; Morgan, J.B.; Avery, G.B.; Kieber, R.J.; Kirk, A.M.; Skrabal, S.A.; Willey, J.D.  
535 Occurrence of the artificial sweetener sucralose in coastal and marine waters of the United States.  
536 *Mar. Chem.* **2009**, 116, 13–17.
- 537 38. International Federation of Pharmaceutical Manufacturers, Associations (IFMPA). The  
538 Pharmaceutical Industry and Global Health: Facts and Figures 2017. ISBN 978-2-940498-50-5, **2017**.
- 539 39. Bernhard, E.S.; Rosi, E.J.; Gessner, M.O. Synthetic chemicals as agents of global change. *Front.*  
540 *Ecol. Environ.* **2017**, 15, 84-90.
- 541 40. Lara-Martín, P.A.; Renfro, A.A.; Cochran, J.K.; Brownawell, B.J. Geochronologies of  
542 Pharmaceuticals in a Sewage-Impacted Estuarine Urban Setting (Jamaica Bay, New York). *Environ.*  
543 *Sci. Technol.* **2015**, 49, 1202–1211.
- 544 41. Salimi, M.; Esrafil, A.; Gholami, M.; Jonidi Jafari, A.; Rezaei Kalantary, R.; Farzadkia, M.;  
545 Kermani, M.; Sobhi, H.R. Contaminants of emerging concern: a review of new approach in AOP  
546 technologies. *Environ. Monit. Assess.* **2017**, 189, 414.
- 547 42. Alygizakis, N.A.; Samanipour, S.; Hollender, J.; Ibáñez, M.; Kaserzon, S.; Kokkali, V.; van  
548 Leerdam, J.A.; Mueller, J.F.; Pijnappels, M.; Reid, M.J.; Schymanski, E.L.; Slobodnik, J.; Thomaidis,  
549 N.S.; Thomas, K.V. Exploring the Potential of a Global Emerging Contaminant Early Warning  
550 Network through the Use of Retrospective Suspect Screening with High-Resolution Mass  
551 Spectrometry. *Environ. Sci. Technol.* **2018**, 52, 5135-5144.
- 552 43. Hernández, F.; Ibáñez, M.; Portolés, T.; Cervera, M.I.; Sancho, J.V.; López, F.J. Advancing  
553 towards universal screening for organic pollutants in waters. *J. Hazard. Mater.* 2015, 282, 86-95.

## 561 **Figure Captions**

562

563 **Fig. 1.** Map showing the location of the Gulf of Cadiz (SW Spain) and sampling sites: GD1-4 =  
564 Guadalquivir River mouth transect, CC1-4 = Cadiz Coast transect, and TF1-5 = Trafalgar Cape  
565 transect. The inset shows the Cadiz Bay and sampling sites: G1-10 = Guadalete Estuary transect,  
566 R1-8 = Rio San Pedro transect, and P1-9 = Sancti Petri transect. The location of the main WWTP is  
567 also depicted (Schlitzer, R. Ocean Data View, [odv.awi.de](http://odv.awi.de), 2020).

568

569 **Fig. 2.** Workflow tree and nodes involved in the non-target screening (NTS) and identification of  
570 sewage-derived compounds in coastal and oceanic samples. Maximum confidence identification level  
571 provided by different nodes is also depicted: 1 (chemical standards), 2 (spectral libraries), 3 (in-silico  
572 fragmentation algorithms), and 4 (online compound databases and suspect lists). A detailed  
573 explanation of each node can be found in Supporting Information.

574

575 **Fig. 3.** Hierarchical cluster analysis (HCA) of potential sewage-derived compounds ( $n = 11,256$ )  
576 along the Guadalete Estuary (G1-10) (A) and selected clusters: potential contaminants from Jerez  
577 WWTP (61%) (B), naturally occurring organic matter (34%) (C), and other pollution sources (5%)  
578 (D). Examples of compounds for each cluster are plotted in red.

579

580 **Fig. 4.** Box plot showing the variability (minimum, first quartile, median, third quartile, and  
581 maximum) in the number of all organic compounds and potential sewage-derived contaminants  
582 detected in sampling stations at the Gulf of Cadiz (GD1-4 = Guadalquivir River mouth transect, CC1-  
583 4 = Cadiz Coast transect, and TF1-5 = Trafalgar Cape transect) and the Cadiz Bay (P1-9 = Sancti  
584 Petri transect, R1-8 = Rio San Pedro transect, and G1-10 = Guadalete Estuary transect). Normalized  
585 signal intensity ranges of sewage-derived contaminants are also plotted.

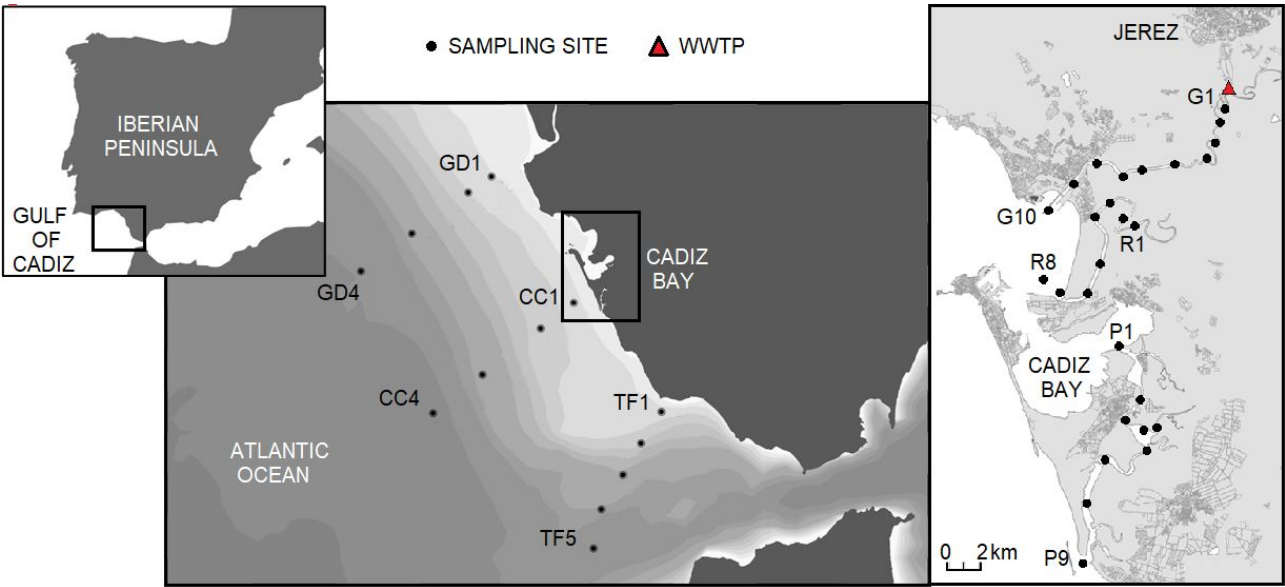
586



587 **Fig. 5.** Characterization of the sewage-derived contaminants ( $n = 537$ ) detected in oceanic waters  
588 from the Gulf of Cadiz: identification confidence levels (1-4, sector graph) and elemental composition  
589 (bar graph) (A), and percentage of these chemicals found in online databases and suspect lists (sector  
590 graph) and known uses and applications (bar graph) (B). For those compounds included in the red  
591 N/A sector and bar in Fig. 5B, information was not found on their identity or uses/applications,  
592 respectively.

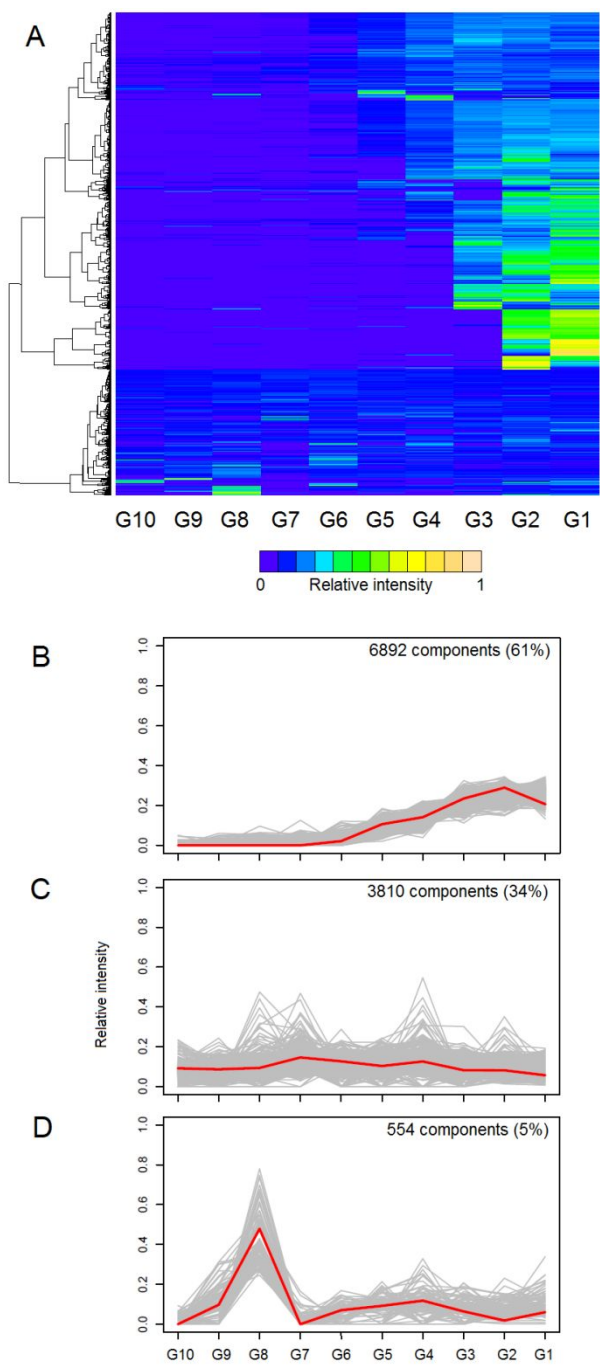
593

594 **Fig. 6.** Maximum normalized signal intensities of the top 10 sewage-derived contaminants in coastal  
595 (Cadiz Bay) and oceanic (Gulf of Cadiz) waters sorted by use/application class. Confidence  
596 identification levels (1 = green, 2 = blue, 3 = orange) are presented on the right of the compound  
597 names.

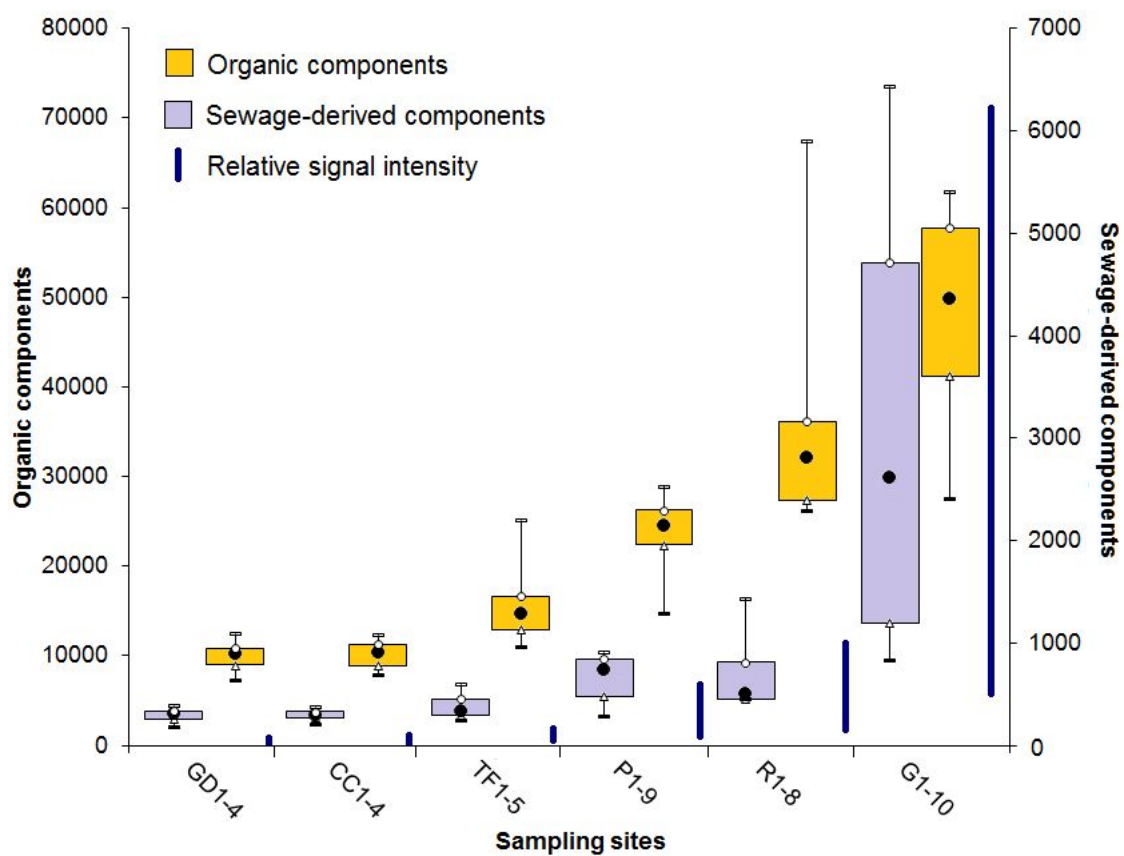


**Fig. 1**





**Fig. 3**

**Fig. 4**

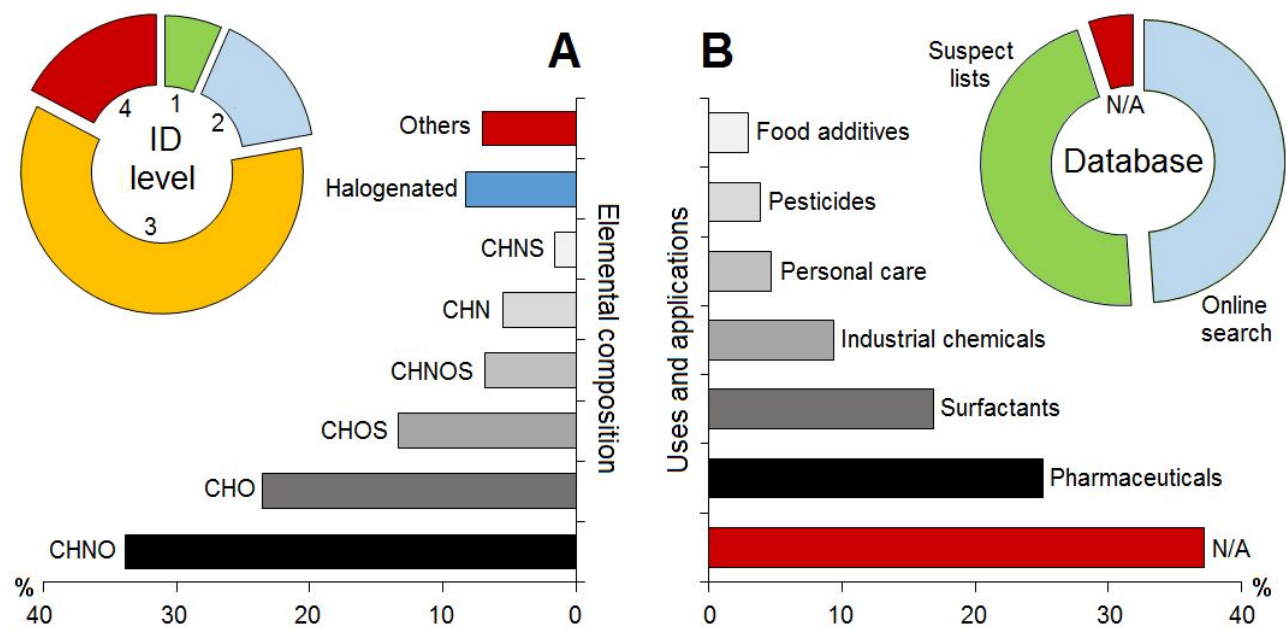


Fig. 5

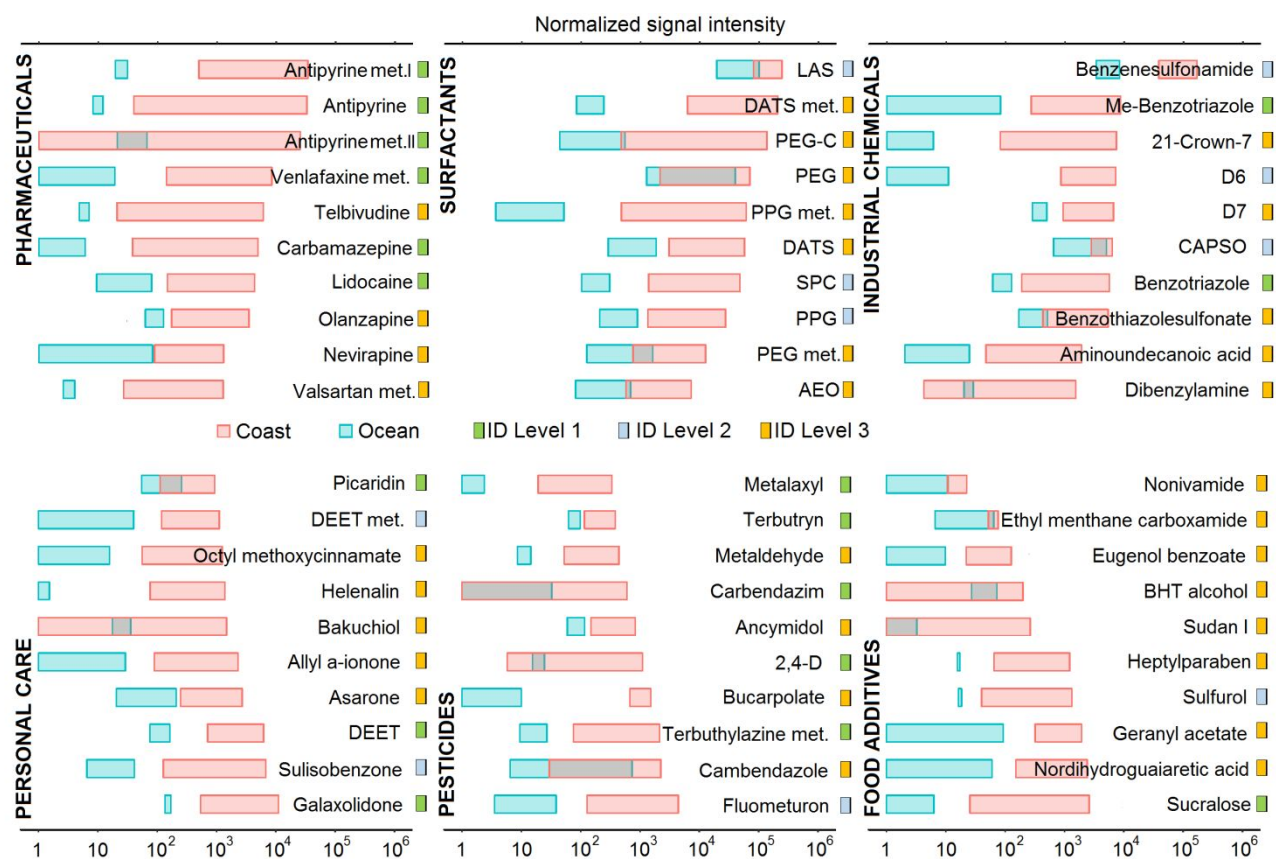


Fig. 6