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Contaminants in Aquatic and Terrestrial Environments

TRACING URBAN WASTEWATER CONTAMINANTS INTO THE ATLANTIC OCEAN BY NONTARGET SCREENING

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27 Abstract

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Oceans are the ultimate sink for many of the over 100 million man-made substances. Until now, 29 monitoring was limited to a reduced number of targeted persistent organic pollutants, reaching open 30 waters mainly via atmospheric deposition. However, the composition and fate of the thousands of 31 pollutants reaching the marine environment though wastewater discharges from coastal sources 32 remain largely unexplored. By combining a newly developed non-target screening (NTS) workflow 33 and high resolution mass spectrometry (HRMS), we have identified over 500 sewage-derived 34 contaminants occurring in the ocean. Samples from the NE Atlantic contained this anthropogenic 35 imprint at distances over 50 km from the coastline and > 500 m depth, beyond the continental margin. 36 The range of identified compounds spans from pharmaceuticals and personal care products to food 37 additives and industrial chemicals, including several that have never been reported in the 38 environment, as they escaped conventional targeted analytical methods. Predicting the effects of the 39 continuous input of this chemical "cocktail" on marine ecosystems is a formidable challenge, since 40 40% of the detected compounds lack information regarding their use and ecotoxicity. 41

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49 **1. Introduction**

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

63

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

2008/56/EC2) that established a limited number of substances of wide concern (named priority 75 substances) to be regularly monitored in the coastline. Most of the target substances included in these 76 legislations are either metals (As, Cd, Hg, etc.) or persistent organic pollutants (POPs) such as 77 78 organochlorine compounds, pesticides, brominated flame retardants, perfluorinated chemicals, and recognized endocrine disruptors (e.g., nonylphenol and phthalates). The most comprehensive list of 79 potential chemical contaminants in the marine environment, based on a compilation of substances 80 from relevant aforementioned legislations, global conventions (e.g., Stockholm Convention), and 81 other international organizations (e.g., US EPA) contains approximately 2700 substances (5). Many 82 of these proposed compounds are considered as contaminants of emerging concern and have not been 83 implemented yet in routine monitoring programs. 84

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86 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) 87 occurring in the ocean. To date, most of the effort has been focused on a targeted analysis of selected 88 POPs, susceptible to long-range atmospheric transport and bioaccumulation in marine organisms (6). 89 Analysis of non-regulated substances such as specific sewage-markers (e.g., coprostanol) and, most 90 recently, selected pharmaceuticals has often been limited to enclosed seas such as the Baltic Sea and 91 the Mediterranean Sea (7, 8). The identification of new contaminants is not only a challenge but also 92 one of the most commonly-mentioned bottlenecks in the advance of disciplines such as environmental 93 chemistry and ecotoxicology (9). Recent advances in high resolution mass spectrometry (HRMS), 94 capable of discerning individual chemicals in complex matrices by accurate mass measurement, 95 coupled with chromatographic separation are seen as key tools to address this challenge. By enabling 96 97 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 98 in the environment. As we aim to explored the whole chemical composition of samples instead of 99 specific target compounds, complexity in the interpretation of the results increases exponentially. The 100

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

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

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120 **2. Materials and Methods**

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Water sampling. All samples were collected in summer 2015 from the inlet and outlet of the WWTP
serving Jerez de la Frontera, the main population in the area, and along 6 different transects: 3 within
the Cadiz Bay area (Guadalete River estuary: G1-G10, Sancti Petri creek: S1-9, Rio San Pedro creek:
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 with glass fiber filters and extracted using Oasis HLB cartridges. Extracts were spiked with a mix of 130 internal standards (Table S1) and analyzed by high performance liquid chromatography coupled to a 131 132 Q Exactive hybrid quadrupole-Orbitrap mass spectrometer equipped with an electrospray ionization source (HPLC-ESI-HRMS) and operating in full-scan mode at a mass resolution of 140,000 FHWM 133 referenced to m/z 400 according to ref. 16. For structural elucidation of prioritized compounds (see 134 next section), targeted MS/MS fragmentation was performed at 17,5000 FHWM. Specific 135 information on SPE conditions and HPLC-HRMS instrumental settings can be found in SI. 136

137

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

(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.

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154 **3. Results and Discussion**

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Prioritization of sewage-derived compounds. HRMS analysis of wastewater samples at the influent 156 and effluent from the WWTP serving Jerez, the biggest coastal city (215,000 inhabitants) in the study 157 area (Fig. 1), revealed over 250,000 different compounds occurring in sewage. By compounds, we 158 mean the integration of all the different MS features such as molecular ions, salt adducts, and isotopes. 159 160 They cover a chemical space containing dissolved polar to semi-polar substances within a mass-to-161 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 162 signals of interest (sewage-derived contaminants) from the background (naturally occurring 163 components making up the dissolved organic matter), the NTS workflow was first calibrated by using 164 a specific subset of samples. More specifically, we conducted a comparative analysis between the 165 aforementioned wastewater samples and sewage-impacted surface waters from Guadalete Estuary 166 (G1-10) (Fig. 1) to screen for common features. This estuary, highly polluted due to continuous 167 sewage inputs from Jerez WWTP and other pollution sources, has been the subject of a series of 168 169 studies on targeted contaminants, mainly surfactants (active ingredients in detergent formulations) (15, 19, 20). 170

171

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

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

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

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

207

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

225

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

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

245

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

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

265

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

281

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

295

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

workers (26), including alterations in the enzymatic and immunological systems of filter feeders. As 307 NTS workflows are not aimed for accurate quantification of the concentrations, we proceeded to re-308 analyze our samples using specific methodology for target determination of pharmaceuticals. Total 309 310 concentrations of these contaminants were between 50 and 200 ng L⁻¹, not currently known to be high enough to pose any foreseen risk towards marine biota (20). This preliminary environmental risk 311 assessment, however, was not without limitations due to poor ecotoxicological data regarding the 312 toxicity of these substances towards marine species, as well as unknown additive or synergistic effects 313 with other chemicals co-existing in the water column. Among these chemicals, pesticides are also 314 bioactive substances on their own, designed to kill specific organisms. Fungicides, such as metalaxyl 315 and carbendazim, and herbicides (terbutryn, 2,4-D, and fluometuron) were identified at different 316 confidence levels. Toxicity towards primary producers such as marine microalgae can be expected 317 318 even at sub-ppb concentrations (27).

319

Surfactants were second in number of identified sewage-derived components, but first in terms of 320 signal intensities. Although their removal in conventional WWTPs is very efficient (25) (> 95%), 321 their current worldwide production is the highest of all synthetic chemicals (after plastics), with over 322 15 million tonnes per year (28). As a consequence, these compounds are reported at the highest 323 concentrations in treated effluents and streams in comparison with other organic contaminants not so 324 efficiently eliminated during wastewater treatment but used in lower amounts (e.g., psychiatric drugs) 325 (29). Linear alkylbenzene sulfonates (LAS), polyethylene glycols (PEG), and polypropylene glycols 326 (PPG) were the main components detected in seawater, together with their respective biodegradation 327 products (e.g., sulfophenyl carboxylic acids, or SPCs, and oxidized PEG) and manufacturing 328 329 impurities (dialkyltetralin sulfonates, or DATS). Rather than individual compounds, they are complex mixtures comprising different homologous and ethoxymer series (Fig. S5) with changing 330 environmental and ecotoxicological properties (30). Although readily biodegradable according to 331 screening tests, their elimination was shown to be considerably slower under marine conditions (31) 332

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

345

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

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sources and the relative contributions of these sources is challenging and certainly would not be 359 possible without using additional analytical techniques (e.g., compound-specific stable isotope 360 analysis). Nevertheless, the highest concentrations observed in urban wastewater ensure that cities 361 362 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 363 carcinogenicity, and benzotriazoles were previously reported in WWTP effluents (32, 33). Both are 364 resistant to biodegradation and very persistent in aquatic environments, and the latter have been 365 proposed as anthropogenic indicators in groundwater and rivers (34). Similarly, the artificial 366 sweetener sucralose, the food additive detected at the highest intensity in oceanic samples, is 367 considered as an ideal marker of sewage contamination in freshwater (35) and, more recently, coastal 368 systems (36) due to its recalcitrance, source specificity, and high solubility. Concentrations up to 70 369 ng L^{-1} were reported in the Gulf Stream (37), the only available data reported from offshore samples. 370 371 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. 372 Due to the low reactivity of these markers, it was mostly attributed to dilution of wastewater in the 373 ocean. Other chemicals more prone to undergo degradation (e.g., antipyrine and its main metabolites) 374 showed a decrease in their concentration of more than 1000-fold from the estuary to the ocean. The 375 transformation of the parent compound into different degradation products during their transport was 376 evident as the relative abundances increased for the latter as we moved away from the contamination 377 source (Fig. S6B). 378

379

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

significant amounts from land-based sources such as WWTPs. Immediate efforts should be focused 385 towards development of specific target methodologies aimed at the accurate determination of the 386 concentrations of those contaminants detected by NTS, as well as ecotoxicity assays and/or modeling 387 388 to discern whether they may pose risks towards marine biota. This is also a long-term task as the number of synthetic chemicals is continuously increasing (e.g., in 2015, 56 new drugs were launched 389 and more of 7000 compounds were in trial) (38). Their global trends in terms of worldwide production 390 and diversification has already outpaced the rates of change of other agents of global change such as 391 rising CO₂ atmospheric concentrations and habitat destruction (39), but pollution by synthetic 392 chemicals has not yet been included in most analyses of global change. In addition, and in spite of 393 nowadays stricter environmental policies, concentrations of many sewage-derived chemicals in the 394 marine environment are expected to keep rising within the next decades as the human population 395 increases while conventional WWTPs are incapable of efficient removal of many contaminants. This 396 growing trend has already been confirmed for pharmaceuticals from recent targeted analyses of dated 397 marine sediment cores (40). Mitigation measures include improving wastewater treatment through 398 the use of additional technologies (e.g., advanced oxidation processes) in current and planned coastal 399 WWTPs, which have proved to increase water quality in sewage-impacted freshwater settings (41). 400

401

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

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 <u>http://pubs.acs.org</u> .
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427	Notes. The authors declare no competing financial interest.
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22 ACS Paragon Plus Environment

561 Figure Captions

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

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

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

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

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

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







Fig. 2



Fig. 3



Fig. 4



Fig. 5



Fig. 6