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Screening for contaminant hotspots in the marine environment of Kuwait using ecotoxicological and chemical screening techniques

A.J. Smith [a,*], T. McGowan [a], M.J. Devlin [b], M.S. Massoud [c], M. Al-Enezi [c], A.S. Al-Zaidan [c], H.A. Al-Sarawi [c], B.P. Lyons [d]

[a] Centre for Environment, Fisheries and Aquaculture Science (Cefas), Lowestoft Laboratory, Pakefield Road, Lowestoft, Suffolk, NR33 0HT, UK
[b] James Cook University, Catchment Reef Research Group, TropWater, Townsville, QLD 4811, Australia
[c] Kuwait Environment Public Authority, P.O. Box 24395, Safat 13104, Kuwait
[d] Cefas, Weymouth laboratory, Barrack Road, Weymouth, Dorset DT4 8UB, UK

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ABSTRACT

Kuwait is a country with low rainfall and highly concentrated industrial and domestic effluents entering its coastal waters. These can be both treated and untreated. In this study we sampled a series of coastal and open-sea sites and used a variety of analyses to identify those sites requiring the most attention. We used a high throughput GC–MS screen to look for over 1000 chemicals in the samples. Estrogen and androgen screens assessed the potential to disrupt endocrine activity. An oyster embryo development screen was used to assess biological effect potential. The chemical screen identified sites which had high numbers of identified industrial and domestic chemicals. The oyster screen showed that these sites had also caused high levels of developmental abnormalities with 100% of embryos affected at some sites. The yeast screen showed that estrogenic chemicals were present in outfalls at 2–3 ng/l E2 equivalent, and detectable even in some open water sites.

1. Introduction

Kuwait is a country with almost 500 km of coastline, which lies along the north eastern end of the Arabian Gulf and comprises Kuwait Bay, a shallow body of water to the North West. The majority of Kuwait's 3.6 million inhabitants reside in Kuwait City which is located along the southern shoreline of the bay and extends south along the Gulf coastline. Although large amounts of contaminants enter the Kuwaiti marine environment via the Shatt Al Arab, the confluence of the Tigris and Euphrates rivers in the northern Arabian Gulf (Beg and Al-Ghandban, 2003), waste effluents from industry and domestic sewage are thought to make up the key components of marine pollution around Kuwait (Al-Ghadban et al., 2002; Al-Abdulghani et al., 2013; Al-Sarawi et al., 2015). In particular, sewage contamination, both from illegal discharges or authorised releases, has been documented as being of particular concern (Al-Omran, 1998; Ghannoum et al., 1991; Lyons, et al., 2015; Saeed et al., 2012). Other effluents from power stations, desalination plants and various sized industrial or commercial processes also reach the marine environment from outfalls. Due to these various inputs, the waters of Kuwait Bay and the Gulf coast are subject to a constant stream of complex effluents varying in volume, constituents and flow. The problems associated with understanding the overall effect of these mixed effluents were recognised by the early 1990s (Matthiessen et al., 1993) and research also showed that effluents from sewage treatment plants may also, through various chemical pollutants, demonstrate endocrine disruption effects (Desbrow et al., 1998; Harries et al., 1996; Jobling et al, 1998; Harries et al., 1999). These effects can be caused by natural steroids and also by industrial chemicals acting as endocrine disruptors with variable potency (Aerni et al, 2004; Duong et al, 2010; Fawell et al., 2001; Pawlowski et al., 2004; Pothitou and Voutsa, 2008; Sohoni and Sumpter, 1998; Thomas et al, 2001). Interest in what was in these complex effluents widened and a wide range of pharmaceutical and personal care product compounds was also identified in the receiving waters (Prasse et al., 2010; Roberts and Thomas, 2004; Thomas et al., 2004). The presence of pharmaceuticals is of particular concern, as they are designed to have specific biological effects and low concentrations, and so are thought to pose high risk to organisms in receiving waters (Gaw et al., 2014). These types of effluents, and their dispersal, dilution and degradation are problematic for chemical analysis because not all the chemicals are identified in the effluent. In addition, reactions that take place within the effluents are hard to predict so reaction products and biotransformation

* Corresponding author.
E-mail address: Andy.smith@cefas.co.uk (A.J. Smith).

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products may or may not be identified or quantified. Predicting the toxicity of such complex mixtures has been one of the hot topics in environmental monitoring so far this century (Backhaus and Karlsson, 2014; Backhaus et al, 2011; Cleuvers, 2004; Flaherty and Dodson, 2005; Thorpe et al, 2001; Thorpe et al, 2003). Effective biological assays can provide data on the overall effects of a complex mixture and help to target sites with problems without any preknowledge of what chemicals might be present. Finding the right combination of suitable biological and chemical analysis can give the capability to monitor for both specific and unknown pollutants and understand how sites are changing in response to changes in climate, industry, hydrodynamics and other ecological pressures. One effective screening assay for effluent based contamination is the yeast estrogen screen (Routledge and Sumpter, 1996). This is a tool for looking at steroid and steroid mimicking chemicals in environmental samples and gives a measure of the overall effect of the sum of active steroid-like chemicals. It is a widely accepted assay and has been used in many countries to look at contamination in effluents, rivers, estuaries and marine areas (Balaam et al, 2010; Beck et al, 2006; Fernandez et al, 2007; Galluba and Oehlmann, 2012; Pawlowski et al, 2004; Thomas et al, 2001, 2002, 2004; Tollefsen et al, 2007; Viganò et al, 2008).

To date no attempt has been made to assess the potential toxicological and endocrine disrupting risks posed by effluents that are discharged into Kuwait's marine environment. To address this a series of water and effluent samples was collected from various points around Kuwait's marine coastline to establish if such a threat exists. Water samples were screened using a GC–MS target based screening approach to identify the main pollutants present and assessed for both toxic and endocrine disruption potential using bioassays.

2. Materials and methods

2.1. Sample location and collection

Over a period of a few days a series of 16 water samples was collected from four known points sources of effluent input, Al Ghazali, Salmiya, Al Bedaa and Al Messela, and from twelve other sites located offshore in Kuwait Bay and along the Gulf coastline adjacent to the city (see Fig. 1). Approximately 2.5 litres of water or effluent were collected at each site using either a stainless steel bucket or Winchester bottles to collect samples from immediately beneath the water surface. All samples were stored in 2.5 litre amber Winchester bottles prior to extraction and analysis.

The Winchesters were transported back to the EPA laboratory at Shuwaikh, Kuwait City, where they were immediately prepped for vacuum extraction.

2.2. Extractions

Phenomenex 200 μg 6 ml Strata X cartridges were used with an 18 ml methanol activation followed by clean seawater rinse (laboratory prepared artificial sea water using Sigma Sea Salts). Samples were extracted under a steady drip of approximately 1 ml/minute. Once the sample was extracted, the cartridges were rinsed with 18 ml deionised water to remove salt residues. Due to the nature of the samples, and the desire to extract them without filtering, it was not always possible to extract the entire sample (2.6 litres) in the time available. In this case the volume of sample that passed through the SPE was recorded for calculation of concentration factor later. It was only possible, for example,

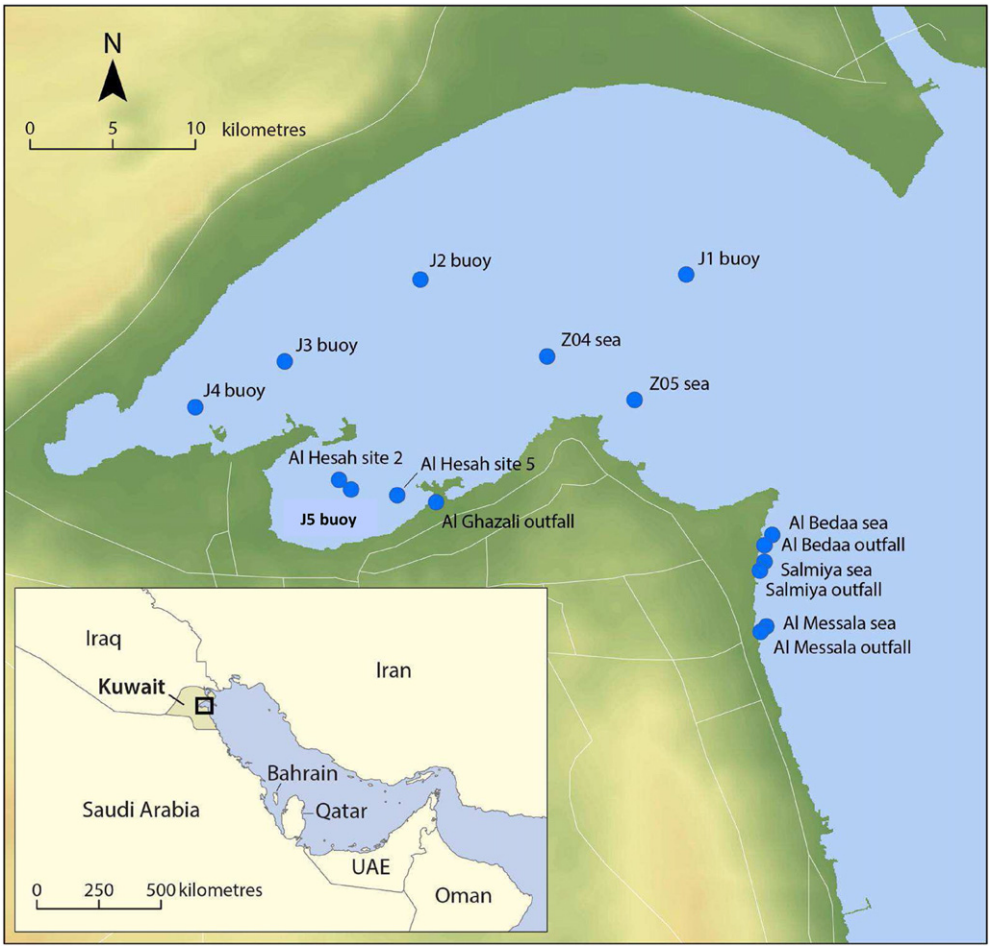

Fig. 1. Map showing the location of the sample sites referred to in the data.

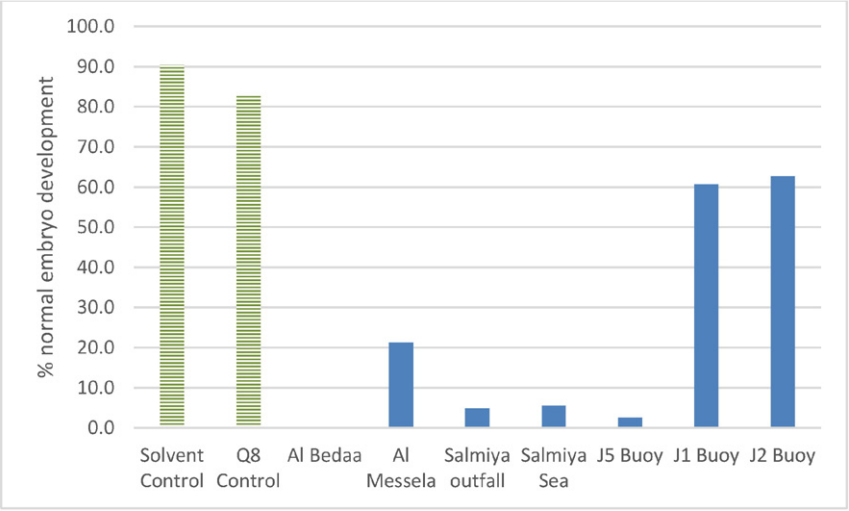

Fig. 2. Oyster embryo development screen. *Y*-axis represents percentage of embryos developing normally. Solid bars represent results from sites of interest. Striped bars indicate control sample results.

to extract 1 litre of the Al Messela sample, whilst other samples ranged from 1.4 up to 2.6 litres for clearer samples which passed through the SPE cartridge faster. A control sample, Kuwait Control, of clean artificial seawater was made in the lab and extracted at the same time using the same process.

2.3. Elution

Elution took place in the Cefas laboratory in the UK. A second control sample of clean artificial seawater, Cefas Control, was made in this lab and extracted at the same time using the same process. SPE cartridges containing the samples were eluted using both polar and nonpolar solvents to ensure that as many chemicals of interest were eluted as possible. Samples were eluted through the addition of 4 ml methanol, which was left to soak for 1 minute, before draining into a 15 ml evaporation tube. This was repeated a further two times. The composite methanol sample was then evaporated to dryness under a flow of nitrogen gas and reconstituted in acetone. The same SPE cartridge was then extracted again with hexane. During the evaporation phase the hexane extracts were evaporated to near dryness before being reconstituted in acetone, using a vortex mixer.

The two fractions for each sample were then mixed to create a combined sample in acetone, made up to a total volume of 10 ml. This extract was used as the primary extracted sample for subsequent screening with the individual bioassays.

2.4. Yeast screening

The samples were tested for androgenic and estrogenic activity using a Yeast-based estrogen screen (YES) and yeast-based androgen screen (YAS) using the methods described in detail by Routledge and Sumpter (1996) and Sohoni and Sumpter (1998), with minor modifications to plate dilutions and arrangements. In these assays, the human estrogen receptor (hER-α) and human androgen receptor (hAR-α) have been integrated into the yeast genome of separate strains of yeast. Expression plasmids carrying estrogen or androgen responsive elements which control the expression of the reporter gene Lac-Z have also been added. In the presence of chemicals with estrogenic or androgenic activity (which bind to, and activate the receptor), β-galactosidase is synthesised and then secreted into the yeast assay medium. The β-galactosidase acts by breaking down the chromogenic substrate chlorophenol red β-galactopyranoside (CPRG). This causes a colour

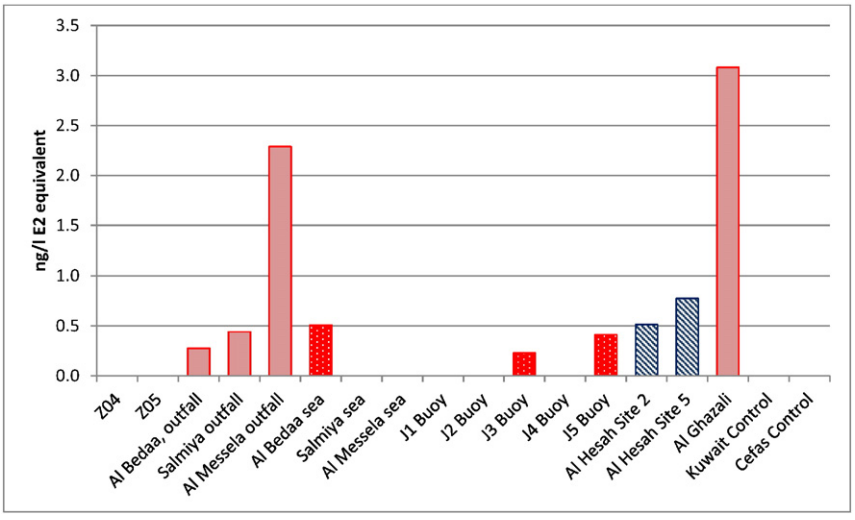

Fig. 3. Response for samples tested using the yeast estrogen screen. The vertical scale represents the amount of E2 in ng/l required to produce a response similar to that cause by the sample. Solid pink data bars represent the concentration in outfalls. Blue diagonally striped bars represent near shore samples. Red spotted bars indicate open water samples.

Table 1
Open water J-buoy sites 1–4. Chemicals identified by GC–MS residue scans. Listed chemicals are toxic, carcinogenic, indicators of other pollutants, indicators of industrial process wastes, pharmaceuticals or personal care products.

CAS number	Name	Approximate concentration μg/l	Possible use
75252	Bromoform	0.04–0.48	Volatile solvent
74953	Dibromomethane	0.01–0.05	Volatile solvent
99763	Methylparaben	0.1–2.3	Antifungal preservative in cosmetics
120478	Ethylparaben	0.8	Antifungal preservative in cosmetics
88755	2-Nitrophenol	0.01–0.02	Manuf dyes, paint coatings, rubber coatings and fungicides
119619	Benzophenone	0.05	Fixative for heavy perfumes
142916	Isopropyl palmitate	7.5	Widely used in cosmetics and personal care products
5466773	Octyl-methoxycinnamate	0.02	Personal care products, sun blocker
118605	2-Ethylhexyl salicylate	0.1–9.7	Used in cosmetics and sunscreens to absorb UVB
6197304	Octocrylene	0.13–9.9	UV-filter
88040	Chloroxylenol	0.06	Biocide/antiseptic
131113	Dimethyl phthalate	0.06	Additives for plastics known as phthalates
3622842	Benzenesulfonamide, *N*-butyl-	0.14–0.3	Intermediate for the synthesis of dyes, photochemicals and disinfectants
80057	Bisphenol A	0.03	In the manufacture of epoxy resins and polycarbonates for food packaging
77907	Tributyl acetylcitrate	0.05	Plasticizer or carrier solvent permitted in the field of food additive, food contact material as well as for polymers especially for cellulosics
10323	1 Bis(2-ethylhexyl) adipate	0.06–0.13	Plasticizer
10544500	Sulphur (S8)	0.03	Acaricide/insecticide
58082	Caffeine	0.04–0.37	Psychoactive stimulant drug
57885	Cholesterol	7.3	Waxy steroid of fat that is manufactured in the liver or intestines

shift from the initial yellow into a chlorophenol red, the concentration of which can be measured by absorbance.

Sample extracts in acetone were blown down under nitrogen until almost dry and then reconstituted to the original volume in ethanol to work with the yeast assay. The sample extracts, and standards prepared in ethanol, were added in duplicate to 96 well microwell test plates as a dilution series and then allowed to evaporate to dryness at room temperature. Growth medium that had been inoculated with yeast cells was combined with CPRG and added to the test plate. The plate was then incubated at 32 °C for 2 days for YAS and 3 days for YES, shaken vigorously for 2 minutes on each day. On the final day for each assay any change in the optical density of the CPRG was read colourimetrically using a spectrophotometric plate-reader (Bio-Tek instruments, Inc.) at an absorbance of 540 nm for colour. The number of yeast cells was estimated from the optical density at 620 nm. A dilution series of 17β-estradiol (E2) or dihydrotestosterone (DHT) was assayed, together with a solvent blank, as part of each multi-well plate as a positive control and androgenic/estrogenic standard.

Sample response in each of the assays was quantified according to how much of the E2 or DHT standards would be required to give the same response. The dose–response curves for the standard and the extracts were considered to be parallel. The equation for the steepest part of the dose response curve is used, together with OD (540 nm) values of the test extract to calculate how much E2 or DHT would be required to create the same response as the sample. All values falling within the linear range of the response curve were averaged to produce equivalent values in ng E2/ml or mg DHT/ml. These equivalence values were divided by the overall concentration factor to calculate final equivalence values for the raw sample.

Table 2
Combined Mishref outfalls. Chemicals identified by GC–MS residue scans. Listed chemicals are toxic, carcinogenic, indicators of other pollutants, indicators of industrial process wastes, pharmaceuticals or personal care products.

CAS number	Name	Approximate concentration μg/l	Possible use
74953	Dibromomethane	0.01- 0.02	Volatile solvent
75252	Bromoform	0.06 - 0.08	Volatile solvent
108689	3,5-Dimethylphenol	0.02–0.08	Used as pesticides and in the manufacture of disinfectants
88040	Chloroxylenol	0.3	Biocide/antiseptic
126863	2,4,7,9-Tetramethyl-5-decyne-4,7-diol	0.15	Surfactant-wetting and foaming control
134623	*N*,*N*-Diethyl-*m*-toluamide	0.03	DEET, insect repellent
84662	Diethyl phthalate	1	Additives for plastics known as phthalates
119619	Benzophenone	0.08	Fixative for heavy perfumes
10543574	*N*,*N*,*N*′,*N*′-Tetraacetylethylenediamine	0.07–0.4	Peroxide bleach activator for household detergents, paper pulp
115968	Tri-(2-chloroethyl) phosphate	0.06	Flame retardant
3622842	Benzenesulfonamide, *N*-butyl-	0.17–0.6	Neurotoxic plasticiser
333415	Diazinon	0.01–0.25	Acaricide/insecticide
118605	2-Ethylhexyl salicylate	0.22	Used in cosmetics and sunscreens to absorb UVB
58082	Caffeine	0.08–3.3	Psychoactive stimulant drug
10544500	Sulphur (S8)	0.03–13	Acaricide/insecticide
206440	Fluoranthene	0.01	PAH
86737	Fluorene	0.01	PAH
129000	Pyrene	0.01	PAH
13674878	Tris-(1,3-dichloroisopropyl) phosphate	0.6	Used as a fire retardant and plasticizer in various plastic foams, resins, and latexes
117817	Bis(2-ethylhexyl)phthalate (DEHP)	2.0	Additives for plastics known as phthalates (mrv 1 μg/l)
6197304	Octocrylene	0.1	UV-filter
106445	*p*-Cresol (4-methylphenol)	0.04–0.06	Phenol
123911	1,4-Dioxane	0.07	Personal care products, stabilizer in chlorinated solvents
102761	Triacetin	0.08	Plasticizers fuel/feed/cigarette additive
136856	1H-Benzotriazole-5-methyl	0.75	Multi-functional petroleum additive, widely used in antirust oils & aircraft de-icing fluids

Table 3
Al Ghazali unlicensed effluent outfall. Chemicals identified by GC–MS residue scans. Listed chemicals are toxic, carcinogenic, indicators of other pollutants, indicators of industrial process wastes, pharmaceuticals or personal care products.

CAS number	Name	Approximate concentration μg/l	Possible use
79016	Trichloroethylene	0.1	Volatile solvent
74953	Dibromomethane	0.1	Volatile solvent
127184	Tetrachloroethylene	0.2	Volatile solvent
108907	Chlorobenzene	0.01–0.03	Volatile solvent
98828	Isopropylbenzene	0.14–0.17	Volatile solvent
103651	*n*-Propylbenzene	0.4–0.48	Volatile solvent
108678	1,3,5-Trimethylbenzene	4.3–5.8	Volatile solvent
95636	1,2,4-Trimethylbenzene	7	Volatile solvent
135988	sec-Butylbenzene	0.15–0.22	Volatile solvent
99876	*p*-Isopropyltoluene	3.1–4	Volatile solvent
108883	Toluene	4.8–6.0	Volatile solvent (reporting above 1 μg/l approx.)
1330207	Total xylene	8.0–9.5	Volatile solvent (reporting above 1 μg/l approx.)
75252	Bromoform	0.6	Volatile solvent
95476	*o*-Xylene	3.77	Volatile solvent
104518	*n*-Butylbenzene	1.3–1.9	Volatile solvent
100414	Ethylbenzene	1	Volatile solvent
3380345	Triclosan	0.02	Antiseptic, bactericide, disinfectant
99763	Methylparaben	0.2	Antifungal preservative in cosmetics
88040	Chloroxylenol	43070	Antimicrobial used to control bacteria, algae and fungi
70553	Benzenesulfonamide, 4-methyl-	1.1	Fungicide and mildewicide
92524	Biphenyl	0.05–0.09	Fungicide
126738	Tributyl phosphate	0.8–1.5	Used as a solvent in inks, synthetic resins, gums, adhesives (namely for veneer plywood) and herbicide and fungicide concentrates
333415	Diazinon	0.32–0.36	Acaricide/insecticide
10544500	Sulphur (S8)	1.9–4.8	Acaricide/insecticide
108689	3,5-Dimethylphenol	1.2–1.6	Used as pesticides and in the manufacture of disinfectants
5989275	d-Limonene	4.5–5.8	EU regulated contact allergen, personal care products
78706	Linalool	0.4–0.5	EU regulated contact allergen, personal care products
118581	Benzyl salicylate	0.24	EU regulated contact allergen, personal care products
91645	Coumarin	0.19–0.3	EU regulated contact allergen, personal care products
127515	α-Isomethyl lonone	0.1–1.2	EU regulated contact allergen, personal care products
94133	Propylparaben	0.16	Personal care products
123911	1,4-Dioxane	1.4–2.1	Personal care products, stabilizer in chlorinated solvents
94133	Propylparaben	0.08	Personal care products
119619	Benzophenone	0.2	Fixative for heavy perfumes
98555	Terpineol	4.8–12	Antiseptic, manufacture perfumes/soaps
101848	Diphenyl ether	0.03	Used to perfuming soaps
131577	Benzophenone	0.05	Fixative for heavy perfumes
80262	Terpinyl acetate	0.16	Fragrant liquid ester
108941	Cyclohexanone	0.5–10.7	Production of precursors to Nylon 6,6 and Nylon 6
55185	*N*-nitrosodiethylamine	0.07	Found in tobacco smoke and various processed foods
106514	*p*-Benzoquinone	0.15	Used as a chemical intermediate, a polymerization inhibitor, an oxidising agent, a photographic chemical, a tanning agent
91203	Naphthalene	0.8–1.1	PAH (used as insecticide)
86737	Fluorene	0.16	PAH
129000	Pyrene	0.02–0.05	PAH
85018	Phenanthrene	0.05–0.12	PAH
206440	Fluoranthene	0.01–0.02	PAH
496117	Indane	0.02–0.6	Petrochemical compound
59483	2H-Indol-2-one, 1,3-dihydro	2.0–7.0	Aromatic heterocyclic organic compound
95136	Indene	0.02	Used as a solvent and raw material for making other organic compounds
2687914	1-Ethyl-2-pyrrolidone	0.42–0.54	Used for refined oil products, pesticides, paints, resins and medicines
88755	2-Nitrophenol	0.06	Manufacture dyes, paint coatings, rubber coatings and fungicides
105602	Caprolactam	2	Precursor to Nylon 6, a widely used synthetic polymer
91576	2-methylnaphthalene	0.6	Used to make other chemicals such as dyes, resins, and, for 2-methylnaphthalene, vitamin K
121335	Vanillin	0.4–1	Used as a flavouring, used to mask bad tastes in medicines
126863	2,4,7,9-Tetramethyl-5-decyne-4,7-diol	0.5–0.6	Surfactant-wetting and foaming control (Background level 0.03 μg/l approx.)
93049	2-Methoxynaphthalene	0.05–0.3	Intermediate for the synthesis of nonsteroidal anti-inflammatory drugs. It is used for soap perfumes
131113	Dimethyl phthalate	1.2–1.5	Additives for plastics known as phthalates
132649	Dibenzofuran	0.03–0.05	Used as an insecticide, in the production of PVC, industrial bleaching and incineration
90437	*o*-Phenylphenol	0.15–0.9	Household disinfectants
84662	Diethyl phthalate	3.0–3.5	Additives for plastics known as phthalates
10543574	*N*,*N*,*N*′,*N*′-Tetraacetylethylenediamine	1.5–2.3	Peroxide bleach activator for household detergents, paper pulp
934349	2(3H)-Benzothiazolone	1.8–2.1	Used in tyre rubber manufacturing to accelerate the vulcanization process
13674878	Tris(1,3-dichloroisopropyl) phosphate	0.32–0.35	Used as a fire retardant and plasticizer in various plastic foams, resins, and latexes
115968	Tri-(2-chloroethyl) phosphate	0.1–0.15	Flame retardant
3622842	Benzenesulfonamide, *N*-butyl-	3.3–4	Neurotoxic plasticiser
84742	Di-*n*-butyl phthalate	4.0–5.0	Plasticizer
1678257	Benzenesulfonanilide	0.07–0.1	Building block
80057	Bisphenol A	0.45–1.1	In the manufacture of epoxy resins and polycarbonates for food packaging
298464	Carbamazepine	0.08	Anticonvulsant and mood stabilising drug

(continued on next page)

Table 3 (*continued*)

CAS number	Name	Approximate concentration μg/l	Possible use
103231	Bis (2-ethylhexyl) adipate	0.1–0.26	Plasticizer
51036	Piperonylbutoxide	0.12–0.16	Insecticide synergist, especially for Pyrethrins and Rotenone
117817	Bis(2-ethylhexyl)phthalate (DEHP)	1.5–4	Additives for plastics known as phthalates (mrv 1 μg/l)
110861	Pyridine	0.8	Used as a precursor to agrochemicals and pharmaceuticals
149575	2-Ethylhexanoic	1	Widely used to prepare metal derivatives that are soluble in nonpolar solvents
102761	Triacetin	0.06	Plasticizers fuel/feed/cigarette additive
77097	Tributyl acetylcitrate	0.06	Plasticizer or carrier solvent permitted in the field of food additive, food contact material as well as for polymers specially for cellulosics
58082	Caffeine	13.0	Psychoactive stimulant drug
57885	Cholesterol	50.0–190.0	Waxy steroid of fat that is manufactured in the liver or intestines

2.5. Pacific oyster (Crassostrea gigas) 48 h embryo development test

The oyster embryo development test was adapted from the technique by Thain (ICES, 1991), conducted for 24 h at 24 ± 1 °C. Conditioned Pacific oysters were purchased from Guernsey Sea Farms Limited and were sexed by the suppliers. Male and female gametes were obtained by stripping the gonads. Samples were prepared by taking an aliquot of the acetone extract, evaporating to almost complete dryness (negligible acetone trace) and then reconstituting in Lowestoft filtered seawater. Solvent controls were prepared in the same way using only acetone instead of a sample in Acetone. The test was carried out using 4.5 ml samples in polystyrene 12-well cell well plates. Oyster embryos are allowed to develop to the 16–32 cell stage of development, and 50 embryos/ml, estimated by volume, are then added to each replicate well. After 24 h the test was terminated using 4% formalin to fix the embryo development stage and the number of healthy developed larvae was assessed under the light microscope. Solvent controls were run at the same time, containing an amount of solvent consistent with the highest amount found in the sample extracts. All wells were incubated under the same conditions with percentage embryos undergoing normal larvae development as the endpoint.

2.6. GC–MS target based MR chemical screen

In parallel to samples collected for bioassay screening 1 litre of water was collected for chemical screening analysis. Water samples were placed on ice and frozen, before being shipped back to the UK under dry ice for chemical analysis. GC–MS analysis was carried out by the UK Environment Agency's National Laboratory Service using their full-scan mass spectrometry GC–MS tool for multi-residue screening of volatile (VOCs) and semi-volatile compounds (SVOCs). The method uses a retention time locked, custom built target MS database with >1050 compounds, allowing for the identification and measurement of a wide range of organic pollutants. The method consists of analysing the samples using a GC–MS operating in full-scan mode. This is paired with the latest deconvolution reporting software (DRS) to perform searches of the sample spectra against the target database. This approach delivers high accuracy target identification for samples with unknown chemical contaminants and allows rapid analysis of a number of samples.

3. Results and discussion

For this project we were trying to understand where pollution effects were most likely to be detectable around the coastline and marine areas of Kuwait. Various effects such as fish kills and deaths of shellfish beds combined with at least one long term discharge of vast amounts of untreated sewage had created a situation where effects were being identified but there was not a clear link to suspected causes. We attempted to use a simple battery of chemical and biological tests to assess a number of sites to see if we could separate those of greatest concern from general background issues. Samples had to be taken and extracted for easy transport to the UK, where they were reconstituted. There will have been some losses at all points in the process, so any responses are likely to be quite conservative, compared to working with raw samples.

Table 4
Sulaibikhat Bay samples not including the Al Ghazali outfall. Chemicals identified by GC–MS residue scans. Listed chemicals are toxic, carcinogenic, indicators of other pollutants, indicators of industrial process wastes, pharmaceuticals or personal care products.

CAS number	Name	Approximate concentration μg/l	Possible use
74953	Dibromomethane	0.02–0.19	Volatile solvent
75252	Bromoform	0.44–8.1	Volatile solvent
103651	*n*-Propylbenzene	0.01	Volatile solvent
108678	1,3,5-Trimethylbenzene	0.08	Volatile solvent
95636	1,2,4-Trimethylbenzene	0.08	Volatile solvent
135988	sec-Butylbenzene	0.03	Volatile solvent
99876	*p*-Isopropyltoluene	0.01	Volatile solvent
98555	Terpineol	0.08–0.28	Antiseptic, manufacture perfumes/soaps
88040	Chloroxylenol	0.06–0.79	Biocide/antiseptic
126863	2,4,7,9-Tetramethyl-5-decyne-4,7-diol	0.1	Surfactant-wetting and foaming control (background level 0.03 μg/l approx.)
126738	Tributyl phosphate	0.06	Used as a solvent in inks, synthetic resins, gums, adhesives (namely for veneer plywood) and herbicide and fungicide concentrates.
3622842	Benzenesulfonamide, *N*-butyl-	0.2	Neurotoxic plasticiser
333415	Diazinon	0.02	Acaricide/Insecticide
58082	Caffeine	0.12–0.37	Psychoactive stimulant drug
10544500	Sulphur (S8)	0.03–0.1	Acaricide/Insecticide
496117	Indane	0.01	Petrochemical compound
108689	3,5-Dimethylphenol	0.01	Used as pesticides and in the manufacture of disinfectants
10543574	*N*,*N*,*N*′,*N*′-Tetraacetylethylenediamine	0.06	Peroxide bleach activator for household detergents, paper pulp.
6197304	Octocrylene	0.02–0.03	UV-filter

Due to the limits of the amount of concentrated sample available, the Oyster embryo screen (Fig. 2), was run on only a subset of the samples, selected to represent the breadth of expected site characteristics. The most polluted sites were expected to be the outfall areas at Al Bedaa, Salmiya and Al Messela. The least polluted sites were expected to be the J-buoy sites in open water areas of the bay. J5 buoy is in an enclosed shallow bay with the Al Ghazali outfall contributing to it, and so was expected to show some response. The Salmiya Sea site was over a hundred metres offshore from the Salmiya and other major outfalls, and so would have received some dilution. The assay showed well defined results, with effects in the sites tested that correlated well with the results of both the yeast screens and the GC–MS chemical screen. Negligible effects on development were seen in the two control samples and only minimal amounts of developmental abnormalities were seen for the two open water J-buoy sites J1 and J2. The effluent outfall sites, Al Bedaa, Al Messela and Salmiya, demonstrated almost no normally developed embryos. This was also true for the J5 buoy site, in Sulaibhikat Bay, possibly due to its proximity to the Al Ghazali effluent channel, which was not in the subset of samples assessed using the Oyster embryo screen. The size of the effect on the Salmiya coastal sample was unexpected and could be related to either the potency of the effluent, or the position that the sea sample was taken in relation to the effluent plume in the Gulf waters.

Yeast estrogen and yeast androgen screens were carried out on all the samples to look for chemicals that interacted with human estrogen and androgen receptors. The androgen screen data showed no positive responses with all sites below the limit of detection for the assay. Positive responses using dihydrotestosterone as a reference standard showed that the assay was working effectively. Data from the yeast estrogen screen (YES) are shown in Fig. 3. The YES data shows positive responses in a number of the samples. This indicates that chemicals in these samples bound with, and activated, the estrogen receptors in the modified yeast cells. Chemicals which do this are considered to be endocrine disruptors as they will have an effect on the expression of receptor based characteristics. Responses indicating higher concentrations of active chemicals are found at sites on, or adjacent to, effluent outfalls, but there were also effects measured in samples from open water at the buoy sites J3 and J5, and also at the coastal sample taken by boat in line with the Al Bedaa outfall. This is offshore from the three outfalls at Al Bedaa, Salmiya and Al Messela. It is uncommon to find these sort of effects in open water marine samples because the amount of dilution available is usually enough to drive all responses below the limits of detection.

The samples were also run through a chemical screening procedure. This looks for over 1000 different xenobiotic, mainly nonpolar chemicals, some of which have not been assessed for estrogenic effects. Various chemicals were picked up at the different sites including caffeine, parabens, bromoform, PAHs and flame retardants, though cholesterol was the largest contaminant by concentration, found at Al Ghazali in particular. Amongst the known endocrine disrupting chemicals found, Al Ghazali sites showed Bisphenol A, and several phthalates at microgram per litre concentrations. The outfalls at Al Bedaa, Salmiya and Al Messela had similar levels of two phthalates, whilst some of the J sites had varying levels of phthalates, or Bisphenol A but not many at any one site. 17-a-Ethinyl estradiol, found in most sewage effluents due to use of the contraceptive pill, did not appear on the list of chemicals detected, but is highly potent even at ng/l levels which would not be picked up by this rapid screening tool. Other common human steroids such as 17-β estradiol, estrone and estriol are also likely to be present in the effluents in low concentrations, adding to the levels of effect detected by the yeast screen. Sites have been grouped together as the offshore J-buoy sites, the Mishref outfalls, the enclosed Sulaibikhat Bay sites and the unlicensed Al Gahazali outfall on its own. Tables 1 to 4 show the identified chemicals for each of these groups of sites. The J-buoy sites and Sulaibikhat bay registered 19 chemicals of interest. Al Ghazali on its own had a list of 78 chemicals. Descriptions of potential uses for the chemicals allow a certain amount of speculation as to how the chemicals arrived in the effluent, but these are not necessarily how these chemicals were used before arriving in the samples.

When assessing the state of the environment in any scenario it is important to consider why we are concerned about the environment in question. Often for marine areas the concern is driven by regulatory need, which is in itself driven by the realisation that there is both a perceived and a real financial value to the marine environment which is based mainly around the ecology that it supports in the various habitats. Whether the value derives from human aesthetic/ecological appreciation, or an ability to harvest resources, it creates a need for protection and assessment to establish whether harm is being done, or recovery is taking place. When you start to consider threats to the species that populate those habitats the most obvious human impact is the addition of harmful chemicals in one form or another. Because the problems are caused by chemicals it is easy to look to chemical analysis to identify the issues and provide the necessary data to start to solve them. The results from the chemical screen used here show that this can be a successful approach for many chemicals; however, this is not the only approach to consider in situations with a wide number of varying inputs, with both known and unknown contaminants in mixtures.

The rapid GC–MS screen provided a lot of useful data which could be assessed in a number of ways including concentrations of known toxicants, identification of specific contaminants from specific sites or just the number of potentially harmful chemicals present at a site, which can be a rapid and simple method to screen sites initially, enhancing understanding of where problem areas are. Sites identified as containing more contaminants in the GC–MS data were generally supported by both the yeast screen data and the Oyster embryo screen.

These two tests show definite differences between the sites and between control samples and contaminated sites. The Oyster embryo test results represent the effects of chemicals which have both toxic and developmental effects on a very sensitive life stage. This could include any number of the chemicals identified as being present by the chemical screen, as pharmaceuticals, agricultural chemicals, plasticisers and petrochemicals all contain chemicals implicated in developmental abnormality. With the YES screen, it has been well established since the 1990s that domestic and industrial sewage effluents potentially contain a number of chemicals which can bind to and activate parts of the endocrine system such as the estrogen receptors used in this test (Harries et al., 1996 and 1999; Desbrow et al, 1998). Chemicals binding to these receptors have been linked to the development of intersex in lab and field trials (Jobling et al, 2003; Kirby et al, 2004; Nash et al, 2004; Thorpe et al, 2009). These range from human and animal hormones such as estrone and 17–β-estradiol, through powerful artificial hormones such as 17-α-Ethinyl estradiol, to industrial process chemicals such as Bisphenol A and various phthalates. Several of the sites are at or very near to sewage effluent outfalls, so the source of these chemicals and the route they take to get into the sample are clear and would be expected to affect both the Oyster and YES screens. Of more concern are the estrogenic responses from samples taken from the J sites (buoys moored in open water section of Kuwait Bay) and offshore as the dilution factor of any effluent stream should be very high at this point, making detection of any but the most highly contaminated effluents very unlikely. The concentrations found were low ng/l, but this is on the high side for open water marine samples (e.g. Atkinson et al, 2003; Beck et al, 2006). Effects on fish can be detected even where concentrations remain below 1 ng/l E2 equivalent (Henneberg et al, 2014).

Use of these relatively simple tests has allowed us to identify certain sites, such as Al Ghazali and Al Messela, as being sites with particular pollution problems, and even to identify where pollution may be entering the system for some of the areas, such as the bay surrounding the Al Ghazali effluent outfall. These are all sites with outfalls which receive part of Kuwait City's mainly domestic sewage effluent once it has been through treatment, and so might be expected to be areas facing more challenges in terms of water quality. This combined rapid testing

approach could be used to regularly monitor sites for change, or for reassessing priority sites after incidents or prolonged periods of little change might prove to be another useful tool for nonextracted samples. There are a number of similar scale tests that could be used to carry out a similar level of biological screen, or to broaden the scope, such as the acute copepod mortality test (ISO 14669, 1999), the 72 h acute Skeletonema assay (ISO 10253, 2006), Microtox, LumiMARA, sea urchin embryo development etc. In a competent laboratory these could also be done rapidly and provide information for samples from a number of sites.

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