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ESTROGENIC POTENTIAL OF THE VENICE, ITALY, LAGOON WATERS

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Abstract—The exposure of the Venice lagoon (Italy) to endocrine-disrupting compounds (EDCs) from different sources was investigated. Spatial and time distribution of EDC concentrations were determined in four sampling sessions (December 2001–May 2002) by solid phase extraction followed by high-performance liquid chromatography separation coupled with mass spectrometry detection via electrospray interface (SPE-HPLC-ESI-MS), which allowed identification of natural (estradiol, estrone) and synthetic estrogenic compounds, both steroidal (ethinylestradiol, mestranol) and nonsteroidal (benzophenone, bisphenol-A, nonylphenol, nonylphenol monoethoxylate carboxylate). No significant differences in the EDC distribution were observed between stations located near selected sources (raw sewage from the historical center of Venice, treated municipal and industrial effluents from sewage treatment plants, and areas undergoing the inflow of rivers). While synthetic nonsteroidal analytes were recorded in the 1 to 1,040 ng/L range (average concentration: 34 ng/L), steroidal EDC (estradiol, ethinylestradiol) concentrations were lower (1–125 ng/L; average concentration: 8 ng/L). The estrogenic activity of lagoon waters was estimated in terms of estradiol equivalent concentration (EEQ) by applying the estradiol equivalency factors (EEFs). Steroidal EDCs (estradiol, ethinylestradiol) contributed >97% to the total potential estrogenicity of the waters, which accounted for 4 to 172 ng/L (average: 25 ng/L), as total EEQs. These levels are likely to pose adverse effects on the Venice lagoon aquatic organisms.

Keywords—Endocrine-disrupting compound Lagoon waters Estradiol equivalent concentration

INTRODUCTION

Environmental concern about chemicals that can alter endocrine functions is increasing in the recent years due to the wide occurrence of such chemicals in the aquatic environment and their potential hazard to both aquatic [1] and human life [2]. An endocrine disrupter is an exogenous substance that alters functions of the endocrine system and consequently causes adverse health effects on an intact organism, its progeny, or on (sub)populations [2]. Estrogenic compounds are the most studied endocrine-disrupting compounds (EDCs), because the relatively low specificity of estrogen receptors makes not only natural estrogens, such as estradiol (E2) and estrone (E1), but also many synthetic chemicals, such as pesticides, alkylphenols, dioxins, and synthetic steroids, capable of estrogenic activity [3–5]. Today, thousands of compounds are demonstrated or suspected to modulate or mimic the actions of steroidal hormones and produce biological responses qualitatively similar to those produced by endogenous hormones [1,2,6]. Reduced fecundity and/or fertility, abnormally elevated levels of plasma vitellogenin, and intersex gonads are the most commonly observed effects produced by EDCs on aquatic wildlife species [5–8]. All these effects could cause impacts at both population and ecosystem level [5]. Because the reproductive cycle of aquatic organisms occurs in water, the aquatic environment presents an ideal medium where the potential effects of EDCs on wildlife populations can be studied. Fish are one of the most thoroughly studied groups of wildlife in terms of effects of chemicals on developmental and reproductive processes [5,6,8,9].

As a result of all such evidence, EDCs are being included in European and international conventions for the protection of the aquatic environment, especially of surface waters. European policy regarding endocrine disrupters is outlined by the Community Strategy for Endocrine Disrupters (COM[1999] 706 final) [2]. In the United States, the U.S. Food Quality Protection Act [10] requires the U.S. Environmental Protection Agency (U.S. EPA) to test all pesticide chemicals for endocrine-disrupting effects, while the Safe Drinking Water Act Amendments [11] authorizes the U.S. EPA to develop and to implement a program for identifying and regulating substances that may have effects on humans similar to those produced by naturally occurring estrogens or other endocrine effects.

The chemicals examined in this work are both of natural and synthetic origin. The E2 is the most potent and biologically active estrogen, naturally synthesized in female ovaries, while E1 is its main degradation product originated in the liver. The synthetic derivatives of natural estrogens, such as ethinylestradiol (EE2) and mestranol (MES), are used extensively in oral contraceptives and for treating both pre- and postmenopausal disorders. Furthermore, EE2 is used in human medicine to treat conditions such as amenorrhea, breast carcinoma, hypogonadism, postpartum breast engorgement, and prostatic carcinoma (Environmental Health Perspectives official website: http://ehp.niehs.nih.gov). Selected synthetic nonsteroidal estrogens exhibit estrogenic activity with no structural similarity with steroidal EDCs. Bisphenol-A (BPA) is an industrial product used as intermediate and additive monomer in plastics; benzophenone (BP) is used as photoinitiator, fragrance enhancer, ultraviolet curing agent, and as additive in insecticides, pharmaceuticals, plastics, coatings, and adhesives. Nonylphenol monoethoxylate carboxylate (NP1EC) and nonylphenol (NP) are the main microbial degradation products of nonylphenol polyethoxylates (NPE), which are nonionic industrial surfactants used worldwide in detergents, paints, herbicides, and cosmetics.

Because E2, E1, EE2, and MES are excreted with urine, final effluents from sewage treatment plants and untreated municipal sewage are the primary sources of natural and synthetic

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steroidal estrogens for the aquatic environment. Treated and untreated industrial effluents are the main sources of synthetic nonsteroidal estrogens.

Based on daily excretion of estrogens by humans and dilution factors, as well as on field observations, sub-ng/L up to μg/L levels of natural and synthetic EDCs are expected to be encountered in any aquatic ecosystem affected by human activities [3,9,12–15].

Many biological experiments have been performed in order to evaluate potential effects of EDCs. Estrogenic potency of an endocrine disrupter commonly is related to E2 by means of estradiol equivalency factors [16]. The estradiol equivalency factor (EEF) is the quotient of values $EC50_{E2}/EC50_{test\ compound}$ (EC50 = median effective concentration) and conventionally it is set to 1 for E2. These factors cover a very wide range of values. The EE2, E1, and MES are the most potent steroidal EDCs: Their EEFs are 1.5, 5.8×10^{-2} and 1.3×10^{-2}, respectively [1,17–19]. Nonsteroidal EDCs indeed are much less potent than E2 (EEFs: 5.7×10^{-4}, 1.0×10^{-4}, 2.0×10^{-5}, 6.1×10^{-7} for BPA, NP, NP1EC, and BP, respectively) [1,5,17–23]. Despite the different applied bioassays reported, EEFs generally are of the same order of magnitude for each tested EDC. Because the additive behavior of the estrogenic activity of EDC mixtures recently has been demonstrated in the E-screen assay [17], the total estrogenic activity of water samples contaminated by EDCs can be evaluated quantitatively in terms of estradiol equivalent concentration, provided that individual concentrations of all active compounds are known. The estradiol equivalent concentration (EEQ), expressed typically as pmol/L or ng/L, is the total amount, E2-normalized, of estrogenic compounds contained in a sample. Literature reports show that when EEQ values, obtained from both laboratory studies or field investigations, are in the 2.5 to 10 ng/L range, an increase of plasma vitellogenin levels can occur in fish [7], while for EEQs ranging between 10 and 100 ng/L, a decrease of testicular growth can be observed [24]. When EEQs >100 ng/L are determined, the production of testis-ova in the testes of male fish can be noticed [25]. However, it is known from literature that EEQs calculated by chemical analysis are always higher (by a 2–4 factor) than those determined by the biological analysis due to detoxification mechanisms taking place at cellular level [1]. Until now, to the best of our knowledge, no EEQ thresholds values over which a contaminated aquatic environment can cause an observed damage to exposed organisms have been proposed. To the best of our knowledge, effects were observed only on fish. No data are available of these effects relative to other aquatic organisms.

The Venice lagoon can be considered quite suitable for investigating occurrence and effects of EDCs. It is a shallow coastal lagoon ecosystem (average depth: ~1 m ± 0.3 m, average salinity: 31 ± 4 ‰) connected with the Adriatic Sea by three inlets (Fig. 1). This lagoon is subjected to heavy anthropogenic pressure of nutrients and pollutants, which increased greatly during the last century, following urban, industrial, and agricultural development. The main potential sources of EDCs in the Venice lagoon are: Raw sewage from the historical center of Venice (~110,000 equivalent inhabitants, over the year, including tourists), final effluents from municipal sewage treatment plants (~400,000 equivalent inhabitants), treated industrial effluents from the large industrial district of Porto Marghera (~3,000 ha), and agricultural runoff and contaminated freshwater from a variety of rivers, canals, and streams (total discharge: 31.5×10^{-6} m³/s) [26]. Despite the several expected inputs of EDCs, no data are available so far about the potential impact of EDCs on lagoon wildlife.

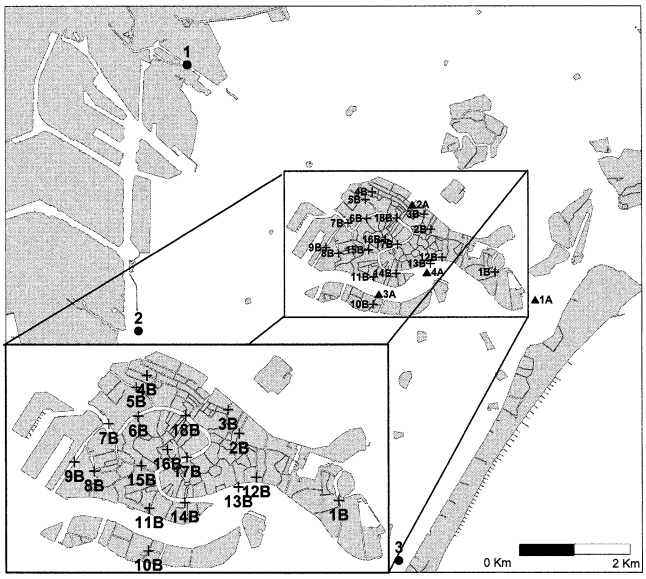

Fig. 1. Location of sampling stations in the Central Venice lagoon and in the historical center of Venice (Italy).

Here we report the results of a survey conducted in the central part of the Venice lagoon, the most exposed to chemical pollutants. Natural (E2, E1), and synthetic, both steroidal (EE2, MES) and nonsteroidal (NP, NP1EC, BPA, BP) estrogenic compounds were analyzed by solid phase extraction followed by high-performance liquid chromatography separation coupled with mass spectrometry detection via electrospray interface [27], with the aim of investigating EDCs occurrence, spatial and time distribution, and calculating EEQs of the lagoon waters.

MATERIALS AND METHODS

Chemicals

Analytical standards of E2, BPA, E1, MES, EE2, NP, and BP (>98% pure) were obtained from Fluka (Büchs, Switzerland). The NP1EC (purity ~90%) was purchased from Ciba Specialty Chemicals (Basel, Switzerland) and further purified by semipreparative HPLC up to a final >99% purity. Isotope-labeled EDCs used as internal standards (*n*-NP-d4, BPA-d16, EE2-d4 and E2-d3) were obtained by Chemical Research 2000 (Rome, Italy). Ammonium acetate ($AcNH_4$), HCl, and NH_3 solutions (32%, v/v in water, and 37%, v/v in methanol, respectively), all >99% pure, were from Fluka. All employed organic solvents were HPLC ultra-gradient grade from Romil (Dublin, Ireland). Water for chromatographic purposes was purified by a MilliQ system (Millipore®, Billerica, MS, USA). Standard stock solutions were prepared for all compounds but MES and E1 at 1 μg/μL by dissolving solid standards in methanol. Both MES and E1 were dissolved in methanol at 0.1 μg/μL. All working solutions (100, 10, 1, 0.1 ng/μL) were weekly prepared by diluting stock solutions in 2 ml Teflon®-capped glass vials from Agilent (Avondale, PA, USA).

Sampling

Grab water samples were collected in dark glass bottles in 25 stations, three of which located in the central Venice lagoon, 22 placed in the inner canals of Venice historical center (Fig.

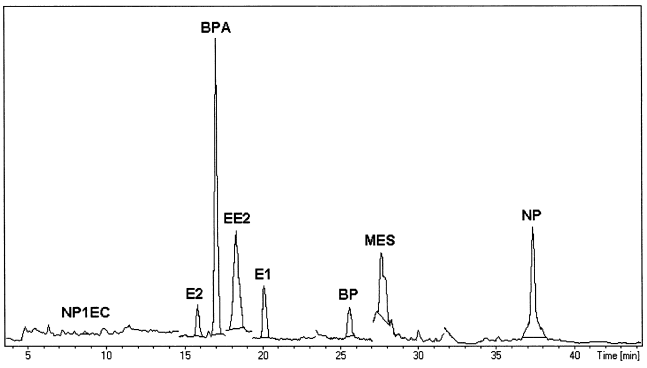

Fig. 2. Chromatogram of a lagoon water extract collected in an inner Venice (Italy) canal during the May sampling session and determined by high-performance liquid chromatography-electrospray interface-mass spectrometry (concentrations: E2: 21 ng/L; BPA: 60 ng/L; EE2: 29 ng/L; E1: 70 ng/L; BP: 118 ng/L; MES: 2.5 ng/L; NP: 10 ng/L). Refer to Table 1 for compound definitions.

1). Selected sites were monitored by four sampling sessions in December 2001, and February, April, and May 2002. Grab water samples were collected under neap tide conditions in order to minimize tidal influence. Just after collection, $HgCl_2$ (100 ppm) was added to each sample. Particulate matter was removed by filtration on 0.7 μm Whatman glass fiber filters (Maidstone, UK). Samples were stored at dark at 2°C before analysis, always performed within 96 h after sampling.

Physico–chemical data (dissolved O_2, pH, temperature, and salinity) were concurrently determined at each station during sampling by a multiparameter probe.

Extraction, chromatographic separation, and detection

Simultaneous determination of selected compounds in lagoon water samples was performed by solid-phase extraction (SPE) on C-18 cartridge followed by HPLC gradient separation on a C-8 column coupled with MS detection via electrospray interface (SPE-HPLC-ESI-MS) [27]. A typical mass-chromatogram of a water sample extract (May sampling campaign, station 23) is presented in Figure 2. Among the detected EDCs, only NP1EC was < method detection limit ([MDL] minimum concentration of an analyte giving to a MS signal-to-noise ratio of 3).

Estimation of EEQ

The EEFs were taken from literature and averaged before application (Table 1) [1,5,15–22]. These EEF values were then multiplied by the measured molar concentration of each analyte detected, resulting in an EEQ for each chemical. The EEQ value of each sample was obtained by adding the individual EEQs.

Statistical analysis

Data treatment was performed with SPSS® for Windows (Ver 10.0, SPSS, Chicago, IL, USA). The EDC concentrations and physico–chemical data of the lagoon waters were compared to highlight a possible seasonal trend. Prior to analysis, data obtained were transformed to improve normality and homogeneity of variance. The preparatory normalization step was achieved by ranking the data and the statistical treatment was based on an R-mode factor analysis. Data were ranked in order to obtain comparable units for variables and to identify outliers; in particular, each value was replaced by a number giving its place in the sequence from the highest to the lowest or vice versa [28]. The adopted approach was selected to take into consideration two aspects: The constant sum problem for some parameters (e.g., chemical and physical parameters) and the comparability of data. Among the normalization procedures, ranking was selected as the most suitable one, because mainly the goal was exploratory and more oriented at evidencing relationships among variables rather than single variable distributions. Among the classification tests, discriminant function analysis was performed in order to disclose possible correlations between physico–chemical parameters and EDC concentrations. Among data-reducing functions, factor analysis was carried out in order to decrease the data dimension without information loss.

RESULTS AND DISCUSSION

Occurrence and spatial distribution of EDCs in lagoon waters

The sampling stations (Fig. 1) were selected in order to evaluate the possible EDC sources in the lagoon of Venice. Station 1 (~3 m deep) was located near the mouth of the Osellino River that carries both treated and untreated sewage

Table 1. Average values of estradiol equivalency factors (EEFs) taken from literature

Compound[a]	EEF[b]	Endpoint[c]	References
E2	1[d]	—	—
E1	5.8×10^{-2}	ES, ER,	[1,15,18]
EE2	1.5 (5.71–0.091)	ES, ER, HGELN	[1,15,18]
MES	1.3×10^{-2} 5.7×10^{-4}	ES	[1]
BPA	$(5.3 \times 10^{-5} - 6.0 \times 10^{-3})$	ES, ER	[1,15,16,18,20]
BP	6.1×10^{-7} 1.0×10^{-4}	ES ES, RTH, ER,	[17]
NP	$(9.0 \times 10^{-6} - 7.0 \times 10^{-3})$ 2.0×10^{-5}	ERC, YES	[1,5,15,18–22]
NP1EC	$(6.3 \times 10^{-6} - 2.5 \times 10^{-4})$	ES, RTH, YES	[1,5,21]

[a] E2 = estradiol; E1 = estrone; EE2 = ethinylestradiol; MES = mestranol; BPA = bisphenol-A; BP = benzophenone; NP = nonylphenol; NP1EC = nonylphenol monoethoxylate carboxylate.
[b] Mean (range).
[c] ES = E-Screen assay; Vtg = plasma vitellogenin induction; ER = estrogen receptor binding; RTH = rainbow trout hepatocytes; ER-C = ER-CALUX assay; YES = recombinant yeast estrogen screen; HGELN = luciferase reporter gene assay using HGELN cells.
[d] Set as conventional reference.

from the mainland, as well as agricultural runoff. Station 2 (located at 7 m depth in a channel) was situated nearby the outlet of a large mechanical–biological sewage treatment plant (~320,000 equivalent inhabitants), receiving both municipal and industrial sewage. Station 3 (~4 m deep), located nearby Lido Island, is affected by municipal sewage from the facing residential area. The remaining 22 stations (average depth: 1 m), located in the inner canals of Venice, receive municipal untreated sewage from the historical center of Venice. These stations were divided into two groups: The former (group A), including stations located on the external side of the historical center (1A–4A), are exposed to a greater sewage dilution due to the tidal exchange; the latter (1B–18B), are located in the narrow canals of the innermost part of the historical center. All stations were monitored during four sessions: December 2001, February, April, and May 2002. Concurrently, typical physico–chemical parameters (dissolved O_2, pH, temperature, and salinity) were monitored. Temperature was the only parameter that changed significantly during sampling period, exhibiting values of 9.2°C (min–max: 7.5–12) in December, 8.4°C (min–max: 6.5–14) in February, 16°C (min–max: 12–19) in April and 22°C (min–max: 21–24) in May, respectively. Dissolved O_2 ranged between 5.4 mg/L in May to 8.9 mg/L in February. A quite high value (18 mg/L) was observed at station 1 during February session. Such high values are not unusual in the Venice lagoon, because of the high primary production, as reported by literature [29]. Other parameters remained fairly constant throughout the sampling period: Ph and salinity values were in their typical ranges (8.0–8.4 and 27–35 mg/L, as NaCl, respectively) all over the sampling period. In Figure 2, a typical HPLC-ESI-MS chromatogram is presented. All determined concentrations in stations 1, 2, 3, as well as in the stations of groups A and B, are reported in Table 2. Remarkably, only <20% of the examined concentration values of the analyzed EDCs were <MDLs.

A preliminary spatial evaluation of the distribution of EDCs showed the systematic occurrence of steroidal estrogens in most of selected stations, with concentrations of 1.2 to 52 ng/L (average concn.: 4.5 ng/L) for E2, 2.3 to 85 ng/L (average concn.: 7.4 ng/L) for E1, 2.3 to 125 ng/L (average concn.: 12 ng/L) for EE2, and 2.3 to 75 ng/L (average concn.: 9.4 ng/L) for MES. While recorded levels for natural EDCs were in the typical concentration range previously reported for treated and untreated sewage effluents, as well as for freshwaters (0.05–88 ng/L and 0.1–132 ng/L for E2 and E1, respectively), synthetic EDCs showed noticeably higher concentrations than those found previously (0.05–31 ng/L and 1–3 ng/L for EE2 and MES, respectively) [3,30–34].

Among nonsteroidal EDCs, BPA, a typical industrial byproduct, was recorded in all the monitored stations, although at concentrations (1.5–88 ng/L; average concn.: 13 ng/L) lower than those recorded previously in surface waters and sewage effluents (10–37,000 ng/L) [19,31,32,35]. The BP, used as a flavor ingredient and additive, exhibited the highest concentrations (up to 1,040 ng/L; average: 81 ng/L) among selected EDCs, far higher than previous literature data (1.3–190 ng/L) (http://ntp-server.niehs.nih.gov /htdocs/Chem_Background/ExecSumm/Benzophenone.html). The two selected biodegradation products of nonylphenol polyethoxylate (i.e., NP and NP1EC) were found at similar concentration levels (2.8–201 ng/L; average: 25 ng/L, and 1.1–256 ng/L; average: 18 ng/L, respectively), which were lower than those reported elsewhere (250–644,000 ng/L) [7,12,21,32].

Table 2. Dissolved concentration of analyzed EDCs in lagoon water samples (1,000 ml) collected during sampling. See Table 1 for definitions; MDL, method detection limit

Month	Station	Average concentration (min–max) (ng/L)[a]							
		E2*	E1**	EE2**	MES***	BPA*	BP***	NP**	NP1EC*
December		E2	E1	EE2	MES	BPA	BP	NP	NP1EC
	1	1.9	2.3	<MDL	1.8	17	99	36	85
	2	2.9	<MDL	<MDL	2.9	18	92	35	72
	3	1.8	<MDL	<MDL	1.7	3.2	25	21	56
	Group A	1.0 (−MDL-1.8)	<MDL	10 (<MDL-28)	5.0 (<MDL-28)	17 (5.7–32)	225 (113–433)	70 (25–144)	23 (1.9–78)
	Group B	3.6 (<MDL-16)	<MDL	28 (<MDL-75)	13 (<MDL-73)	31 (11–66)	313 (53–1,040)	78 (5.2–201)	34 (<MDL-187)
February	1	2.7	<MDL	22	7.3	52	20	10	83
	2	1.5	4.2	<MDL	11	10	31	20	87
	3	2.1	<MDL	2.0	7.3	7.3	17	44	18
	Group A	1.3 (<MDL-2.7)	5.0 (<MDL-16)	1.7 (<MDL-8.3)	8.5 (<MDL-22)	3.0 (<MDL-10)	8.4 (<MDL-25)	13 (<MDL-68)	4.8 (<MDL-27)
	Group B	2.0 (<MDL-7.3)	13 (<MDL-43)	5.6 (<MDL-35)	4.0 (<MDL-29)	7.3 (<MDL-59)	7.9 (<MDL-38)	24 (<MDL-135)	16 (<MDL-256)
April	1	1.7	<MDL	2.1	6.5	8.6	7.3	<MDL	7.3
	2	2.2	4.2	3.0	2.0	4.6	5.5	<MDL	3.0
	3	1.5	<MDL	7.2	<MDL	3.2	5.6	<MDL	4.2
	Group A	0.9 (<MDL-2.7)	<MDL	8.2 (<MDL-13)	<MDL	3.1 (<MDL-8.0)	26 (5.0–60)	<MDL	7.7 (<MDL-28)
	Group B	4.0 (<MDL-31)	4.7 (<MDL-32)	7.1 (<MDL-25)	9.2 (<MDL-66)	7.3 (<MDL-39)	24 (<MDL-259)	<MDL	21 (<MDL-199)
May	1	3.9	<MDL	112	<MDL	8.0	20	<MDL	<MDL
	2	50	<MDL	37	<MDL	11	20	<MDL	<MDL
	3	42	<MDL	6.3	<MDL	1.4	14	<MDL	<MDL
	Group A	<MDL	<MDL	3.9 (<MDL-6.7)	12 (6.4–32)	2.2 (1.3–4.3)	20 (8.4–41)	15 (6.8–23)	<MDL
	Group B	8.3 (MDL-51)	18 (<MDL-85)	11(<MDL-56)	18(<MDL-75)	15(1.1–60)	43(<MDL-117)	9.0(2.8–21)	<MDL

[a] * MDL, 1 ng/L; ** MDL, 2 ng/L; *** MDL, 5 ng/L.

In some more detail, according to different EDC sources affecting the Venice lagoon, an accurate station-by-station evaluation highlighted some peculiarities. At station 1, located near the mouth of the Osellino River and affected by municipal sewage, agricultural runoff, and, to some extent, industrial sewage, EE2 was the dominant EDC with average concentration of 34 ng/L, while E2 and MES exhibited systematically lower average concentrations: 2.6 ng/L and 3.9 ng/L, respectively, and E1 was < MDL. Based on the daily human excretion of E1, E2, and EE2, these compounds were expected to be found according to the following concentration order: E1 > E2 > EE2 [30]. Vice versa, EE2 resulted the dominant steroidal EDC occurring in station 1, probably due to the fact that it is more persistent in the studied environment than natural estrogens E1 and E2 [31]. Moreover, all selected nonsteroidal EDCs (BP, BPA, NP1EC, and NP) were found systematically (average concentrations: 37 ng/L, 21 ng/L, 44 ng/L, and 12 ng/L, respectively) indicating that both municipal and industrial effluents affect station 1.

At station 2 (located near the outfall of the Fusina municipal/industrial sewage treatment plant), all analytes also were recorded. Among steroidal EDCs, E2 and EE2 were the dominant, with average concentrations of 14 ng/L and 10 ng/L, respectively, while E1 and MES concentrations were lower: 2.1 ng/L and 4.0 ng/L, respectively. Among nonsteroidal EDCs, BP and BPA were recorded at 37 ng/L and 11 ng/L, respectively. The NP1EC was found dominant over NP, with average concentration of 41 ng/L versus 14 ng/L, according to the expected higher concentration of carboxylated NPE biointermediates in the effluents from mechanical–biological sewage treatment plants, with respect to neutral ones [12].

At station 3, mainly affected by municipal raw sewage, nonsteroidal EDCs were more abundant than steroidal ones. Average concentrations were 16 ng/L for NP, 20 ng/L for NP1EC, 15 ng/L for BP, and 3.8 ng/L for BPA, versus 3.9 ng/L for EE2, 112 ng/L for E2, and 2.3 ng/L for MES. The E1 systematically was <method detection limit (MDL) in all sampling sessions at this station. Such evidence indicates that station 3 is affected by both domestic and industrial sewage.

Nonsteroidal EDCs were dominant in the 22 stations located in the inner lagoon canals and affected by raw sewage, suggesting nondomestic wastes as main carriers of these chemicals. Benzophenone was the dominant EDC both in group A and B stations, with concentration values of 70 ng/L and 97 ng/L, respectively. Also NP and NP1EC values were higher in group B than in group A stations, according to a greater sewage dilution characterizing the latter; in particular, NP was recorded at 28 ng/L and NP1EC at 18 ng/L for group B, while average concentrations in group A stations were 25 ng/L and 8.9 ng/L, respectively. The occurrence of high concentrations of NP and NP1EC in the inner Venice canals can be ascribed to nondomestic activities that use industrial detergents, because NPE have not been used in Italian household detergents since the mid-1980s [12]. The BPA also was recorded at higher levels in the group B stations (15 ng/L) compared to the group A stations (6.3 ng/L). Among the steroidal EDCs, the synthetic ones were dominant in A stations, with concentrations of 7.9 ng/L for EE2 and 6.4 ng/L for MES, respectively, while the natural ones (E2, E1) were both <MDL. In group B stations, steroidal EDC concentrations were higher than those recorded in group A, with average concentrations: 4.5 ng/L for E2, 8.9 ng/L for E1, 13 ng/L for EE2, and 11 ng/L for MES.

The overall spatial distribution clearly indicates that all selected EDCs are widespread contaminants of the Venice lagoon waters. Moreover, both municipal and industrial sources were found to contribute significantly to the overall EDC contamination, which appears to be redistributed inside the central lagoon by hydrodynamics.

Seasonal trend

The concentration data were treated statistically to disclose possible correlation with physico–chemical parameters. Temperature was the discriminant variable between winter (December–February) and spring (April–May) sessions, according to the factor structure matrix (loading = −0.417). Recorded average water temperatures were 9.2°C, 8.4°C, 16°C, and 22°C in December, February, April, and May, respectively. Such differences were expected to cause significant changes in the residual water concentrations of the selected compounds, because the biodegradation rates of E2, E1, and NP are strongly temperature-dependent [12,31]. Because reported half-lives for NP, NP1EC, and EE2 in surface waters are in the 10- to 30-d range [31–32], longer than water residence time, typically 2 to 10 d in the Venice lagoon [26], such chemicals are not expected to undergo a significant biodegradation. The other examined EDCs for which half-life data are available (BPA: 2.5–4 d; E2: 2.8 d; E1: 3 d) [31,36] instead are expected to biodegrade to a certain extent. However, a correlation with temperature was noticeable only for NP, by means of both discriminant function analysis (Wilks' *lambda* = 0.245, F = 97.5) and factor analysis (first principal component = 0.832). Taking into account that NP formation results from the anaerobic degradation of NPE, the inverse correlation between NP concentration and temperature would suggest a preferential occurrence of the hydrolytic biodegradation mechanism at lower temperatures, compared with the hydrolytic oxidation mechanism. This is confirmed by the NP/NP1EC concentration ratios (Table 2) [37].

Estimation of estrogenic potential

The EDC exposure concentrations permitted to assess the possible endocrine effects on the Venice lagoon biota. The EEQs of lagoon waters were calculated in order to obtain a cumulative evaluation of the estrogenic potential. Because no EEF values were calculated for saltwater samples, reported EEFs for freshwaters were used for each individual EDC. Moreover, because many different bioassays have been developed to determine EEF values, EEF values reported by literature were averaged and applied for the calculation of the EEQ of each sample (Table 1). The determined EEQ values ranged between 3.4 and 172 ng/L (average value: 25 ng/L) for all samples, with no marked seasonability. Time-averaged values and ranges for every station are reported in Table 3. The highest levels were recorded in May, stations 1 and 2 (172 ng/L and 106 ng/L, respectively), while the lowest EEQ value was found in February, station 2 (3.4 ng/L). In Figure 3, the average EEQs calculated in the four sampling sessions for stations 1 to 3, groups A and B, respectively, are reported. The average values calculated for December, February, and April, were very similar (13 ng/L, 12 ng/L, and 11 ng/L, respectively), while in May the average EEQ was much higher (72 ng/L). The EEQ estimation also allowed identification of the compounds contributing most to the total estrogenic potential of the lagoon waters (Table 3). Synthetic steroids (EE2 and MES) accounted for a median 77% (with EE2 accounting for ~76%) of the total EEQ values, while natural steroids

Table 3. Relative contribution of all individual endocrine disrupting compounds to the total estrogenicity (EEQs) of the Venice lagoon waters. See Table 1 for definitions

	Station				
	1	2	3	A	B
Compound	Contribution (%) to estradiol equivalent concentration				
EE2	11	44	24	13	17
E1	0.3	2.6	0.7	1.1	2.2
E2	88	52	75	84	80
MES	0.3	0.6	0.2	1.3	0.8
BPA	<0.1	0.1	<0.1	<0.1	0.1
BP	<0.1	<0.1	<0.1	<0.1	<0.1
NP	<0.1	<0.1	<0.1	<0.1	<0.1
NP1EC	<0.1	<0.1	<0.1	<0.1	<0.1
EEQ[a] (ng/L)	54 (5–172)	30 (3.4–106)	18 (3.4–51)	10 (4.2–16)	23 (11–38)

[a] Mean value (min–max).

accounted for a median 23% (with E2 accounting for ~22%). Both E2 and EE2 were the EDCs that mostly contributed to the total EEQ because of their EEFs (average values: 1.5 and 1, respectively) and their elevated recorded concentrations. Both E2 and EE2 contributed to an extent >97% to the total estrogenic activity of all examined samples, and were responsible for the high EEQ scores determined for stations 1 and 2 during the May session (172 ng/L and 106 ng/L, respectively). Although synthetic nonsteroidal EDCs were found systematically, even at high peak concentrations (NP: 201 ng/L, group B; NP1EC: 256 ng/L, group B; BPA: 88 ng/L, station 1; BP: 1,040 ng/L, group B), they only accounted for a negligible portion of the total EEQ (<0.1%) because of their much lower EEFs (1×10^{-4}, 2×10^{-5}, 6×10^{-4}, 6×10^{-7}, respectively) with respect to steroidal EDCs.

The EEQ threshold values over which a contaminated aquatic environment can cause an observed damage to exposed organisms are not yet available. However, laboratory biological tests, usually performed with one or two EDCs, reported that EEQ values in the 0.1 to 5 ng/L (as nominal concentration range) can already induce effects in certain fish [1,7,25,31,38,39]. For example, the in vivo estimated concentration of NP responsible for vitellogenin induction in rainbow trout is between 1 and 10 μg/L (corresponding to EEQ = 0.1–1.1 ng/L) [7]. Studies with EE2 on medaka have shown the production of testis-ova in testes of males exposed to concentrations > 63.9 ng/L (corresponding to EEQ > 96 ng/L). The Danish Environmental Protection Agency reported testicular effects at E2 concentration between 10 to 50 ng/L (same value for EEQ) [31]. In another study the observed exposure concentrations for plasma vitellogenin accumulation in sheepshead minnows were 100 ng/L (EEQ = 150 ng/L) for EE2 and 200 ng/L (EEQ = 200 ng/L) for E2 [39]. Based on the comparison between literature effect concentrations [7,24,25] and determined EEQs, the Venice lagoon waters show a significant potential to induce biological effects on the endocrine system of aquatic organisms, if the exposure to these levels are of sufficient duration. In fish these expected effects would be an increase in vitellogenin levels at stations with EEQs >2.5 ng/L, a decrease in testicular growth at stations with EEQs >10 ng/L, and the development of testis-ova at stations with EEQs >100 ng/L. Current work is focusing on the determination of predicted effects in selected lagoon organisms such as fish and filter feeders.

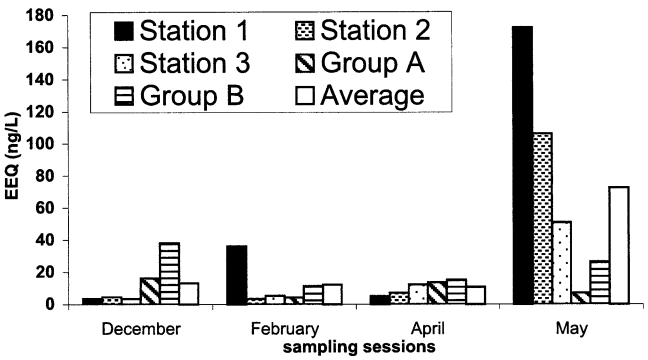

Fig. 3. Average estradiol equivalent concentration ([EEQs] ng/L) calculated for stations 1 through 3 and the group of stations A and B (see Fig. 1) in the four sampling sessions.

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REFERENCES

1. Körner W, Spengler P, Bolz U, Schuller W, Hanf V, Metzger JW. 2001. Substances with estrogenic activity in effluents of sewage treatment plants in southwestern Germany. 2. Biological analysis. *Environ Toxicol Chem* 20:2142–2151.
2. Commission of the European Communities. 1999. Communication from the Commission to the Council and the European Parliament. COM 706. Brussels, Belgium, pp 9–19.
3. Desbrow C, Routledge EJ, Brighty G, Sumpter JP, Waldock M. 1998. Identification of estrogenic chemicals in STW effluent. I: Chemical fractionation and in vitro biological screening. *Environ Sci Technol* 32:1549–1558.
4. Larsson DGJ, Adolfsson-Erici M, Parkkonen J, Pettersson M, Berg AH, Olsson PE, Förlin L. 1999. Ethinyloestradiol—An undesired fish contraceptive? *Aquat Toxicol* 45:91–97.
5. U.S. Environmental Protection Agency. 1997. Special report on environmental endocrine disruption: An effects assessment and analysis. EPA/630/R-96/012. Risk Assessment Forum, Washington, DC.
6. Jobling S, Nolan M, Tyler CR, Brighty G, Sumpter JP. 1998. Widespread sexual disruption in wild fish. *Environ Sci Technol* 32:2498–2506.
7. Solé M, Lòpez de Alda MJ, Castillo M, Porte C, Ladegaard-Petersen K, Barcelò D. 2000. Estrogenicity determination in sewage treatment plants and surface waters from the catalonian area (NE Spain). *Environ Sci Technol* 34:5076–5083.
8. Jobling S, Casey D, Rodgers-Gray T, Oehlmann J, Schulte-Oehlmann U, Pawlowski S, Baunbeck T, Turner AP, Tyler CR. 2003. Comparative responses of mollusks and fish to environmental estrogens and an estrogenic effluent. *Aquat Toxicol* 65:205–220.
9. Ternes TA, Stumpf M, Mueller J, Haberer K, Wilken RD, Servos

M. 1999. Behavior and occurrence of estrogens in municipal sewage treatment plants—I. Investigation in Germany, Canada, and Brazil. *Sci Total Environ* 225:81–90.
10. U.S. Code. 1996. Food Quality Protection Act: PL 104–170: et seq.
11. U.S. Code. 1996. Safe Water Drinking Act: PL 104–182: et seq.
12. Marcomini A, Pojana G, Sfriso A, Quiroga Alonso JM. 2000. Behavior of anionic and nonionic surfactants and their persistent metabolites in the Venice Lagoon. *Environ Toxicol Chem* 19: 2000–2007.
13. Johnson AC, Belfroid A, Di Corcia A. 2000. Estimating steroid oestrogen inputs into activated sludge treatment works and observations on their removal from the effluent. *Sci Total Environ* 256:163–173.
14. Ferguson PL, Iden CR, Brownawell BJ. 2001. Distribution and fate of neutral alkylphenol metabolites in a sewage-impacted urban estuary. *Environ Sci Technol* 35:2428–2435.
15. Staples CA, Dorn PB, Klecka GM, O'Block ST, Branson DR, Harris LR. 2000. Bisphenol A concentrations in receiving waters near U.S. manufacturing and processing facilities. *Chemosphere* 40:521–525.
16. Legler J. 2001. Development and application of in vitro and in vivo reporter gene assays for the assessment of (xeno-)estrogenic compounds in the aquatic environment. PhD thesis. Wageningen University, Utrecht, The Netherlands.
17. Körner W, Hanf V, Schuller W, Kempter C, Metzger J, Hagenmaier H. 1999. Development of a sensitive E-Screen assay for quantitative analysis of estrogenic activity in municipal sewage plant effluents. *Sci Total Environ* 225:33–48.
18. Körner W, Schuller W, Hagenmaier H, Hanf V. 1999. Entwicklung und praktische Erprobung eines einfachen Screening-Systems für estrogenartig wirkende Umweltchemikalien. Projekt Umwelt und Gesundheit, Baden-Württemberg, Germany.
19. Gutendorf B, Westendorf J. 2001. Comparison of an array of in vitro assay for the assessment of estrogenic potential of natural and synthetic estrogens, phytoestrogens, and xenoestrogens. *Toxicology* 166:79–89.
20. Legler J, Dennekamp M, Vethaak AD, Brouwer A, Koeman JH, van der Burg B, Murk AJ. 2002. Detection of estrogenic activity in sediment-associated compounds using in vitro reporter gene assay. *Sci Total Environ* 293:69–83.
21. Körner W, Bolz U, Sïûmuth W, Hiller G, Schuller W, Hanf V, Hagenmaier H. 2000. Input/output balance of estrogenic active compounds in a major municipal sewage plant in Germany. *Chemosphere* 40:1131–1142.
22. Routledge EJ, Sumpter JP. 1996. Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. *Environ Toxicol Chem* 15:241–248.
23. Thomas KV, Hurst MR, Matthiessen P, Waldock M. 2001. Characterization of estrogenic compounds in water samples collected from United Kingdom estuaries. *Environ Toxicol Chem* 20:2165–2170.
24. Harries JL, Sheahan DA, Jobling S, Matthiessen P, Neall P, Sumpter JP, Tylor T, Zaman N. 1997. Estrogenic activity in five United Kingdom rivers detected by measurement of vitellogenesis in caged male trout. *Environ Toxicol Chem* 16:534–542.
25. Seki M, Yokota H, Matsubara H, Tsuruda Y, Maeda M, Tadokoro H, Kobayashi K. 2002. Effect of ethinylestradiol on the reproduction and induction of vitellogenin and testis-ova in medaka (*Oryzias latipes*). *Environ Toxicol Chem* 21:1692–1698.
26. Vazzoler S, Costa F, Bernardi S. 1987. Lagoon of Venice and transfer of freshwater and pollutants. Istituto Veneto di Scienze ed Arti, Venice, Italy.
27. Pojana G, Busetti F, Collarin A, Bonfà A, Marcomini A. 2004. Determination of natural and synthetic estrogenic compounds in coastal lagoon waters by HPLC-electrospray-mass spectrometry. *Intern J Environ Anal Chem* (in press).
28. Swan ARH, Sandilands MH. 1995. *Introduction to Geological Data Analysis.* Blackwell Science, Oxford, UK.
29. Sfriso A, Facca C, Ghetti PF. 2003. Temporal and spatial changes of macroalgae and phytoplankton in a Mediterranean coastal area: The Venice lagoon as a case study. *Mar Environ Res* 56:617–636.
30. Baronti C, Curini R, D'Ascenzo G, Di Corcia A, Gentilizi A, Samperi R. 2000. Monitoring natural and synthetic estrogens at activated sludge sewage treatments plants and in a receiving river water. *Environ Sci Technol* 34:5059–5066.
31. Christiansen LB, Winther-Nielsen M, Helweg C. 2002. The effect of estrogenic compounds and their fate in sewage treatment plants and nature. Environmental Project 729. Danish Environmental Protection Agency, Copenhagen, Denmark.
32. Spengler P, Körner W, Metzger JW. 2001. Substances with estrogenic activity in effluents of sewage treatment plants in southwestern Germany. 1. Chemical analysis. *Environ Toxicol Chem* 20:2133–2141.
33. Ferguson PL, Iden CR, McElroy AE, Brownawell BJ. 2001. Determination of steroid estrogens in wastewaters by immunoaffinity extraction coupled with HPLC-electrospray-MS. *Anal Chem* 73: 3890–3895.
34. Belfroid AC, Van der Horst A, Vethaak AD, Schäfer AJ, Rijs GBJ, Wegener J, Cofino WP. 1999. Analysis and occurrence of estrogenic hormones and their glucuronides in surface waters and wastewater in The Netherlands. *Sci Total Environ* 225:101–108.
35. Fürhacker M, Scharf S, Weber H. 2000. Bisphenol A: Emission from point sources. *Chemosphere* 41:751–756.
36. Klecka GM, Gonsior SJ, West RJ, Goodwin PA, Markham DA. 2001. Biodegradation of bisphenol A in aquatic environments: River die-away. *Environ Toxicol Chem* 20:2725–2735.
37. Ahel M, Giger W, Koch M. 1994. Behaviour of alkylphenol polyethoxylate surfactants in the aquatic environment—I. Occurrence and transformation in sewage treatment. *Water Res* 28: 1131–1142.
38. Folmar LC, Hemmer MJ, Denslow ND, Kroll K, Chen J, Cheek A, Richman H, Meredith H, Grau EG. 2002. A comparison of the estrogenic potencies of estradiol, ethynilestradiol, diethylstilbestrol, nonylphenol, and methoxychlor in vivo and in vitro. *Aquat Toxicol* 60:101–110.
39. Folmar LC, Hemmer M, Hemmer R, Bowman C, Kroll K, Denslow ND. 2000. Comparative estrogenicity of estradiol, ethinyl estradiol, and diethylstilbestrol in an in vivo, male sheepshead minnow (*Cyprinodon variegatus*), vitellogenin bioassay. *Aquat Toxicol* 49:77–88.