Detection of estrogenic substances and their distribution patterns in the marine environment

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Carina Deich

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Abstract

Female sex hormones, i.e., estrogens, belong to the messenger substances of organisms. In addition to endogenous steroidal estrogens such as estrone (E1), 17β-estradiol (E2) and estriol (E3), there are substances mimicking the estrogen function (xenoestrogens) such as genistein (GEN), daidzein (DAI) or zearalenone (ZEN), which are produced by plants or fungi. Other known xenoestrogens are synthetically produced estrogens such as 17α-ethinylestradiol (EE2) or chemical substances whose hormonal activity was not intended and has only been proven in retrospect. It has been observed that the increased occurrence of these substances can have a lasting (adverse) effect on aquatic organisms. Estrogen-active substances can lead to the disturbance of the hormone balance and thus belong to the endocrine disruptors.

The present thesis addresses the analysis of estrogenic substances as well as the analysis of the estrogenic activity in marine systems since the majority of existing studies focused on the inflows and outflows of sewage treatment plants or river waters. In order to detect estrogenic substances, an instrumental analysis method using LC-MS/MS technology was first established. Furthermore, a effect-based bioassay was used to determine estrogenic activity in the marine system.

It was found that estrogenic substances and estrogenic activity are present in the Baltic Sea (BS) as well as in the northern South China Sea (SCS) around the Pearl River Estuary (PRE). It was also shown that natural estrogens and synthetic estrogens were found in both systems. Seasonal variability and regiospecific distribution patterns were revealed by the analysis of annual cycles in the BS as well as from the analysis of surface water samples from the SCS. A risk assessment was performed based on the method of risk quotients to classify the measured estrogen concentrations. It was shown that there is a high potential risk for marine organisms due to the presence of the synthetic estrogen EE2 in both the BS and the SCS.

Kurzfassung

Weibliche Sexualhormone (Östrogene) gehören zu den Botenstoffen des Körpers. Neben den körpereigenen steroidalen Östrogenen Estron (E1), 17β-Estradiol (E2) und Estriol (E3) gibt es östrogene Substanzen, welche die Wirkung von Östrogenen nachahmen (Xenoestrogene). Dazu gehören beispielsweise von Pflanzen und Pilzen produzierte Substanzen wie Genistein (GEN), Daidzein (DAI) und Zearalenon (ZEN). Ebenfalls bekannte Xenoestrogene sind synthetisch hergestellte Östrogene wie 17α-Ethinylestradiol (EE2) sowie weitere chemische Substanzen, deren hormonelle Aktivität nicht beabsichtigt und erst im Nachhinein nachgewiesen worden ist. Durch das vermehrte Auftreten dieser Substanzen in aquatischen Ökosystemen können die darin lebenden Organismen nachhaltig beeinflusst werden. Östrogen aktive Substanzen können zur Störung des Hormonhaushaltes führen und zählen somit zu den endokrinen Disruptoren.

Die vorliegende Arbeit befasst sich mit der Analyse von östrogenen Substanzen sowie mit der Analyse der östrogenen Aktivität im marinen System, da sich die Mehrzahl der bereits existierenden Studien auf die Analyse von Zu- und Abflüssen von Kläranlagen oder auf Flüssen fokussierten. Um östrogene Substanzen zu detektieren, wurde zunächst eine instrumentelle Analysemethode mittels LC-MS/MS Technik etabliert. Weiterhin wurde ein effektbasierten Bioassay genutzt, um die östrogene Aktivität im marinen System zu bestimmen.

Es konnte gezeigt werden, dass östrogene Substanzen und östrogene Aktivität sowohl in der Ostsee als auch im nördlichen Südchinesischem Meer rund um das Perlflussästuar präsent sind. Es zeigte sich hierbei ebenfalls, dass sowohl natürliche Östrogene als auch synthetische in beiden System zu finden waren. Aus der Analyse von Jahresgängen in der Ostsee und der Analyse von mehreren Proben innerhalb des Südchinesischen Meeres gehen sowohl saisonale als auch regionsspezifische Verteilungsmuster hervor. Es wurde eine Risikoabschätzung mittels Risikoquotienten durchgeführt, um die gemessenen Konzentrationen der östrogenen Substanzen einzuordnen. Dabei zeigte sich, dass sowohl in der Ostsee als auch im Südchinesischem Meer ein hohes potentielles Risiko aufgrund des Vorkommens des synthetischen Östrogens EE2 für marine Organismen besteht.

Contents

List of Abbreviations

A. adeninivorans	*Arxula adeninivorans*
A-YES	Arxula-yeast estrogen screen
BS	Baltic Sea
CAF	Caffeine
CBM	Carbamazepine
CEC	Contaminant of emerging concern
Chl *a*	Chlorophyll *a*
CTD	Conductivity, temperature, depth
DAI	Daidzein
DL	Dilution level
DOC	Dissolved organic carbon
E1	Estrone
E1-d_4	Estrone, deuterated
E2	17β-estradiol
E2-d_5	17β-estradiol, deuterated
E3	Estriol
EC_{50}	Half maximal effective concentration
EDC	Endocrine disrupting compound
EE2	17α-ethinylestradiol
EE2-d_4	17α-ethinylestradiol, deuterated
EEF	Estradiol equivalent factor
EEQ	Estradiol equivalent quotient
FLD	Fluorescence detector
GC	Gas chromatography
GEN	Genistein
GF/F	Glass fiber filter
HD	Heiligendamm Pier
HESI	Heated electrospray ionization
HPLC; LC	High pressure liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantification

M	Molecular mass
MEC	Measured environmental concentration
MET	Metoprolol
MS; MS/MS	Mass spectrometry; tandem mass spectrometry
PNEC	Predicted no-effect concentration
p-NP	*para*-nitrophenol
p-NPP	*para*-nitrophenylphosphate
POC	Particulate organic carbon
PPCP	Pharmaceuticals and personal care products
PRE	Pearl River Estuary
RE	Relative error/Accuracy
RSD	Relative standard deviation/Precision
$RQ_{(cum.)}$	Risk quotient (cumulative)
S	Salinity
S. cerevisiae	*Saccharomyces cerevisiae*
SCS	(Northern) South China Sea
SMP	Sulfamethoxypyridazine
SMT	Sulfamethazine
SMX	Sulfamethoxazole
SMZ	Sulfamerazine
SPE	Solid Phase Extraction
SPM	Suspended particulate matter
T	Temperature
UV/Vis	Ultraviolet–visible spectroscopy
WFD	Water Framework Directive
WWTP	Wastewater treatment plant
YES	Yeast estrogen screen
ZEN	Zearalenone
α/β-ZEL	α/β-zearalenol

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1. Introduction

1.1. Endocrine disrupting compounds

The Anthropocene era is synonymous with the influence of humankind on the environment and its living beings. Today, over 7 billion people live on earth, and about 60 % of them live in coastal areas, thereby pressuring the marine ecosystem (UN, 2019). In recent years, our oceans have become increasingly polluted. For example, high nutrient inputs have led to strongly eutrophicated waters, and elevated nitrate levels are already observed in drinking and groundwater. Oceans and the organisms living in them are recipients of microplastic due to the increasing use of plastics and the insufficiently regulated recycling processes. In addition to this, pollution is caused by the ongoing input of chemicals, which are often transported into the oceans via the river system. These include heavy metals, industrial chemicals, pharmaceuticals and chemicals used in daily life. Another anthropogenic stressor to the marine ecosystem is the continuous input of carbon dioxide, which results in ocean acidification (Wilhelmsson et al., 2013). Yet, the consequences to the marine environment and its response to the anthropogenic pressure in the long-term perspective remain to be elucidated.

Environmentally relevant pollutants such as polycyclic aromatic hydrocarbons and polychlorinated biphenyls have long been the subject of regular monitoring programs (El-Shahawi et al., 2010; HELCOM, 2018). However, there is a continuous rise in studies that report on substances whose harmfulness to the environment has only lately been discovered or which are simply of new origin. These substances are referred to as contaminants of emerging concern (CECs), and the list of CECs continues to grow. CECs comprise, for example, pharmaceuticals and personal care products (PPCPs) as well as other products of daily use (Dulio et al., 2018; UNESCO and HELCOM, 2017). Among them are substances that have been observed to profoundly distress the hormone system (endocrine system) of living organisms in short-term and long-term perspectives, which are therefore named as endocrine disrupting compounds (EDCs): *"An endocrine disrupting compound is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in intact organism, or its progeny, or (sub)populations"* (IPCS, 2002).

This category includes all substances that, due to their structural resemblance to the messengers of the endocrine system (hormones), bind to the specific hormone receptors. The interaction and interference with the reproduction system can be in form of an estrogen-

agonist, estrogenic-antagonist, androgen-agonist or androgen-antagonist (Bergman et al., 2013). Interfering EDCs can lead to amplification of the receptor response or inhibition by competing or occupying the receptor's binding sites. The list of EDCs comprises mainly exogenous and, thus, mainly synthetic substances. Recently, however, natural substances such as plant- and fungi-derived compounds have also been added as they were shown to interact with hormone receptors and potentially result in endocrine disruption (Diamanti-Kandarakis et al., 2009). Furthermore, steroidal estrogens have been included as well despite their natural occurrence and command of regulatory functions of the endocrine system. It was shown that increased estrogen concentration in the environment can also impair the hormone balance of exposed organisms (Sumpter & Johnson, 2005).

1.2. Estrogenic compounds

1.2.1. Estrogens

Sources

Natural estrogens, together with progestogens, are primarily female sex hormones and belong to the class of steroid hormones (Beck, 2006). The most important endogenous estrogens are 17β-estradiol (E2), estrone (E1) and estriol (E3; Fig. 1.1).

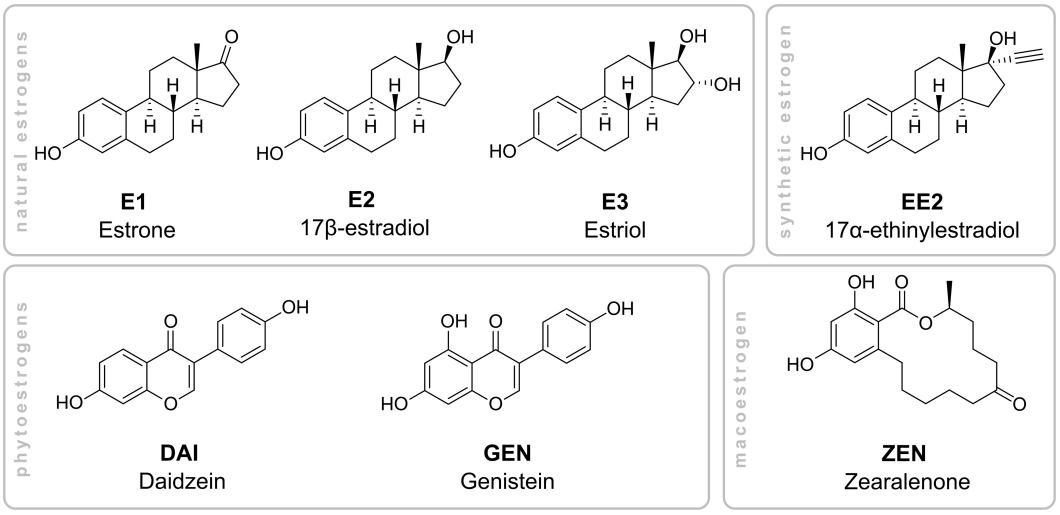


Figure 1.1.: Molecular structures of the natural estrogens (E1, E2 and E3), synthetic estrogen (EE2), phytoestrogens (DAI, GEN) and mycoestrogen (ZEN) in focus of this thesis.

They are also present in male bodies, however, to a lesser extent (Table 1.1, Johnson et al., 2000; Ying et al., 2002). The primary function of estrogens is the regulation of the reproduction system. Furthermore, they control the postnatal development, the cardiovascular system

and contribute to fat and bone metabolism. Their biosynthesis occurs in ovaries or testicles of vertebrates as well as in their adrenal glands and fatty tissue (Beck, 2006). Estrogens are transported within the body via the bloodstream to the target organ, where they can pass easily through the cell membranes due to their lipophilic character. Estrogens bind to specific estrogen receptors (estrogen-α, estrogen-β) in the cells of vertebrates and invertebrates. Hormones leave the body as conjugates via urine and bile. For example, E2 is oxidized in a first step to E1. A subsequent hydroxylation reaction turns E1 into E3. Eventually, estrogens are metabolized and excreted as glucuronic acid and sulfonic acid conjugates (Adeel et al., 2017; Shore & Shemesh, 2003; Tsuchiya et al., 2005).

Another important steroid hormone is the synthetic 17α-ethinylestradiol (EE2), in which an ethynyl group has been introduced at the 17-C position of E2 (Fig. 1.1). This modification improves the oral bioavailability of this hormone. EE2 is the main active ingredient in contraceptive pills. The daily dose of EE2 in these pills is between 10–50 µg d^{-1} (Table 1.1). Within the body, EE2 is partially deacetylated into E2, but the majority is metabolized and excreted as a conjugated analogous of the natural estrogens (Adeel et al., 2017; Beck, 2006; Shore & Shemesh, 2003). It has been estimated that the human population excretes over 30 000 kg of natural hormones (E1, E2 and E3) annually. The consumption of contraceptive pills containing EE2 is assumed to cause 700 kg of EE2 discharges per year (Adeel et al., 2017).

Table 1.1.: Daily excretion of steroidal estrogens, adapted from Johnson et al., 2000; Johnson & Williams, 2004; Kostich et al., 2013; Ying et al., 2002.

	E1 [µg d^{-1}]	E2 [µg d^{-1}]	E3 [µg d^{-1}]	EE2 [µg d^{-1}]
Female (cycling)	8–9.3	3.5–6.1	4.8–17.4	-
Female (menopausal)	2.9–4.0	1.5–2.3	1–3.9	-
Female (pregnant)	600–787	259–277	6000–9850	-
Contraceptive pill	-	-	-	4.5–13.1
Males	3.9	1.6	1.5	-

Transportation into the aquatic environment

The majority of estrogenic substances is released into the aquatic environment via wastewater treatment plants (WWTP) because of insufficient removal rates during the clean-up process (Tang et al., 2021; Ying et al., 2002, 2008). As a consequence, estrogenic compounds have been observed worldwide in influents and effluents of WWTP and also in the receiving riverine waters (Fig. 1.2). Further sources of estrogenic compounds are improper disposal of industrial and household waste as well as recreational activities (Jiang et al., 2020; Xu et al., 2014). For example, Jin et al. (2008) compared the inflows and outflows of WWTPs and

reported removal rates below 77 % for E1 and 97 % for E2. In most of the studies concerning removal rates, E3 is removed more sufficiently (85–99 %; Jin et al., 2008; Johnson & Sumpter, 2001; Ying et al., 2002). Among the steroidal estrogens, EE2 was observed to be far more resistant to removal (−58–85 %, Baronti et al., 2000; Jiang et al., 2020). Jiang et al. (2020) assumed that low or negative removal efficiency occurred due to the breakdown of conjugated estrogens into their natural form during wastewater treatment. Thus, disregarding estrogen conjugates removal rates may lead to overestimating and underestimating the actual removal or both. The supposedly low degradation rate of E1 can be explained by the fact that it is produced by the degradation of E2 in WWTPs (Svenson et al., 2003; Ternes et al., 1999). Furthermore, improper disposal of industrial and domestic waste, river run-off from agricultural fields with livestock as well as the application of manure can contribute to the release of estrogenic substances into the aquatic environment (Adeel et al., 2017; Jiang et al., 2020; Kolodziej et al., 2004; Xu et al., 2014). Concentrations of natural estrogens in aquatic environments have been subject to many research campaigns and were observed worldwide (Du et al., 2020; Jin et al., 2008; Wang et al., 2011, 2012). In this regard, Pawlowski et al. (2004) detected up to 5.6 ng L^{-1} of E2, 19 ng L^{-1} E1 and 1.5 ng L^{-1} of EE2 in effluents of a WWTP located at the river Rhine (Germany).

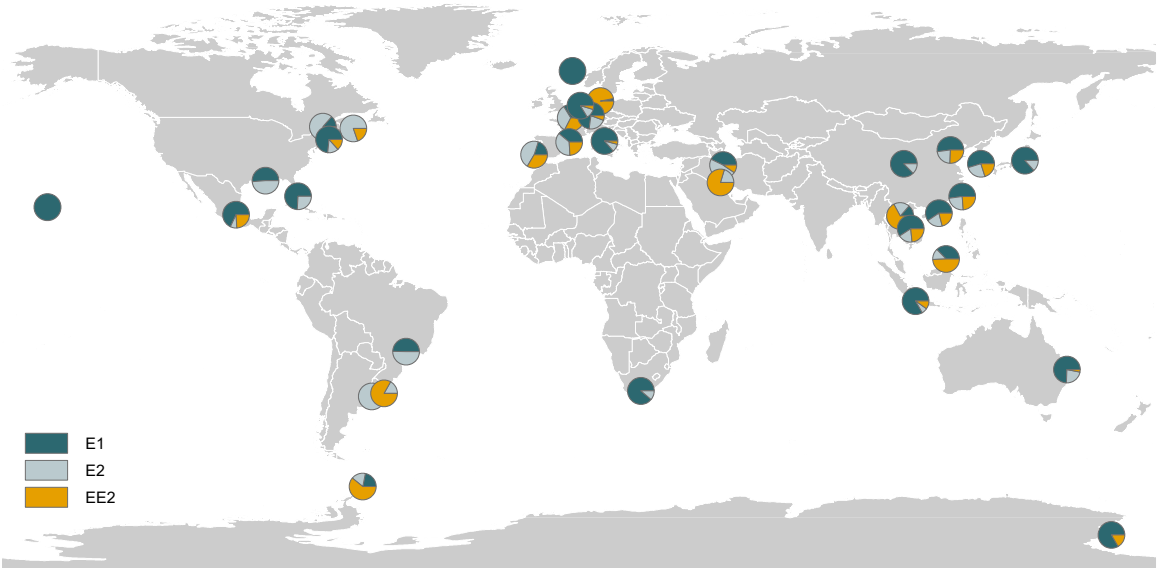


Figure 1.2.: Exemplary regions for the detection of estrone (E1), 17β-estradiol (E2) and 17α-ethinylestradiol (EE2) in surface waters. Pie charts show the proportions of E1, E2 and EE2 determined in the samples from the respective sampling sites. A detailed overview of the concentrations and the corresponding literature is summarized in the appendix (Table A.1).

Next to river system, studies focused on the presence of estrogens in estuaries and coastal areas (Atkinson et al., 2002; Singh et al., 2010; Saeed et al., 2017). Beck et al. (2005) reported E1 and EE2 concentrations in the western Baltic Sea of 0.53 and 17.2 ng L^{-1}, re-

spectively. In the Pearl River Estuary (PRE; China), Xu et al. (2014) observed E1 and E2 with high concentrations at the estuary's head and low concentrations at its mouth. Although it has been shown that estrogens are released into the marine environment, data on their occurrence in the open seas are scarce. Zhang et al. (2014) gave a first overview of the situation in the northern South China Sea and reported up to 11.4 ng L^{-1} of E1, 3.7 ng L^{-1} of E2, 21.6 ng L^{-1} of E3 and 3.99 ng L^{-1} of EE2.

Natural steroids were shown to undergo degradation within hours up to a few days in the aquatic environment. However, the synthetic EE2 was shown to be more stable, and half lives of 4-80 days were reported. The higher half-lives reflect the resistance of synthetic hormones to biodegradation, resulting in environmental concentrations as high as natural hormones despite the lower input (Aris et al., 2014; Adeel et al., 2017; Duong et al., 2010; Ying et al., 2002). To the best of the author's knowledge, half-lives of estrogenic substances have not yet been determined for the marine system in particular. However, from the detection of these substances in rivers, estuaries, coastal areas as well as in remote regions, it can be concluded that they are not completely degraded during transport and may be more persistent than previously assumed.

Toxicological concerns

Although estrogens take over regulatory functions in many organisms, additional exogenous exposure can result in adverse health effects (Czarny et al., 2017). For instance, Morthorst et al. (2014) observed abnormalities in embryos of the viviparous eelpout, and Lu et al. (2010) reported elevated E2 plasma concentration as well as induction of vitellogenin (egg yolk precursor) in adult male goldfish after exposure to E1 and E2.

Furthermore, first incidences of vitellogenin induction in rainbow trouts were reported arising from EE2 concentrations of 0.1–1 ng L^{-1} (Purdom et al., 1994). It was also shown that chronic exposure to EE2 at concentrations of 5–6 ng L^{-1} causes high vitellogenin levels, impacts gonadal developments and has almost led to the near extinction of a fathead minnow population (Kidd et al., 2007). Almeida et al. (2020) recently summarized that EE2 accumulates in mussels exposed to this estrogen. Among the steroidal estrogens, EE2 was reported to be the most effective estrogen, followed by natural E2, while E1 and E3 are classified as estrogens with low estrogenic activity (Czarny et al., 2017). Concerns about human and animal health effects in combination with the presence of estrogenic substances in the aquatic environment have been the reason for the implementation of E1, E2 and EE2 on the 2nd Watch-List (European Commission Decision 2018/840) to be monitored within the Water Framework Directive (WFD, 2008/105/EC, amended by 2013/39/EU) (Loos et al., 2018).

1.2.2. Plant- and fungi-derived estrogenic compounds

Sources and transportation into the aquatic environment

As mentioned above, there are other natural substances that can act as agonists and antagonists of the estrogen receptor due to their structural resemblance to hormones. So-called phyto- and mycoestrogens are derived from plants and fungi (Benassayag et al., 2002).

Phytoestrogens are substances from secondary metabolism that protect plants from predators. The main representatives are steroidal phytoestrogens and the more ubiquitous phenolic phytoestrogens, to which isoflavones, coumestans and lignans belong. The most important representatives of isoflavones are genistein (GEN) and daidzein (DAI; Fig. 1.1). Both are mainly associated to legumes such as soy and clover. In addition, they have been observed in algae and cyanobacteria species and their exudates (Gong et al., 2014; Jarošová et al., 2015; Procházková et al., 2017; Sychrová et al., 2012). Pathways of DAI and GEN are release from human and animal excretion and were thus detected in influents and effluents of WWTP (Bacaloni et al., 2005). Furthermore, they were observed in effluents of wood processing industries (paper and pulp mills) as well as in drainage water and surface run-off from agricultural fields with cultivated crops (Erbs et al., 2007; Kiparissis et al., 2001; Kolpin et al., 2010). Rocha et al. (2014) examined surface water samples obtained from the Mondego Estuary (Portugal), amongst others, for GEN and DAI. Both phytoestrogens were present at all stations in the estuary, and concentrations were up to $5.1\,\mu g\,L^{-1}$ for GEN and $11.9\,\mu g\,L^{-1}$ for DAI at the estuary's mouth.

Mycoestrogens (or mycotoxins) are produced by the *Fusarium* species, which infect crops such as maize, wheat and barley. An important representative of this group is zearalenone (ZEN; Fig. 1.1). In the human body, it is rapidly metabolized into the major breakdown products α-zearalenol (α-ZEL) and β-zearalenol (β-ZEL), which are reported of being even more estrogen active that ZEN (Jarošová et al., 2015; Laranjeiro et al., 2018; Molina-Molina et al., 2014). In the past, α-ZEL has been widely used as growth promoters for cattle, but its application has been banned in the European Union since the late 1980s (Bucheli et al., 2008; Jarošová et al., 2015). ZEN was found in WWTP and receiving river waters up to $60\,ng\,L^{-1}$. It was assumed that ZEN is primarily released by excretion from cattle treated with growth promoters. However, later on, it was concluded that the occurrence of ZEN in the environment rather occurs due to run-off from *Fusarium* infected fields (Hartmann et al., 2007). An investigation of surface water samples from the Douro River (Portugal) revealed the presence of ZEN and its metabolites in the estuary (Ribeiro et al., 2016). Consequently, the determination of GEN, DAI and ZEN in river waters and estuaries implies the transport pathway of these substances into the marine system.

Toxicological concerns

Phytoestrogens and mycoestrogens have been shown to be less estrogenic active than steroid hormones by a factor of $10^2 - 10^6$, but it has already been proven that they can affect the reproduction system of organisms (Gong et al., 2014; Jarošová et al., 2015). Meanwhile, it is being discussed whether phytoestrogens have a beneficial effect, for example, on cancer treatment, the cardiovascular system and the treatment of menopausal symptoms, or if they actually may provoke cancer (Erbs et al., 2007). An extensive study conducted between 1940 and 1970 could show that cattle, sheep and rodents displayed adverse effects in their reproduction system after ingestion of high levels of clover (Benassayag et al., 2002; Humfrey, 1998). In addition, exposure to phytoestrogens may lead to higher egg production, gonadal abnormalities, embryo mortality or a reduced number of oocytes of aquatic organisms (Clotfelter & Rodriguez, 2006; Kiparissis et al., 2003; Rearick et al., 2014; Sarasquete et al., 2020; Shao et al., 2019).

It has been observed that the exposure of fish to ZEN led to intersex in males, decreased sperm production and motility as well as decreased fertilization success. In addition, impaired gonadal development, testicular degeneration and induction of vitellogenin were associated to ZEN exposure (Bakos et al., 2013; Schwartz et al., 2010; Woźny et al., 2020).

1.3. Analysis of estrogenic substances and estrogenic activity in the environment

Environmental concentrations of estrogenic substances and estrogenic activity can be determined by instrumental analytical methods or effect-based bioassays. The focus of the instrumental analysis methods is on the determination of the concentration of known estrogens selected in advance of the analysis (Barreiros et al., 2016; Tomšíková et al., 2012). In contrast, effect-based bioassays can be used to evaluate the cumulative effect of all compounds present in the sample. Thus, this type of analysis takes into account known as well as unknown substances in the environmental sample (Hettwer et al., 2018; Kunz et al., 2015).

1.3.1. Instrumental analysis

The instrumental analytical methods for determining hormones in waters are versatile, but the most commonly applied methods are reverse-phase liquid chromatography (LC-MS/MS) and gas chromatography coupled to tandem mass spectrometry (GC-MS/MS; e.g, Barreiros et al., 2016; Locatelli et al., 2016; Tomšíková et al., 2012, see Table 1.2). Here, the mixture

of target analytes is separated on a chromatography column by interactions of the compound between the stationary and the mobile phase.

An advantage of LC-MS/MS is the direct processing of the sample extract, whereas the GC-MS/MS method requires a derivatization step prior to chromatographic separation due to the insufficient volatility of steroidal estrogens. Numerous methods have already been published, with limits of detection (LOD) and quantification (LOQ) usually in the $\mathrm{ng\,L^{-1}}$-range (Table 1.2). However, such detection limits can only be achieved by a preceding enrichment step such as solid phase extraction (SPE). In this process, estrogenic substances are retained from the water sample by interaction with a hydrophobic adsorbing material and are afterwards eluted with a lower volume of a solvent in which the target analytes are highly soluble.

In addition to the most common combination of mass-selective detection of hormones by MS or MS/MS and LC or GC, hormones and other estrogenic substances are also detected by flame ionization. The photoactive phenolic group also permits the application of diode array, ultraviolet detectors (UV) and fluorescence detectors (FLD). However, these methods are not as specific compared to MS coupling methods, making them less suitable for trace analysis of estrogenic substances in environmental samples (Barreiros et al., 2016).

1.3.2. Bioassays

As mentioned above, instrumental analysis only determines the concentrations of individual, known analytes. Neither the analysis by LC-MS/MS nor by GC-MS/MS consider the interaction mechanisms between the targeted substances or their combined effects and, thus, on the estrogenic activity potentially acting on any living organism. In order to test the actual effect of mixtures on organisms, *in vivo* tests are often carried out involving living organisms. For example, the occurrence of vitellogenin in the blood plasma of male fish serves as a reliable indicator for estrogenic activity. The disadvantage, however, is that the tests are cost and time-consuming and additionally involve animal testing (Purdom et al., 1994; Sumpter & Johnson, 2005).

Alternatively, combined effects can be investigated with *in vitro* techniques, which usually involve recombinant single cell or whole-cell organisms. For example, ligand binding assays mainly measure the affinity of a ligand to the estrogen receptor. Receptors used in this kind of assay are usually derived from human or animal cells, animal tissues or genetically modified bacteria. However, ligand binding assays do not observe whether the receptor is activated or not (Streck, 2009). The advantages of these tests are that they do not involve animal testing and that they are comparably inexpensive and fast (Giesy et al., 2002). A second type of assay measures only cell proliferation induced by estrogen active compounds versus cell

proliferation in a positive control (Demirpence et al., 1993).

The most commonly used bioassays are receptor-reporter assays, which utilize recombinant yeast strains, mammalian or fish cells. Here, DNA sequences for the human estrogen receptor and the estrogen response element located within a promoter region for a reporter gene are transfected into the yeast cells (Streck, 2009). Examples include the yeast estrogen screen (YES) developed by Routledge & Sumpter (1996) based on the *Saccharomyces cerevisiae* strain (*S. cerevisiae*) and the A-YES test (Hettwer et al., 2018) based on the salt-tolerant yeast *Arxula adeninivorans* (*A. adeninivorans*). Upon the binding of a suitable ligand (estrogenic substance), a receptor response is induced, leading to the reporter gene's transcription and ultimately to the production of the reporter protein. In case of the A-YES test system, the amount of reporter protein produced can be followed spectrophotometrically and correlates with the amount of ligands present in the sample. The estrogenicity of a sample is expressed relative to the effect of the natural estradiol in the same testing, thus, as estradiol equivalent quotient (EEQ).

Although detection levels of such bioassays are in the low ng per liter range, they are often not sensitive enough to detect estrogen activity levels in environmental samples. Therefore, a preceding enrichment step such as SPE is required for these tests as well. It should also be emphasized that the results derived from *in vitro* tests are not necessarily representative of the response of living organisms, but they provide a first overview of the potential estrogenic activity in the environment.

Both the instrumental analysis and the application of biological tests show disadvantages regarding the complexity of their conclusions. Hence, the combined use of both types is a helpful approach to obtain a comprehensive overview of estrogen load in environmental samples. More precisely, instrumental analysis provides specific information about the composition of the sample, while analysis by bioassay represents the interaction of the targeted analytes as well as other substances present in the sample. In this way, conclusions can be drawn as to whether the analytes found are decisive for the estrogenic activity or whether there are other substances (agonistic, antagonistic) influencing the estrogen receptor.

Table 1.2.: Exemplary methods for the determination of single estrogenic compounds in environmental water samples.

Method	Matrix	LOD [ng L^{-1}]	Reference
HPLC-FLD	Ground water	E1: 11 E2: 0.41 EE2: 5.2 E3: 7.18	Liu et al., 2015
HPLC-UV	River water	E1: 290 E2: 280 EE2: 350	Hu et al., 2013
LC-MS/MS	Sea water	E1: 0.02 E2: 0.3 E3: 1.0 EE2: 0.45 DAI: 0.43 GEN: 0.61	Beck et al., 2005
LC-MS/MS	River water	E1: 2.5 E2: 5.0 E3: 0.25 EE2: 5.0	Petrovic et al., 2002
LC-MS/MS	WWTP influents, effluents and receiving river water	E1: 0.1–1.2 E2: 0.2–1.9 E3: 0.3–7.0 EE2: 0.4–1.6 DAI: 0.2–3.5 GEN: 0.7–4.3	Laganà et al., 2004
LC-MS/MS	river water WWTP effluent	ZEN: 0.3–29.0 ZEN: 0.4–47.7	Schenzel et al., 2010
LC-MS/MS	river water WWTP effluent	ZEN: 0.4–1.1 ZEN: 0.8–12.4	Hartmann et al., 2007
GC-MS[a]	River water	E1: 1.5 E2: 1.1 E3: 3.2 EE2: 3	Gong et al., 2016
GC-MS	River water, sea water	E1: 0.97 E2: 0.86 EE2: 1.34 DAI: 1.25 GEN: 1.14	Rocha et al., 2013

[a] LOQ are presented here as LOD were not available.

2. Aim of study

In 1991, participants of the Wingspread Conference concluded that there is evidence of man-made chemicals and a few natural substances that can profoundly disturb the endocrine system of animals and humans. As a result, this topic has come into focus of many research studies over the past three decades, including the investigation of natural and synthetic estrogens as well as further substances with an effect on the estrogen receptor (Colborn et al., 1993; Hotchkiss et al., 2008).

Since the primary input of estrogenic substances is the excretion from humans and animals, the vast majority of the studies deal with the analysis of these substances in inland water bodies such as influents and effluents of wastewater treatment plants, river systems and lakes (Adeel et al., 2017; Aris et al., 2014; Barreiros et al., 2016; Czarny et al., 2017; Jarošová et al., 2015; Du et al., 2020; Tomšíková et al., 2012). In contrast, data on the occurrence of estrogenic substances in marine ecosystems are still limited, although there is evidence that marine organisms are prone to adverse health effects (Kidd et al., 2013; Laranjeiro et al., 2018; Schenzel et al., 2012), and little is known about the distribution patterns or seasonal variability.

In this context, the aim of this thesis was

a) to quantify and assess the presence of natural and synthetic estrogenic substances as well as estrogenic activity in the marine ecosystem,
b) to investigate the possible seasonal and regional distribution patterns and
c) to determine possible drivers of the observed distribution patterns.

In the first step, a suitable method for the analysis of water samples for estrogenic substances with the LC-MS/MS technique was established to identify substances of natural or synthetic origin (Deich et al., 2021a,b, and Deich et al., not published).

The areas of interest were the western Baltic Sea (BS) and the northern shelf of the South China Sea (SCS). The Baltic Sea has been in the focus of scientists for years. The limited water exchange with the North Sea, the resulting high residence time of the water, and in addition the input of large amounts of freshwater transporting industrial, urban, and agricultural wastes, has led to the accumulation of various potentially hazardous pollutants in the Baltic Sea (HELCOM, 2018). Furthermore, the investigation area is easily accessible and as such a suitable test system for method development. The regular sampling experiments and ongoing research cruises provided a good opportunity to investigate whether the distribution patterns of estrogenic substances and estrogenic activity are prone to seasonal and regional

variability (Deich et al., 2020, and Deich et al., not published).

The second sampling area was the northern shelf of the South China Sea, close to the Pearl River Estuary. This area allows the study of the transport of estrogenic substances from an estuary over the continental shelf towards the open, deep SCS. In addition, this sample area is of interest due to its high population density and resulting high anthropogenic pressure. Furthermore, two research cruises in two consecutive years allowed the investigation of distribution patterns within the summer East Asian monsoon (wet season) and the inter-annual variability of the estrogenic substances. Eventually, the obtained data are used to identify possible drivers to explain the observed distribution patterns of estrogenic substances in the SCS (Deich et al., 2021a,b).

3. Methodological approach

3.1. General sampling procedure

Water samples from the Baltic Sea were collected either with a pre-rinsed bucket (Heiligendamm Pier) or were obtained with a CTD SBE 911 rosette system operating on the R/V Elisabeth Mann Borgese. The sampled water was collected in pre-rinsed polyethylene or amber glass bottles. In the laboratory, samples were acidified directly with a 5 M HCl to a pH of 2–3 and stored at 4 °C in order to prevent biodegradation. For instrumental analyses, samples were spiked with 1 ng of E1-d_4 and 2 ng of E2-d_5 and EE2-d_4 (see Deich et al., 2020 and Deich et al., not published).

Water samples from the PRE were collected using a towed CTD pumping system ("towed fish") operating on board of the R/V Hai Yang Di Zhi Shi Hao (H10), and collected in pre-rinsed amber glass bottles. Samples were acidified with 5 M HCl and stored at 4 °C if not subsequently processed with SPE. Samples were spiked with 20 ng of E1-d_4, E2-d_5 and EE2-d_4 for instrumental analyses (see Deich et al., 2021b).

Water samples obtained from the SCS were collected using a CTD SBE 911 rosette system operating on board of the R/V H10 and the R/V Sonne (SO269). Water samples were collected with pre-rinsed amber glass bottles and acidified with a 5 M HCl. In 2018 (H10), samples were spiked with 20 ng of E1-d_4, E2-d_5 and EE2-d_4 for instrumental analyses, and with 1 ng of E1-d_4 and 2 ng of E2-d_5 and EE2-d_4 in 2019 (SO269). The samples were immediately processed with SPE or stored at 4 °C until further processing (see Deich et al., 2021b and Deich et al., 2021a).

3.2. General sample pre-treatment

In order to analyze estrogenic substances and estrogenic activity in the aquatic environment, samples had to be enriched due to the fact that environmental concentrations of estrogenic substances are usually below the detection limits of applied bioassays and instrumental methods. For the first annual cycle at Heiligendamm Pier 2017, a sample volume of 400 mL of surface water was selected. For the second cycle, the sample volume was increased to 1000 mL due to the observed low levels of concentrations during the first annual course. For Baltic Sea and South China Sea samples, a sample volume of 1000 mL was enriched for samples examined by LC-MS/MS to determine the concentration of targeted estrogenic

compounds. A volume of 2000 mL was enriched for samples examined with the A-YES system to determine the pooled estrogenic activity.

The enrichment of estrogenic compounds was conducted by SPE. Samples were passed through glass fiber filters (GF/F, 0.7 µm) to separate the particulate phase of the water sample from the dissolved one and to avoid clogging of the SPE cartridge. Subsequently, the water samples were passed through the SPE cartridge with a flow of approximately 10 mL min^{-1}. After loading of the SPE columns, they were washed with 2.5 mL of a 5 % aqueous methanol solution and with 2.5 mL of analytical grade water. Subsequently, the cartridges were dried gently with N_2 and stored at −20 °C until further processing.

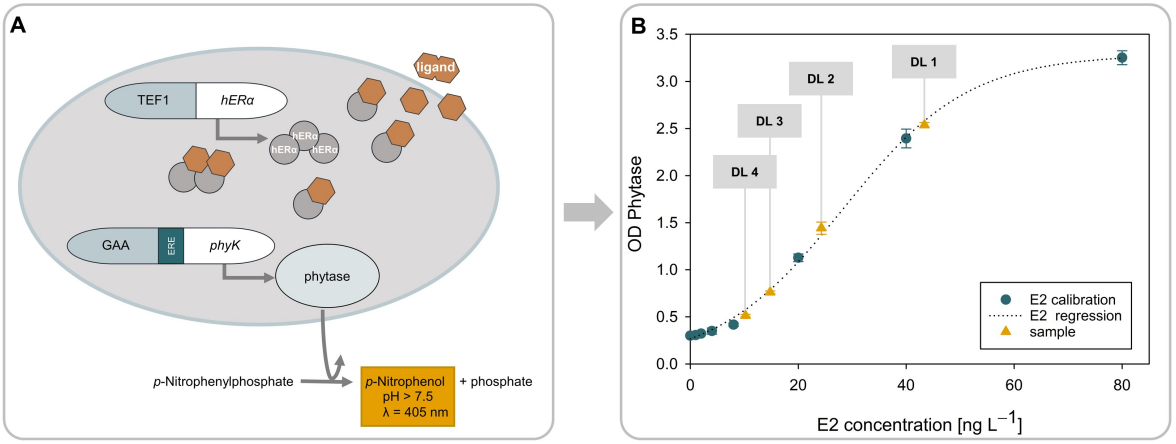
The analytes were eluted from the SPE cartridges with 10 mL and 15 mL of MeOH:EtOAc (70:30, v:v) and dried under clean air in a 40 °C water bath. Then, the sample was redissolved either in 1 mL of analytical grade water: ACN (70:30, v:v) for the analysis of the individual estrogenic substances by LC-MS/MS or in 6 mL of analytical grade water for the assessment of the estrogenic activity by the A-YES bioassay.

3.3. Analysis of estrogenic activity – A-YES® test principle

Estrogenic activity was determined using the A-YES® bioassay, which had been successfully established at the Leibniz Institute for Baltic Sea Research Warnemünde by A. Schoop within the framework of a master thesis (Schoop, 2016) and validated in an international ring trial (Hettwer et al., 2018). A detailed description of the experimental work is given in Hettwer et al. (2018) and in Deich et al. (2020) (**Publication I**), however, the test principle is discussed briefly in the following.

The utilized bioassay is a receptor-based transactivation assay that is working with the genetically modified yeast strain *Arxula adeninivorans* (Fig. 3.1). In this yeast strain, the human estrogen receptor *hERα* is constitutively expressed by the *TEF1* promoter. Upon the presence of suitable agonists (ligand) of the estrogen receptor, the ligands bind to the estrogen receptor protein (receptor-ligand-dimers) and, subsequently, the transcription of the reporter gene *phyK* is induced. *PhyK* is the reporter gene encoding the enzyme phytase. The enzyme catalyzes the cleavage of the substrate *p*-nitrophenylphosphate (*p*-NPP) to *p*-nitrophenol (*p*-NP) and phosphate. The reaction process of *p*-NPP (colorless) to *p*-NP (yellow) can be detected spectrophotometrically and its color intensity correlates to the amount of ligands bound to the *hERα* (Hettwer et al., 2018).

The obtained data were evaluated using the software BioVAL®, which provides a statistical assessment of the samples concerning outliers, method-specific limits of detection and



C

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank Sample 1	NC	Sample 1 (D4)				NC	Sample 3 (D4)				NC
B	Blank Sample 2	E2, CL 1	Sample 1 (D3)				E2, CL 1	Sample 3 (D3)				E2, CL 1
C	Blank Sample 3	E2, CL 2	Sample 1 (D2)				E2, CL 2	Sample 3 (D2)				E2, CL 2
D	Blank Recovery	E2, CL 3	Sample 1 (D1)				E2, CL 3	Sample 3 (D1)				E2, CL 3
E	Blank NC	E2, CL 4	Sample 2 (D4)				E2, CL 4	Recovery (D4)				E2, CL 4
F	Blank NC	E2, CL 5	Sample 2 (D3)				E2, CL 5	Recovery (D3)				E2, CL 5
G	Blank NC	E2, CL 6	Sample 2 (D2)				E2, CL 6	Recovery (D2)				E2, CL 6
H	Blank R_{CL7}	E2, CL 7	Sample 2 (D1)				E2, CL 7	Recovery (D1)				E2, CL 7

Figure 3.1.: A Schematic of the A-YES test principle with promoter sequences (TEF1, GAA), human estrogen receptor gene (*hERα*), human estrogen receptor (hERα), estrogen responding element (ERE), reporter gene (*phyK*), adapted from A-YES® manual by new_diagnostics GmbH. **B** E2 sigmoidal concentration curve, dilution level (DL) and an exemplary sample based on the spectrophotometric measurements. **C** Examplary plate design of a deep-well and microtiter plate containing blanks, E2 standard calibration (E2, CL), sample dilutions, recovery samples and negative controls (NC).

quantification as well as quantification of EEQ based on an E2 sigmoidal calibration curve (Fig. 3.1). For each test, a set of E2-standard dilution series was prepared as well as a series of suitable dilution levels of the environmental samples to overcome potential matrix effects on the human estrogen receptor, i.e., competitive inhibition, overloading of the receptor's binding site. For each test, the highest quantifiable sample dilution was selected to calculate the EEQ (Fig. 3.1).

3.3.1. Estrogenic activity of individual analytes as a function of the receptor activation assay

Due to structural differences, hormones and hormone-like substances affect the hormone receptor differently compared to the female sex hormone estradiol. Also, it was shown that the estrogenic potency expressed as estradiol equivalent factors (EEFs) differs between various bioassays as well (Beck et al., 2006; Chen et al., 2016; Jarošová et al., 2015; Wang et al., 2011; Yao et al., 2018). Therefore, it is necessary to determine the estrogenic potential of the individual estrogenic compounds with the utilized A-YES test system. For this purpose, dilution series of the target analytes E1, E2, E3, EE2, GEN, DAI and ZEN were prepared in Milli-Q water. In the following, the dilution series were processed with the bioassay. From the resulting sigmoidal four-parameter logistic function, the concentration at half of the maximum response (EC_{50}) was derived for each compound (Fig. 3.2).

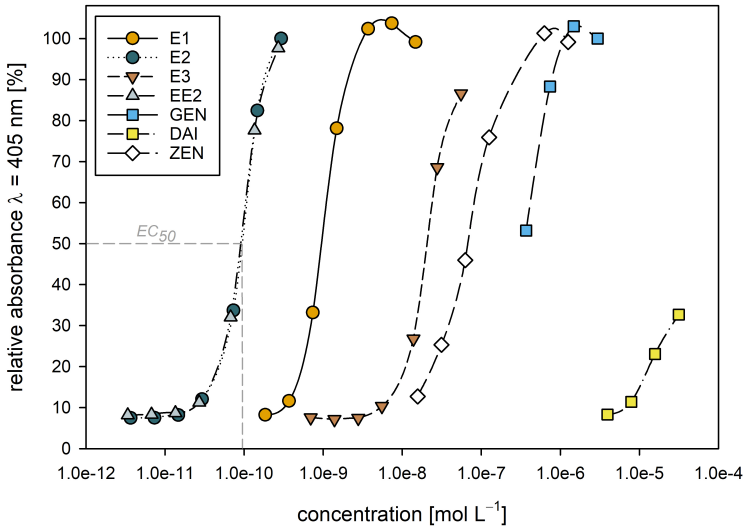


Figure 3.2.: Sigmoidal concentration curves for the targeted analytes. Displayed are the arithmetic means (n = 4) of each concentration level. The gray dashed line represents the EC_{50} of E2.

The estrogenic potential, i.e., efficiency relative to the female sex hormone E2, of the target analyte (EEF_i) is then calculated by the ratio of the EC_{50} value of estradiol ($EC_{50;\,E2}$) and the EC_{50} value of the respective compound ($EC_{50;\,i}$) according to Eq. 3.1 (Table 3.1; Deich et al., 2021b).

$$EEF_i = \frac{EC_{50;E2}}{EC_{50;i}} \tag{3.1}$$

Table 3.1.: Molecular masses (M) of targeted estrogenic substances as well as calculated EC_{50} and estradiol equivalent factors (EEFs) according to Eq. 3.1 obtained with the A-YES test system, adapted from Deich et al. (2021b).

Compound (i)	M [g mol^{-1}]	$EC_{50;i}$ [mol L^{-1}]	EEF_i
E2	272.40	9.26×10^{-11} [a]	1
EE2	296.40	9.68×10^{-11}	0.97
E1	270.37	1.05×10^{-9}	0.09
E3	288.38	2.02×10^{-8}	4.47×10^{-3}
ZEN	318.36	7.41×10^{-8}	1.32×10^{-3}
GEN	270.24	3.91×10^{-7}	2.49×10^{-4}
DAI	254.23	1.46×10^{-5}	6.32×10^{-6}

[a] EC_{50} for E2 = Arithmetic mean of all tests used to determine EEF (number of tests = 5, number of replicates per concentration level = 3)

3.4. Instrumental analysis of estrogenic compounds – Method development

In addition to the analysis of estrogenic activity, this study aims to detect single estrogenic substances in marine water samples. Prior to this, a suitable method for the determination of hormones and hormone-like substances using the liquid-chromatography coupled to tandem mass spectrometry (LC-MS/MS) technique had to be established. The method was developed on the basis of already established procedures from the literature (e.g., Beck et al., 2005; Kumar et al., 2009; Tomšíková et al., 2012). The following parameters were optimized in order to obtain the highest possible signal intensity: Mass transitions as well as mass spectrometer (MS) parameters, injection volumes, chromatographic separation including solvent and stationary phase, resuspension solvent as well as enrichment and processing of the environmental water samples. The final method is described in Deich et al. (2021b) and Deich et al. (2021a) (**Publications II and III**).

Firstly, suitable mass transitions within the mass spectrometer were determined for each estrogenic compound. Deuterated standards of E1, E2 and EE2 were used as internal standards for quantification purposes to counteract analyte loss during the sample's clean up procedure (see Table 2 in Deich et al., 2021b). In the following, ionization parameters (interface voltage, temperature and desolvation line temperature, resolution of quadrupoles and collision energies) were optimized.

Secondly, a chromatographic separation method using the LC was developed. For this, different injection volumes (5 µL, 10 µL and 50 µL) of a solution of target estrogens were tested on two reverse-phase columns: *Gemini NC* (3 µm, 150 × 2.0 mm) and *Kinetex Biphenyl* (2.6 µm, 150 × 4.6 mm). Based on signal intensities, 50 µL and the *Kinetex Biphenyl* were

chosen. Furthermore, different analytical grade mobile phases (H_2O, MeOH, ACN) and additives (NH_4CH_3COO, NH_4OH, NH_4HCO_3, NH_4F) were tested in a gradient program to separate the analytes. Of the solvents mentioned, the combination of H_2O as solvent A, ACN as solvent B and an aqueous solution of NH_4F as solvent C showed the strongest intensification of the peak areas of estrogens. Subsequently, the percentage of NH_4F solution was optimized, resulting in the gradient flow program (flow rate = 0.2 mL min^{-1}): 69.3 % A, 30 % B and 0.7 % C for 1 minute, reduced to 9.9 % A, 90 % B and 0.1 % C within 7 minutes, to 100 % B in 15 minutes, was held for 2 minutes and back to 69.3 % A, 30 % B and 0.7 % C within 2 minutes with subsequent equilibration for 2 minutes (Deich et al., 2021a,b). Although solvents mixed with organic acids (i.e., formic acid) and NH_4CH_3COO have been reported in the literature as suitable for the analysis of hormones (e.g., Beck et al., 2005; Tomšíková et al., 2012), these mixtures resulted in a significant reduction of peak areas of the target analytes in this study.

3.4.1. Sample treatment

Different solvent mixtures for the resuspension of the residues were tested to minimize possible side effects in the LC such as tailing or fronting of peaks. Among the tested four different aqueous solutions that contained 0 %, 10 %, 20 % and 30 % of ACN, the mixture with 30 % ACN provided the most intense signal and best peak shape.

Studies showed that the determination of hormones by heated-electrospray-ionization coupled to a tandem mass spectrometer (HESI-MS/MS) might be influenced by the presence of other substances (Busetti et al., 2012; Białk-Bielińska et al., 2016; Petrovic, 2014; Trufelli et al., 2011). Therefore, it was tested if an additional clean-up step during the sample treatment can improve the signal intensity and reduce any potential influence by co-eluted substances. In a first approach, the eluate was loaded onto a second column which was packed with combusted silica (400 °C for 15 h). In a second approach, a pre-packed NH2 Sep-Pak cartridge was attached to the SPE cartridge (Kumar et al., 2009). Both materials were supposed to retain co-eluted substances such as humic acids, while the target analytes were eluted from the adsorbent with a mixture of EtOAc and MeOH in a second step. Both approaches revealed enhanced signal intensities in the MS. NH2 cartridges were employed for the final method as they showed higher signals in the MS and were more convenient in handling.

The group of Svahn & Björklund (2019) developed a method for easy determination of estrogenic substances in the dissolved and particulate phase together (whole water sample) as demanded by the 2nd Watch-List of the WFD. For this, SPE cartridges were equipped with 2 g of sea sand (Merck AG, Darmstadt, Germany), which was previously heated for

Figure 3.3.: Left side: Schematic of SPE with GF/F filter. Right side: Schematic of SPE with sea sand.

15 h at 400 °C to remove any organic material. The sea sand supposedly retains any particles within the water sample and hinders the clogging of the SPE material (Svahn & Björklund, 2019). To adapt this method, six water samples were collected from Heiligendamm Pier at the Baltic Sea. The water samples were separated into two sub-samples. One sub-sample was filtrated through a GF/F filter 0.7 µm, the other sub-sample was treated with the SPE columns prepared with combusted sea sand (Fig. 3.3). The results show that there is no significant difference between both filtration methods. This may result from the low particle abundance and consequently from the resulting low amount of adsorbed estrogens. It should also be considered that the overall amount of adsorbed estrogens may be only minor, and that the elution step may not be sufficient to extract even small amounts of adsorbed estrogens. The SPE columns packed with sea sand were employed for further analysis of samples from the Baltic Sea to analyze the whole water sample. Table 3.2 provides an overview of the sampling series and the applied methods.

3.4.2. Quantification and method validation

Internal standard calibrations with E1-d_4, E2-d_5 and EE2-d_4 were utilized to quantify estrogenic substances in the water samples. Calibrations were prepared in Milli-Q water over the concentration range of 0–0.8 ng L^{-1} for samples obtained in September 2018 from the South China Sea (H10) and 0–1.0 ng L^{-1} for samples from Baltic Sea, Heiligendamm Pier and South China Sea in August 2019 (SO269). Method detection and quantification limits (LOD, LOQ) were calculated according to the standard DIN 32645:2008-11 (Table 3.3). Furthermore, a calibration for assessing matrix effects was prepared with an environmental water sample to assess the matrix influence on the detection of estrogens (matrix-matched calibration). For this, surface water was obtained from Heiligendamm Pier ($S = 9.4, T = 19.9$ °C, 13

Table 3.2.: Overview of the sampling series Heiligendamm Pier, Baltic Sea, Pearl River Estuary and South China Sea as well as the applied methods to determine estrogenic substances and estrogenic activity.

Region	Analysis	Sample volume [mL]	Filter type	SPE material [mg]	Elution volume [mL]	NH2 column	Resuspension solvent
Heiligendamm Pier							
2017[a]	A-YES	400	GF/F	200	10	-	H_2O
2018[a]	A-YES	1000	GF/F	1000	10	-	H_2O
2019	LC-MS/MS	1000	Sand	1000	10	+	H_2O:ACN (70:30)
Baltic Sea							
2017[a]	A-YES	2 x 1000	GF/F	2 x 1000	15	-	H_2O
2018[a]	A-YES	2 x 1000	GF/F	2 x 1000	15	-	H_2O
2019	LC-MS/MS	1000	Sand	1000	10	+	H_2O:ACN (70:30)
Pearl River Estuary							
2018[b,c]	A-YES	1000	GF/F	1000	15	-	H_2O
	LC-MS/MS	1000	GF/F	1000	10	+	H_2O:ACN (70:30)
South China Sea							
2018[b,c,d]	A-YES	2 x 1000	GF/F	2 x 1000	15	-	H_2O
	LC-MS/MS	1000	GF/F	1000	10	+	H_2O:ACN (70:30)
2019[d,e]	LC-MS/MS	1000	GF/F	1000	10	+	H_2O:ACN (70:30)

[a] in Deich et al., 2020
[b] in Deich et al., 2021b
[c] obtained on board of the H10 in September 2018
[d] in Deich et al., 2021a
[e] obtained on board of the SO269 in August 2019

August 2019) and spiked with estrogen concentrations between 0.05–1.0 ng L^{-1}. It could be shown that the matrix of the environmental water sample significantly reduces the sensitivity, i.e., signal loss of internal standards, of the LC-MS/MS method, which has been emphasized in other studies as well (Busetti et al., 2012; Białk-Bielińska et al., 2016; Petrovic, 2014). Therefore, LOD and LOQ derived from the matrix-matched calibration were used for further analyses (Table 3.3).

The method was validated using Milli-Q and environmental water samples, which were spiked with 0.4 ng L^{-1} and 0.8 ng L^{-1} of each targeted estrogen (three replicates per concentration level). A detailed description is given in Deich et al. (2021a,b). Generally, the accuracy (*RE*, deviation between spiked and measured concentration) of estrogenic compounds in the environmental water samples was ±20 %. Precision (*RSD*, deviation between the measured concentration of the replicates) for steroidal estrogens was < 7 %. RSD for targeted phyto- and mycoestrogens were 15 % for DAI, 39 % for GEN and 14 % for ZEN.

Based on these data, it was concluded that the method is specific for the analysis of steroidal estrogens, but further consideration is needed for the phyto- and mycoestrogens. It appears that the structural differences of steroidal estrogens and plant- and fungi-derived estrogens affect the behavior during the clean-up procedure and the following LC-MS/MS measurements significantly. As such, the use of the E1-d_4 standard for quantification may result in less precision for the phytoestrogens and mycoestrogens as for the steroidal estrogens. It needs to be mentioned that the method was not sufficiently reliable for E3 since this estrogen could not be detected in previously spiked environmental water samples with $0.4\,\mathrm{ng\,L^{-1}}$ and $0.8\,\mathrm{ng\,L^{-1}}$ of E3. Therefore, E3 was excluded from further evaluation of the data.

Table 3.3.: Limits of detection based on a calibration prepared with Milli-Q water (LOD_{water}, LOQ_{water}), and detection and qualification limits based on the matrix matched calibration using an environmental water sample (LOD_{matrix}, LOQ_{matrix}). Adapted from Deich et al. (2021a,b).

	E1	E2	EE2	GEN	DAI	ZEN
				$[\mathrm{ng\,L^{-1}}]$		
LOD_{water}	0.04	0.07	0.09	0.1	0.44	0.18
LOQ_{water}	0.08	0.14	0.17	0.2	0.88	0.36
LOD_{matrix}	0.13	0.17	0.31	0.36	0.26	0.1
LOQ_{matrix}	0.25	0.34	0.62	0.73	0.51	0.21

4. Results and Discussion

4.1. Occurrence of estrogenic compounds in environmental water samples

This thesis focuses on the determination of estrogenic compounds and estrogenic activity in the marine environment. For this purpose, the developed LC-MS/MS method and the A-YES reporter gene activity assay were first applied in the Baltic Sea. Subsequently, samples from the northern South China Sea around the Pearl River Estuary were analyzed for the estrogen content.

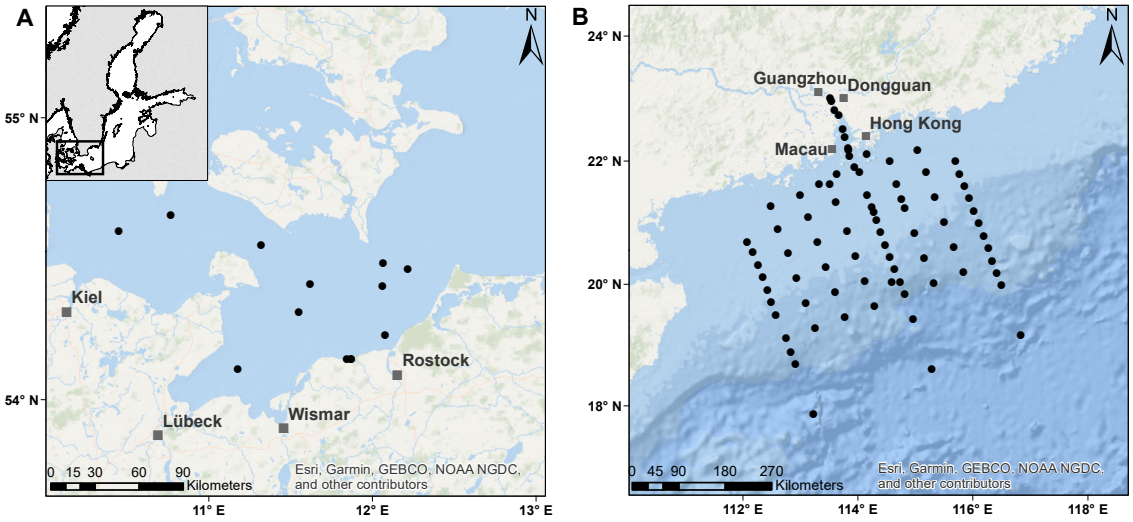


Figure 4.1.: Overview of sampling areas in the Baltic Sea (**A**) and northern South China Sea (**B**). More details are presented in Deich et al. (2020, 2021a,b).

4.1.1. Baltic Sea

Gercken & Sordyl (2002) studied wild fish species in the Baltic Sea to assess the quality of coastal waters. In that study, male species exhibited inter-sexuality characteristics which implied the abundance of feminizing pollutants in the investigated coastal areas. Thereupon, Beck et al. (2005, 2006) examined surface water samples from the same sampling sites for estrogenic substances as well as for estrogenic activity. Their findings supported the con-

clusions made by Gercken & Sordyl (2002) that male fish in the Baltic Sea were exposed to estrogenic substances. Based on these studies, the Baltic Sea was selected as a sampling and testing area for establishing a method for the determination of estrogenic substances by instrumental analysis. The access to the Baltic Sea allowed the analysis of estrogenic substances at sampling sites close to the coast (Heiligendamm Pier, western Baltic Sea) on a regular basis, which also provided insights on seasonal distribution patterns as well as inter-annual variability.

For this purpose, surface water samples were collected bi-weekly from 2016 to 2017 and on a monthly basis from 2017 to 2018 at the coast of the Baltic Sea at Heiligendamm Pier. All samples were tested for estrogenic activity using the yeast-based test system A-YES, and the results are discussed in Deich et al. (2020) (**Publication I**).

Starting in November 2016, EEQs were below the LOQ and only started to rise at the end of May 2017 to $0.2\,\mathrm{ng\,L^{-1}}$. After a short period of elevated EEQ, concentrations decreased abruptly below the LOD within two weeks at the end of July of the same year. The same pattern was observed for the second sampling period in 2018. A pronounced increase from $<$ LOD to $0.2\,\mathrm{ng\,L^{-1}}$ was also observed from May to June 2018, and the estrogenic activity reached its maximum in August with $0.38\,\mathrm{ng\,L^{-1}}$. Shortly thereafter, EEQ decreased sharply from September onward ($< 0.1\,\mathrm{ng\,L^{-1}}$).

In the same study (**Publication I**), additional samples were collected from the sampling sites within the Baltic Sea during six cruises (August 2017 – July 2018) and analyzed for estrogenic activity. Here, concentrations varied from $<$ LOD to $0.11\,\mathrm{ng\,L^{-1}}$, which is comparable with EEQ detected by Beck et al. (2006) in the Baltic Sea (0.01–$0.82\,\mathrm{ng\,L^{-1}}$; see also Table 1 in Deich et al., 2020). Observed EEQs fluctuated strongly within the individual sampling periods, and a regional pattern could not be derived. A comparison of the sampling campaigns revealed a seasonal pattern for the Baltic Sea, which is comparable to the annual cycle detected at Heiligendamm Pier.

Both sampling sites show a seasonal pattern and correlation to water temperature was significant ($R^2 = 0.41, p < 0.001$). This seasonal variability suggests that estrogenic activity in the Baltic Sea might have been influenced by the onset of the vegetation period in spring and increased touristic and recreational activities at the respective sampling sites in summer. A similar behavior was previously described for Portuguese Rivers (Rocha et al., 2014, 2016). The utilized yeast test in the study was shown to be suitable for providing a first insight into the estrogenic activity that might act on marine organisms in the Baltic Sea. However, conclusions on the contributors to the estrogenic activity cannot be derived from this cumulative parameter. A comparison to Beck et al. (2005, 2006) led to the assumption that the presumed major contributors to the detected estrogenic activity in this study were the natural E1 and the synthetic EE2. The presence of the synthetic estrogen also led to the conclusion that the

respective area is under anthropogenic influence.

In a second study conducted in both coastal areas and the open Baltic Sea and also at Heiligendamm Pier, surface water samples were investigated with the previously established LC-MS/MS method (see Section 1.3) to identify major contributors to the estrogenic activity (Deich et al., data not published).

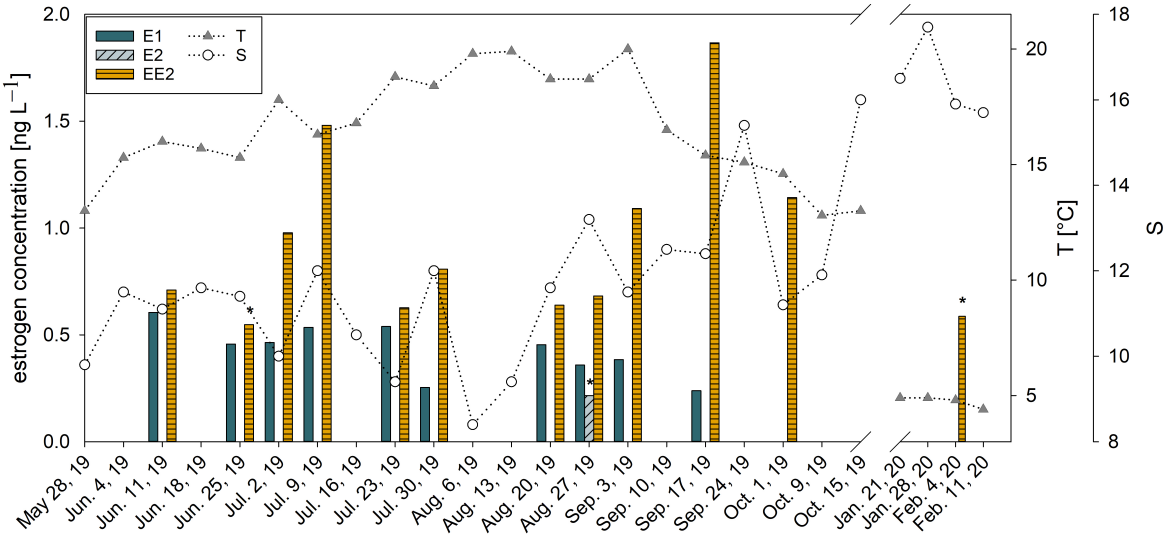


Figure 4.2.: Concentration of single estrogenic compound (E1, E2 and EE2) as well as temperature (T) and salinity (S) of samples obtained from Heiligendamm Pier, Baltic Sea in 2019, * LOD < concentration < LOQ (Deich et al., data not published).

Twelve surface water samples were collected at Heiligendamm Pier between June 2019 and February 2020. Estrogen concentration rose in early summer, as it has been observed for the estrogenic activity from 2016 to 2018 (Fig. 4.2). A second pronounced increase was observed in early autumn. As already assumed by comparing the results obtained from 2016 to 2018 with the literature, the main contributors among the target analytes to the estrogenic activity were E1 and EE2. Concentrations reached up to 0.6 ng L^{-1} and 1.9 ng L^{-1}, respectively. Natural E2 was only observed once (August 2019). Analysis of the Baltic Sea samples also determined E1 and EE2 with concentrations of 0.7 ng L^{-1} and 1.3 ng L^{-1}, respectively. Furthermore, a small tributary to the Baltic Sea was investigated for estrogenic substances. Concentrations of E1 were elevated compared to samples from the coastal Baltic Sea (1.7 ng L^{-1}). In contrast to the coastal sampling sites, both phytoestrogens and the mycoestrogen were detected (DAI: 4 ng L^{-1}, GEN: 1.7 ng L^{-1}, ZEN: 0.9 ng L^{-1}, Deich et al., data not published).

4.1.2. Pearl River Estuary and northern South China Sea

The results from the Baltic Sea showed that the utilized methods were able to detect individual estrogens as well as estrogenic activity in the environmental samples. As part of this work, the next step was to apply the established methods to samples from the northern South China Sea close to the Pearl River Estuary. Here, it was aimed to investigate a marine system that is influenced by the freshwater of the Pearl River, which has a highly urbanized catchment area. This work contributed to the MEGAPOL project that focuses on the investigation of pollutant fingerprints and dispersal in the South China Sea. Surface water samples were obtained during a first sampling campaign in September 2018 on the Chinese R/V H10 and during a second campaign on the German R/V SONNE in August 2019. The results of both sampling campaigns are discussed in more detail in Deich et al. (2021b) and Deich et al. (2021a) (**Publications II and III**).

Pearl River Estuary

During the first cruise, samples were collected along the salinity gradient of the PRE to investigate a possible origin of estrogenic substances in the SCS (see Fig. 2 in Deich et al., 2021b). Within the estuary, E1 was the most frequently detected estrogen with peak concentrations observed at the estuary's head close to the city of Guangzhou, and concentrations of E1 declined towards the estuary's mouth (0.3–3.6 $\mathrm{ng\,L^{-1}}$). Synthetic EE2 was only detectable at the station with the highest salinity ($S = 25$, PR-1).

Plant- and fungi-derived compounds were also detected within the estuary. Highest concentrations occurred upstream (DAI = 0–12 $\mathrm{ng\,L^{-1}}$, GEN = 0–13.6 $\mathrm{ng\,L^{-1}}$, ZEN = 0.2–1.9 $\mathrm{ng\,L^{-1}}$) and generally followed the same declining trend as observed for E1. However, station PR-1 showed a peak in phytoestrogen concentrations.

Generally, it was assumed that estrogenic substances are diluted within the estuary towards the sea. Nevertheless, the PRE is fed by different waterways that may also influence estrogenic substances' occurrence and distributional patterns in the estuary due to varying concentration levels. This could explain the varying, non-linear concentration trends. Additionally, heterogeneity or patchiness within water bodies should be considered.

It has been shown that some algae and cyanobacteria species can produce and release phytoestrogens (Gong et al., 2014; Jarošová et al., 2015; Procházková et al., 2017; Sychrová et al., 2012). A *Pearson* correlation of estrogenic substances with chlorophyll *a* was significant for DAI ($R^2 = 0.74, p < 0.01$) and a relationship was observed for GEN as well ($R^2 = 0.72, p = 0.07$). This may indicate that the elevated phytoestrogen concentrations at these stations (PR-1) result from increased algal growth (Deich et al., 2021b).

Northern South China Sea

At the sampling sites located within the SCS, i.e., shelf towards open sea; SCS-1 – SCS-77, E1 and EE2 were the most abundant estrogens in 2018 with maximum concentrations of 1.0 ng L^{-1} and 0.6 ng L^{-1}, respectively (see Table 1 in Deich et al., 2021a). The natural E2 as well as the plant- and fungi-derived compounds were not observed at the marine sampling sites. A similar pattern was observed in 2019. Maximum concentration of E1 and EE2 were comparable to the previous year (1.1 ng L^{-1} and 0.6 ng L^{-1}, respectively), and E2 was determined with up to 0.7 ng L^{-1}. Although both sample sets were taken during the wet season and thus under the influence of the southwestern monsoon, significant differences were found in the modeled surface currents and measured surface density (see Fig. 2,3 in Deich et al., 2021a). While in September 2018 the Pearl River plume was directed westwards, model surface currents indicated an eastwards directed plume in August 2019, which was also partially entrained into the open sea towards the Dongsha Islands. In 2018, increased concentrations were observed at the coastal stations and in the western part of the working area. In comparison, the concentration pattern in 2019 showed a concentration maximum at eastern stations and at those close to the coast. This implies an influence of the Pearl River plume on the distribution pattern of estrogens since the observations correlate well with the simulated sea surface currents and the sea surface density in both years.

DAI, GEN and ZEN occurred in the PRE, but were detected only once at a marine sampling site during both sampling campaigns (see Table 1 in Deich et al., 2021a). It was assumed that the compounds transported by the river water were below the detection limit due to dilution within the SCS or by degradation during the transport. Except for the shelf, the SCS is an oligotrophic system. Cyanobacteria or algae species that could produce phytoestrogens may be less abundant which results in low concentrations of phytoestrogens. Furthermore, within this thesis, only surface water samples were investigated, but the maximum primary production occurs at 50–60 m depth. Therefore, phytoestrogens may be less abundant at the surface and more present at the chlorophyll maximum. Like steroidal estrogens, phyto- and mycoestrogens are moderately lipophilic and can adsorb to particles. However, as this thesis focused on the detection of estrogenic substances only in the dissolved phase of the water samples, substances adsorbed to particles were not studied.

One aspect of this thesis was the determination of anthropogenic and natural input to the estrogenic activity. At both sampling sites, the natural substances E1 and E2 were detected. Yet, it could not be precisely determined to what extent the concentrations found correspond to a natural signal, i.e., if the concentrations refer to the natural amount excreted by marine organisms, or whether they are elevated due to an anthropogenic input. The presence of the synthetic hormone EE2 suggests that the sampling sites themselves are anthropogenically in-

fluenced and that the natural hormones in it could be of similar origin. For example, elevated E1 concentrations, which were detected in samples from the PRE with decreasing concentration towards the estuary's mouth, may indicate a source in the city center of Guangzhou. Comparable studies of the same sampling area by Gong et al. (2016) and Xu et al. (2014) also show that the concentration of the natural hormone decreases along the PRE, and it was assumed that they originate from local sewage treatment plants.

Indicators of (treated) sewage were detected in the Fisch et al. (2021) study. Here, the focus was on the detection of pharmaceutical and personal care products (PPCPs) such as caffeine (CAF), carbamazepine (CBM) and metoprolol (MET). All three were detected in the PRE, while only CAF and MET were detected at the stations close to the coast of the SCS, and significant correlations (*Pearson*) were found with E1, EE2 and DAI (see Table A.2). The same study and a previously conducted one by Fisch et al. (2017) also investigated the presence of pharmaceuticals (sulfonamide antibiotics) used in veterinary medicine. Residues of these substances have been detected in the PRE, which could indicate an influence of livestock farming. However, no significant correlations between these substances and estrogenic compounds were observed (Table A.2).

Accordingly, one could argue that the measured concentrations of the natural hormones may be partially under an anthropogenic influence, and that they originate from similar sources as PPCPs. Nevertheless, it should be emphasized at this point that a causal relation should not be derived from these considerations.

4.1.3. Risk assessment

The principle of risk quotients (RQs) was employed to estimate the ecotoxicological effects of estrogenic substances (**Publications I-III**). Here, the measured environmental concentration of a compound was considered in relation to its predicted no-effect concentration (PNEC). Since it is assumed that the steroidal hormones cause the largest contribution to the resulting estrogenicity (Jarošová et al., 2014), the risk assessment was carried out for E1, E2 and EE2 in the following (Fig. 4.3). The respective PNECs are $6\,\mathrm{ng\,L^{-1}}$ for E1, $2\,\mathrm{ng\,L^{-1}}$ for E2 and $0.1\,\mathrm{ng\,L^{-1}}$ EE2 as determined by Caldwell et al. (2012) for long-term exposures ($> 60\,\mathrm{d}$).

A low risk for aquatic organisms is considered if $\mathrm{RQ} < 0.1$, a moderate risk if $0.1 < \mathrm{RQ} < 1$ and a high risk if $\mathrm{RQ} > 1$ (Hernando et al., 2006). In this regard, RQs derived from the Baltic Sea indicated a low to moderate potential risk for aquatic organisms arising from natural steroids E1 and E2 (Fig. 4.3A). Calculated RQs based on EE2 concentrations were significantly higher than 1 ($\mathrm{RQ_{EE2,median}} = 7.6$) in most cases. These findings are comparable to the study of Beck et al. (2005), where the highest RQs also arose from EE2 concentra-

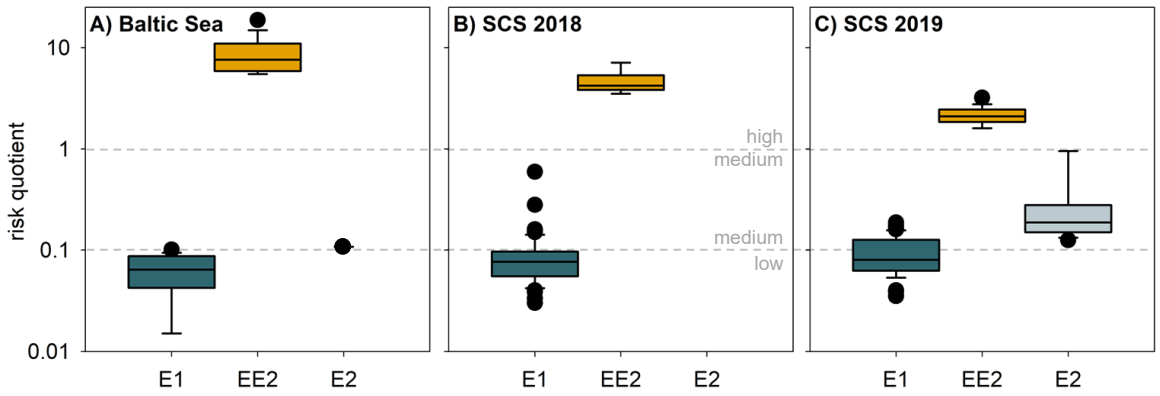


Figure 4.3.: Risk quotients for estrone (E1), 17α-ethinylestradiol (EE2) and 17β-estradiol (E2) calculated for **A)** the Baltic Sea including samples from Heiligendamm Pier (n = 19), **B)** the South China Sea in September 2018 including samples from the Pearl River Estuary (n = 66) and **C)** the South China Sea in August 2019 (n = 44), • represent outliers (Deich et al., 2021a,b, Deich et al., data not published).

tions ($RQ_{EE2,median} = 25$). A similar behavior was observed for the samples obtained from the SCS (Fig. 4.3B, C). RQ calculated for the PRE for E1 were slightly higher compared to those arising at the marine sampling sites and potentially affect aquatic organisms at a moderate level (Table 4 in Deich et al., 2021b, Fig. 5 in Deich et al., 2021a). It can be concluded that all marine sampling sites are at high risk considering EE2 concentrations. A comprehensive review of articles and review papers regarding estrogenic compounds (published between 2015–2018) reported moderate to high risks arising for natural water bodies of Asian countries (Du et al., 2020). That study also emphasized the important role of EE2, of which derived RQs occasionally exceeded even 1 by one or two orders of magnitude.

The studies in the Baltic Sea as well as in the South China Sea and the Pearl River Estuary indicate that the major part of the ecotoxicological risk arises from the presence of the synthetic estrogen EE2. The ratio of PNECs indicates that EE2 poses a 20 to 60 times higher risk to aquatic organisms than the natural substances E1 and E2, meaning that even the lowest concentrations of the synthetic hormone could have negative consequences for aquatic organisms.

However, it should be taken into account that living organisms are often exposed not only to single substances in the environment, but rather to a mixture of various natural and chemical substances. In this context, cumulative risk ratios ($RQ_{cum.}$) were calculated for the estrogens E1, E2, and EE2 determined in this study and from studies around the world (Fig. 4.4). The $RQ_{cum.}$ corresponds to the sum of all RQs of the individual substances (according to Gustavsson et al., 2017 and Posthuma et al., 2019).

In this study, $RQ_{cum.}$ indicate occasionally high risks for aquatic organisms in the BS and

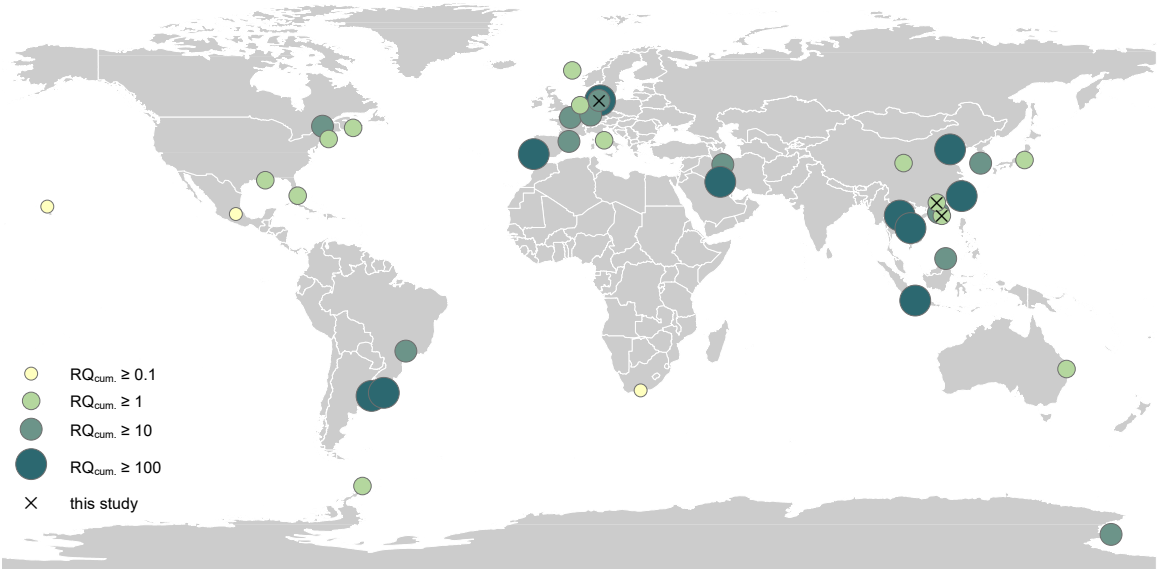


Figure 4.4.: Categorized cumulative risk quotients ($RQ_{cum.}$) calculated from estrogen concentrations around the world and this study. $RQ_{cum.}$ base on risk quotients of E1, E2 and EE2 at the respective sampling site. $RQ_{cum.} \geq 0.1$ indicates medium risk for aquatic organisms, $RQ_{cum.} \geq 1$ and above indicates high risk. A detailed overview of the concentrations and the corresponding literature is summarized in the appendix (Table A.1).

SCS. For comparison, $RQ_{cum.}$ even higher than 100 can be observed at sampling sites across the globe. Based on the considerations above, the high risk results mainly from the presence of the synthetic EE2. These high concentrations of EE2 responsible for the high $RQ_{cum.}$ were measured mainly in rivers, in particular in effluents of wastewater treatment plants.

By means of the (cumulative) risk quotient, the potential risk of individual substances can be estimated. However, this approach does not provide information on the combined effect of all substances since the PNECs used are only determined for individual estrogenic substances and not for mixtures.

4.2. Assessment of the A-YES® test system and instrumental analysis

In this work, two different methods have been used to investigate environmental water samples for estrogenic activity (**Publications I–III**). While the LC-MS/MS technique aims to detect individual substances (target analysis), the A-YES test system regards all substances in the water sample that have an affinity to the estrogen receptor and elicit a response, including mixture effects between the substances. Hence, the estrogenic activity calculated from the LC-MS/MS method may differ considerably from the activity determined by bioassay

(Du et al., 2020).

Instrumental analysis is only as comprehensive as the list of targeted analytes. Therefore, the calculation of an EEQ highly depends on the analyzed compounds and can only provide an estimate of the estrogenic activity. Previous studies concluded that discrepancies could arise from antagonizing effects of co-eluted compounds that interact with the binding sites of the receptor or inhibit the yeast growth, which would thus result in reduced $EEQ_{A\text{-}YES}$ (Chen et al., 2016; Wang et al., 2011). *Vice versa*, a limited list of target analytes can result in underestimates of the EEQ_{calc}, as further agonists of the estrogen receptor may also be present in the environmental sample (Könemann et al., 2018). Likewise, effects may differ in varying mixtures of estrogens due to their affinity and estrogenic potency (Yao et al., 2018).

Within this thesis, the estrogenic activity in the PRE and the SCS was determined on the one hand with the LC-MS/MS technique and, on the other hand, measured with the A-YES test system ($EEQ_{A\text{-}YES}$, see Deich et al., 2021b). With the data obtained from target analysis, EEQs were calculated considering the concentrations of single compounds and their specific estrogenic potency compared to E2 (EEQ_{calc}, see Fig. 4.5).

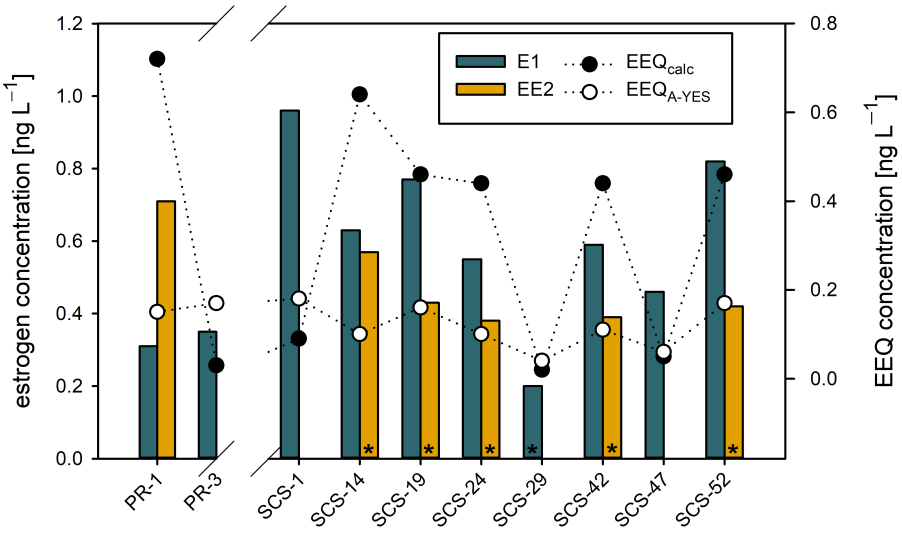


Figure 4.5.: Calculated (EEQ_{calc}) and measured ($EEQ_{A\text{-}YES}$) estrogenic activity in the PRE and SCS, *LOD < concentration < LOQ, adapted from Deich et al. (2021b).

In the PRE, $EEQ_{A\text{-}YES}$ could be quantified only at two stations up to 0.17 ng L^{-1} (PR-1, PR-3). The analysis of the samples from the marine stations (SCS-1, -14, -19, -24, -29, -42, -47 and -52) revealed similar $EEQ_{A\text{-}YES}$ in the range of 0.04–0.18 ng L^{-1}. In contrast, the calculation of EEQ_{calc} based on the measured individual components shows concentrations from 0.1–0.7 ng L^{-1} (Deich et al., 2021b). At the investigated sampling sites, measured and calculated EEQ diverged from each other in both ways: $EEQ_{calc} > EEQ_{A\text{-}YES}$

and $EEQ_{calc} < EEQ_{A\text{-}YES}$ (Fig. 4.5). As already mentioned, the composition of the sample or the completeness of the target analytes list are of great importance. For example, the samples in the SCS show that the $EEQ_{A\text{-}YES}$ correlate significantly with the measured E1 concentrations ($R^2 = 0.9, p < 0.01$). In contrast, the combination of the detected estrogenic compounds and the resulting EEQ_{calc} do not correlate significantly with the $EEQ_{A\text{-}YES}$ ($R^2 = 0.12, p = 0.4$). This is in line with the findings of Beck et al. (2006), whereas Wang et al. (2011, 2012) reported that determined $EEQs_{A\text{-}YES}$ correlate significantly with $EEQs_{calc}$ ($R^2 = 0.52$, $R^2 = 0.85$, $p < 0.0001$). They stated that estrogenic activity based on bioassay analysis was generally lower than the activity detected with instrumental analysis. This raises the question to what extent other estrogenic compounds potentially influenced the yeast-based assay. Also, it should be mentioned that only a small data set ($n = 8$) was regarded here. Thus, a more concise statement regarding the relationship between measured and calculated activity could be achieved by evaluating a larger data set.

Moreover, it needs to be emphasized that the A-YES test used in this study was always combined with a previous enrichment step (SPE). This clean-up procedure might have biased the outcome of the assay since the chemical composition of a sample is altered by using adsorption materials that were selected to be the most efficient for retaining the targeted estrogenic substances. By this, other substances with an affinity to the estrogen receptor but with different adsorption behaviors were neglected in further assessment of the estrogenic activity. Thus, a prior SPE may result underestimating EEQs if the discarded substances could cause a receptor response or overestimating EEQs if the discarded substances would exert an anti-estrogenic or agonistic effect on the receptor. In either case, the actual mixing effects present in the raw sample may not be fully displayed after SPE. Until now, however, enrichment steps such as SPE are practically indispensable for environmental research since concentrations in the environment are usually in the pg to ng L^{-1}-range.

Non-proportional EEQ Another important aspect to consider by enriching environmental samples with SPE is non-proportional EEQs in the bioassay. More specifically, the EEQs of the corresponding dilution levels are not linear (proportional) but increase despite higher dilution. This was observed for both the Baltic Sea samples and those from the South China Sea (**Publication I and II**).

It is assumed that non-linear responses within the bioassay result from a complex sample composition. For example, a high amount of estrogenic substances can lead to overloading and thus to competition for the binding sites of the receptor. Furthermore, masking effects and competitive inhibition may result from the presence of anti-estrogenic compounds that are co-enriched during the SPE procedure (Frische et al., 2009). Such interference could eventually lead to an underestimation of the estrogenic activity (Jarošová et al., 2014; Neale

et al., 2015). The non-proportional behavior can be mitigated using appropriate dilution levels, thereby restoring the linear dependence between dilution level and the receptor's response (non-proportional EEQ in Fig. 4.6).

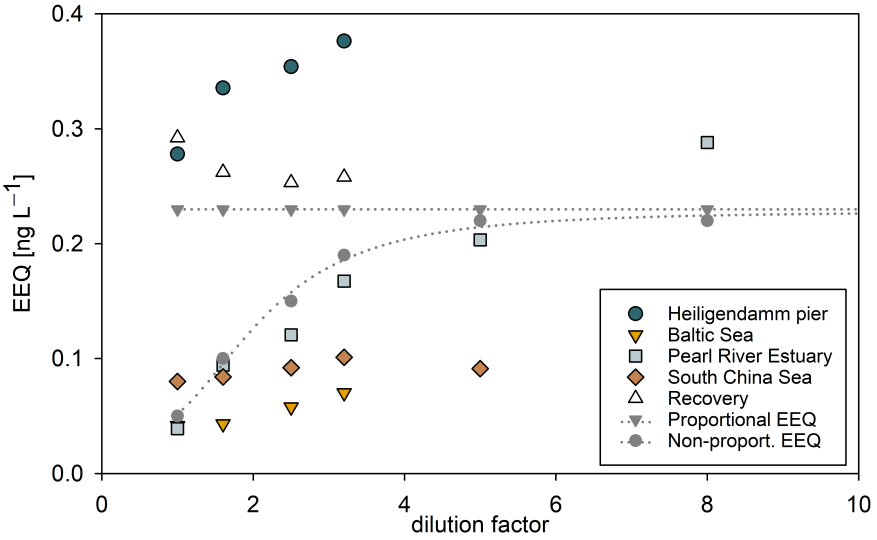


Figure 4.6.: Non-proportional EEQ at the prepared dilution levels for samples from Heiligendamm Pier, Baltic Sea, Pearl River Estuary, South China Sea as well as Milli-Q water spiked with E2 (recovery). Gray symbols represent proportional EEQ and non-proportional EEQ, adapted from Deich et al., 2020, 2021b.

In this work, non-proportional EEQ were predominately observed in the samples with elevated EEQ levels in both the Baltic Sea (Deich et al., 2020) and the Pearl River Estuary samples (Deich et al., 2021b; Fig. 4.6). In comparison, Milli-Q samples spiked with an E2 concentration of 0.2 ng L^{-1} (recovery samples) showed less non-linear relationships. Hence, it could be concluded that the samples' dilution levels were partially insufficient to mitigate the effects arising from the sample composition, and that the herein determined EEQ may have been underestimated. Furthermore, these data demonstrate that only a series of appropriate dilution levels can assess the actual estrogenic activity. However the above mentioned, bioassays still provide a first overview of potential estrogenic effects, in particular when applied simultaneously to target compound analysis.

5. Summary and Outlook

This work aimed to determine estrogenic substances and estrogenic activity in the marine environment for which only limited data sets are available, and further serves as a starting point for future work in this field. Within this study, an LC-MS/MS method was established in a first step to enable the analysis of single estrogenic compounds. It was shown that the method is precise for the steroidal estrogenic substances E1, E2 and EE2, which are known for their high estrogenic potential concerning their impacts on organisms. Furthermore, in addition to single compound analysis, the A-YES reporter gene activation assay was selected to determine the estrogenic activity that can act on marine organisms. The two methods detected estrogenic substances and estrogenic activity in both the Baltic Sea and the South China Sea around the Pearl River Estuary.

Among the targeted estrogenic substances in this thesis, the steroid hormones E1, E2, and EE2 were more frequently detected in the Baltic Sea. According to their estrogenic potential, they contributed the most to the observed estrogenic activity. The phytoestrogens (GEN, DAI) and the mycoestrogens (ZEN) were not abundant in the BS, but were detected in a small tributary to it. Analysis of two annual cycles showed that the concentration of estrogenic compounds and the estrogenic activity increased in summer. This pattern may be related to the beginning of the growing season at this time of year and the associated agricultural use, the increase in tourist activities or both.

The application of both methods in the area around the PRE revealed traces of E1 and EE2 as well as of GEN, DAI and ZEN. Concentrations decreased from the head of the estuary towards its mouth. The analysis of the SCS surface water samples showed that the substances E1, E2 and EE2 were the most abundant. In the PRE and SCS, estrogenic activity was determined both by yeast-based assay and by calculation based on single compound analysis. It was found that the estrogenic activity of the investigated PRE stations were similar to those measured at stations in the SCS close to the coast. The differences between measured and calculated estrogenic activity implied the presence of other substances with an affinity to the estrogen receptor or mixture effects between agonists and antagonists of the estrogen receptor, which were not considered in the LC-MS/MS analysis.

In this study, estrogen concentrations were compared with modeled sea surface currents from the SCS. Inter-annual variability affected the direction of major surface currents in the SCS. The influence and intensity of the summer monsoon have resulted in different directions of the Pearl River Plume. Based on literature data, the PRE is supposedly a major estrogen

deliverer in this region. It was concluded that the current direction of the plume might be responsible for the observed distribution patterns of estrogenic substances.

A first risk assessment revealed that a high potential risk should be considered for both sampling sites. This is mainly dominated by the presence of EE2, a man-made estrogen that could also profoundly disturb the endocrine system of organisms. Furthermore, it was shown that the steroid hormones were frequently detected in the BS and in the SCS, whereas the phyto- and mycoestrogens were rarely found in the marine system. As only the dissolved phase of surface water samples was investigated, particle-bound hormones or hormones at different depths of the water column were not considered.

This work showed that the respective analysis technique by LC-MS/MS is prone to the influence of co-eluting substances with similar chemical behavior. Limits of detection and quantification varied significantly if different sampling matrices, i.e., Milli-Q water and environmental water sample, were used. Overcoming this interference presumably lies in selecting a representative calibration method with the respective water sample, in the analysis of a standard addition sampling series and in further sample purification prior to LC-MS/MS analysis. This is most important for sampling sites with a complex sample composition, i.e., river sites such as the PRE.

Furthermore, this study targeted the most important steroidal estrogens, two selected phytoestrogens and one mycoestrogen. Although the simultaneous detection of synthetic EE2 implies an anthropogenic influence, it is still challenging to attribute elevated concentrations of natural substances such as E1 and E2 to their sources. Therefore, an essential aspect for future research will be the distinction between natural and anthropogenic sources of estrogenic substances. Implementing more substances may help to understand the distribution patterns observed in this study and to further elucidate on their origin. Alternatively, an isotope ratio analysis could provide further insights into the origin of estrogenic substances in the marine environment. Besides the selected estrogens, there are additional substances that also have the ability to elicit estrogenic effects in organisms and might therefore be added to the target list. However, such multi-screening analyses must be selected with great care, as a large number of analytes demand compromises regarding the accuracy, reproducibility, and robustness of the method.

Based on the available studies, to date, no reliable conclusions can be drawn regarding the long-term behavior of estrogenic substances in the marine environment. In addition to regular monitoring of these compounds, which would provide information also on seasonal and year-to-year variability, data on their persistence in the environment would be of great importance. The degradation rates or half-lives in the marine system should be considered in particular. In order to provide detailed information on the distribution of estrogenic substances in the marine system, it is necessary to further investigate coastal areas and estuaries

as a major source of estrogen input to the oceans. The use of suitable models could predict their transport considering the degradation rates and surface currents within the marine system, which might allow to estimate a possible risk for organisms even at remote locations.

In addition to the horizontal distribution of estrogens in the marine system, little focus has been placed on their vertical distribution although the *n*-octanol:water partition coefficients of these substances ($\log K_{OW}$ = 2.8–4.1, Adeel et al., 2017; Hansch et al., 1995) reflect their potential to adsorb to suspended particulate matter. This could contribute to the vertical transport within the water column, eventually resulting in the remaining of estrogenic substances in the sediment. Thus, not only would organisms at the surface be exposed to the potential adverse effects that can arise from estrogenic substances, but also benthic organisms. Therefore, examining the particulate phase of the water samples as well as sediments for such substances should be considered. A prerequisite for future analysis of the particulate phase is a modified and optimized method that quantitatively extracts those estrogens adsorbed to particles and sediment.

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Contributions to manuscripts

Publication I

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This work was conceptualized by C. Deich, M. Kanwischer and J.J. Waniek. C. Deich conducted all the experiments, evaluated the data and wrote all versions of the manuscript. M. Kanwischer evaluated the data and revised the manuscript. J.J. Waniek revised the manuscript. M. Jähne supported the method development. J.J. Waniek secured funding and supervised the study. Approximate contribution to this work by C. Deich = 80 %

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This work was conceptualized by C. Deich and J.J. Waniek. C. Deich conducted all the experiments, evaluated the data and wrote all versions of the manuscript. M. Kanwischer evaluated the data and revised the manuscript. R. Zhang provided Pearl River Estuary environmental data and revised the manuscript. J.J. Waniek revised the manuscript, secured funding and supervised the study. Approximate contribution to this work by C. Deich = 90 %

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This work was conceptualized by C. Deich and J.J. Waniek. C. Deich and J.-S. Appelt analyzed all samples. C. Deich evaluated and visualized the data and wrote all versions of the manuscript. H. C. Frazão visualized the data and revised the manuscript. W. Li and T. Pohlmann provided the model data and revised the manuscript. J.J. Waniek revised the manuscript, secured funding and supervised the study. Approximate contribution to this work by C. Deich = 75 %

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Patterns of estrogenic activity in the Baltic Sea

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Abstract Compounds such as estradiol and ethinylestradiol belong to contaminants of emerging concern, as they can disrupt the endocrine system of an organism with a hormonal system. The determination of such compounds is still challenging due to required low detection and quantification limits. Bioassays have proved to be sensitive tools for investigating the full potential of all compounds that can elicit an estrogenic response. In this study, surface water samples from different sampling sites and seasons in the Baltic Sea were analyzed for estrogenic activity with the *Arxula adeninivorans* yeast estrogen screen. Observed estradiol equivalent concentrations were in the range of $<$LOD – 0.38 ng L^{-1}. In general, a seasonal trend was observed, i.e., with an increase in water temperature in late spring, estradiol equivalent concentrations rose suddenly and decreased as abruptly when the temperature declined in autumn. An initial risk assessment shows that observed estradiol equivalent concentrations potentially affect organisms at a medium risk level based on determined risk quotients.

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Patterns of estrogenic activity in the Baltic Sea

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HIGHLIGHTS

- Two-year analysis of estrogenic activity in Baltic Sea surface water samples.
- Estradiol equivalent concentrations were up to 0.38 ng L^{-1} showing seasonal patterns.
- Observed concentrations at the coast might affect organisms at a medium risk level.

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ABSTRACT

Compounds such as estradiol and ethinylestradiol belong to contaminants of emerging concern, as they can disrupt the endocrine system of an organism with a hormonal system. The determination of such compounds is still challenging due to required low detection and quantification limits. Bioassays have proved to be sensitive tools for investigating the full potential of all compounds that can elicit an estrogenic response. In this study, surface water samples from different sampling sites and seasons in the Baltic Sea were analyzed for estrogenic activity with the *Arxula adeninivorans* yeast estrogen screen. Observed estradiol equivalent concentrations were in the range of < LOD – 0.38 ng L^{-1}. In general, a seasonal trend was observed, i.e., with an increase in water temperature in late spring, estradiol equivalent concentrations rose suddenly and decreased as abruptly when the temperature declined in autumn. An initial risk assessment shows that observed estradiol equivalent concentrations potentially affect organisms at a medium risk level based on determined risk quotients.

1. Introduction

Over 40% of the world population is living in proximity to the coast, and stresses the marine ecosystem by exploiting marine resources, fishing, eutrophication and pollution through waste disposal (Carpenter, 2005). Pollutants are transported into the marine ecosystems and elicit adverse health effects in its organisms, as these are often very sensitive to external influences. Frequently investigated contaminants are, for example, persistent organic pollutants such as polychlorinated biphenyls and polycyclic aromatic hydrocarbons, and restrictions have been implemented to reduce their abundance (El-Shahawi et al., 2010). However, new compounds of emerging concern, e.g., personal care products and pharmaceuticals, have not been included in routine monitoring programs (Dulio et al., 2018). Nevertheless, it is not only synthetically produced substances that are now being viewed critically, but also natural substances, such as steroidal hormones (Jarošová et al., 2014), that are more intensively introduced into the marine environment as a result of anthropogenic pressure.

Recent studies also focus on substances which have the potential to interact with and disrupt the endocrine system in humans and wildlife, referred to as endocrine disrupting compounds (EDCs) (Campbell et al., 2006; Deemoon et al., 2016; Xu et al., 2014; Yao et al., 2018). The endocrine system is an essential part of wildlife and human physiology. It regulates the development of organisms and significantly influences biological processes, making this system very vulnerable to external disturbances even in very low concentration ranges.

In fact, it has already been shown that biological processes can be influenced by EDC concentrations in the range of a few ng L^{-1}. In this regard, elevated vitellogenin levels were found in male trouts after exposure to 17α-ethinylestradiol (EE2) at concentrations as

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low as 0.1 ng L^{-1} (Purdom et al., 1994). At present, several natural and anthropogenic compounds are known for disturbing endocrine systems. In some studies, it is assumed that the majority of endocrine potential in the environment is derived from natural and synthetic hormones (Jarošová et al., 2014), which are estrone (E1), 17β-estradiol (E2) and estriol (E3), endogenous to and excreted from all organisms harboring endocrine function. The synthetic hormone, EE2, is mostly used in contraceptives (Adeel et al., 2017) Additionally, plant-derived estrogens, known as phytoestrogens, have also been shown to have an estrogenic effect (Benassayag et al., 2002). Moreover, endocrine activity was shown for ubiquitously distributed compounds, such as for alkylphenolic substances or bisphenol A (e.g., Beck et al. (2006); Cespedes et al. (2004); Peré-Trepat et al. (2004); Petrovic et al. (2004)) commonly used in plastic materials.

Potential EDCs are released into the aquatic environment mainly through domestic wastewater treatment plants where removal rates are not sufficient (Ying et al., 2002, 2008). EDCs have been found in rivers and inland water bodies (Chen et al., 2016; Pawlowski et al., 2004) but also in coastal areas (Atkinson et al., 2002; Beck et al., 2005; Zhang et al., 2014) and sediments (Wang et al., 2011, 2012). Therefore, the estrogens E1, E2 and EE2 are under observation and were also included in the 2nd EU-Watch list under the Water Framework Directive (Loos et al., 2018).

Although several investigations have focused on the determination of single compounds (Krein et al., 2012; Pojana et al., 2007), organisms are affected by the entirety of endocrine active compounds present (Kunz et al., 2017) and their combined agonistic and antagonistic effects (Petrovic et al., 2004). The resulting estrogenic activity can either be calculated through the amount of single compounds present and their estrogenic potency or determined by using *in vitro* tests sensitive to hormonal activity. Receptor-based transactivation assays, such as the *Arxula* yeast estrogen screen (A-YES), have presented themselves as a suitable tool, as they are fast, comparably cost-effective and give a comprehensive overview of estrogenic activity, since they consider agonistic, antagonistic and competitive mechanisms as well (Giesy et al., 2002; Hettwer et al., 2018; Routledge and Sumpter, 1996).

The Baltic Sea, one of the largest brackish seas (Snoeijs-Leijonmalm et al., 2017), has an area of about 412,500 km^2 and stretches from the Kattegat to the Gulf of Bothnia (HELCOM, 2010). Rivers transport large amounts of freshwater into the Baltic Sea, where the residence time is about 30 years (Stigebrandt, 2001) due to limited water exchange with the North Sea (HELCOM, 2010). As the catchment area of the Baltic Sea is home to approximately 85 million people (HELCOM, 2010), industrial, urban and agricultural waste is transported within rivers into the sea. This waste includes chemicals which enter the marine environment and serve as a potential threat for marine organisms. In this regard, in a study by Gercken and Sordyl (2002) conducted in surface waters alongside the coast of Mecklenburg-Western Pomerania, intersex characteristics in the eelpouts *Zoarces viviparus* were observed. At the same sampling sites, Beck et al. (2005) investigated surface waters for estrogenic compounds in two sampling periods in 2003 and 2004 and observed concentrations of up to 17 ng L^{-1} of the strongly estrogenic EE2. This concentration already exceeds the predicted-no-effect-concentration (PNEC) of 0.1 ng L^{-1} for EE2 estimated by Caldwell et al. (2012).

The present study focuses on the determination of estrogenic activity in samples from the western Baltic Sea, where, to the best of our knowledge, only one study by Beck et al. (2006) reports estrogen levels. We are interested in seasonal patterns of estrogenic activity and the determination of the possible risk for marine organisms in this study area.

2. Experimental

2.1. Chemicals

17β-estradiol (E2) was purchased from Sigma Aldrich. Culture medium as well as the recombinant yeast *Arxula adeninivorans* were obtained from new_ diagnostics GmbH (Berlin/Dresden, Germany) within the A-YES® test kit. Analytical grade water was obtained from Merck KGaA (Darmstadt, Germany), and Milli-Q water was prepared with Milli-Q® reference water purification system from Merck Millipore (Darmstadt, Germany). The substrate *para*-nitrophenyl phosphate (*p*-NPP) was obtained from AppliChem GmbH (Darmstadt, Germany). Ethylacetate and methanol LC-MS/MS grade were purchased from Promochem (Wesel, Germany). NaOH and HCl were obtained from VWR International GmbH (Hannover, Germany).

2.2. Sampling area and sampling strategy

All sampling sites are located in the western part of the Baltic Sea (Fig. 1), and samples were collected to estimate the annual state of estrogenic activity in this area. Surface water samples were collected at Heiligendamm pier (HD, Table S1) located at the Mecklenburg-Western Pomeranian coast. This sampling site has been chosen, as Heiligendamm pier is known for being influenced by tourism, especially during the summer season. In close distance, the small river Mühlenfließ flows into the Baltic Sea through the Jemnitz-floodgate. There are no wastewater treatment plants in the immediate vicinity. However, the Baltic Sea is mainly fed by rivers which also transport wastewater. In the first sampling period between November 2016 and October 2017, samples were taken twice a month, whereas only one sample per month was taken in the second period between February 2018 and November 2018. Surface water was collected via bucket sampling at approximately 0.5 m depth.

During six research cruises in the Baltic Sea from August 2017 to November 2018 (Table S2), which are part of the regular Baltic Sea monitoring program, surface water samples were collected at ten different sampling sites (Fig. 1). Stations TF0040, TF0041 and TF0046 are located in the Bay of Mecklenburg and represent the eastern boundary in this study. Sampling sites TF0011, TF0012, TFO5 and TF0022 are also located in the Bay of Mecklenburg. TFO5 is located close to the Warnow estuary, while TF0022 is located in the Lübeck Bay influenced by larger cities such as Wismar and Lübeck as well as the river Trave. TF0010 is located between the Bay of Mecklenburg and Bay of Kiel at the Fehmarn Belt. TF0361 and TF0360 lie in the Bay of Kiel and display the western border. Surface water was collected with a CTD rosette system (SBE911) operated on R/V Elisabeth Mann Borgese at 1–2.5 m depth.

Sampled water was stored in pre-rinsed polyethylene bottles or canisters and was acidified with 5 M HCl to pH 2–3 directly on board or after arriving at the laboratory. Samples were stored at 4°C. If samples needed to be stored longer than two weeks, they were kept at −20°C.

2.3. Sample extraction

Extraction of the samples was conducted according to the procedure described by new_ diagnostics. In general, samples were filtered (Whatman™GF/F filters 0.7 m) and simultaneously enriched through solid phase extraction (SPE) on Chromabond® C18 ec from Macherey-Nagel (Dueren, Germany). Surface water samples were passed through the SPE columns with a flow rate of approximately 10mLmin^{-1}. For the first sampling period at Heiligendamm pier, 400 mL was passed on to SPE columns with 200 mg

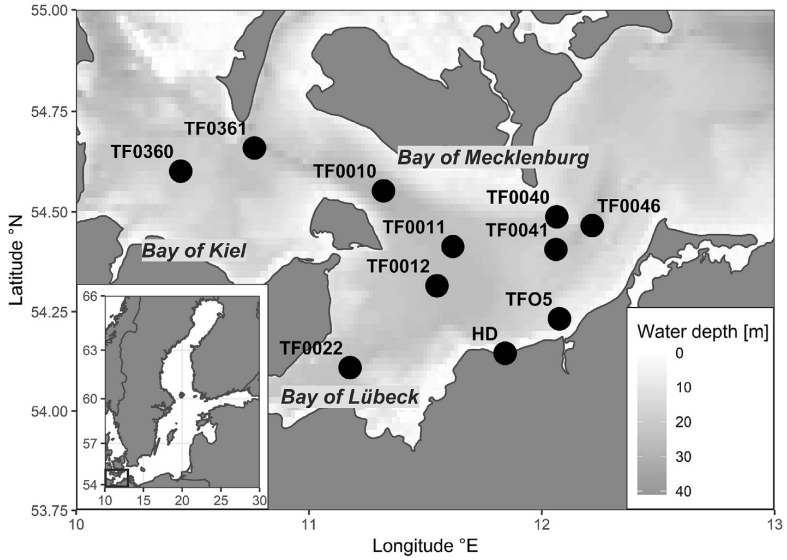

Fig. 1. Baltic Sea (insert) and the location of ten sampling sites in the western part of the Baltic Sea as well as the location of Heiligendamm pier (HD).

of adsorbent material. SPE columns with 1000 mg of adsorbent material were used for the enrichment of 1 L sample volume from the second sampling period. For Baltic Sea samples, a sampling volume of 2 L was divided evenly, and each liter was passed through a 1000 mg SPE column separately to avoid overloading as well as clogging of the column. The columns were washed afterwards with 1.5 mL of 5% MeOH in Milli-Q and 1.5 mL of Milli-Q (3 mL each for 1000 mg C18 ec). After the washing step, columns were dried and then stored at 4 °C until further analysis the day after.

Elution was conducted with 10 mL (15 mL for 1000 mg C18 ec) of EtOAc:MeOH (30:70, v:v). For Baltic Sea samples, both extracts of the two SPE columns were combined. Extracts were vaporized to dryness under clean air in a 40 °C water bath and subsequently reconstituted in 6 mL of analytical grade water.

2.4. Biological analysis

The utilized receptor-based transactivation assay is working with the genetically modified halo-tolerant yeast strain *Arxula adeninivorans*, in which the human estrogen receptor *hER* is constitutively expressed. Upon the presence of a suitable ligand in the aqueous sample, i.e., estrogen affecting compound, the expression of the reporter gene is initiated and the enzyme phytase is produced. Substrate cleavage through phytase can be quantified spectrophotometrically and correlates to the concentration of ligands. The estrogenic activity is expressed as estradiol equivalent concentrations (EEQs), thus, in relation to the endocrine activity of the 17β-estradiol (Hettwer et al., 2018).

The biological analysis was conducted as published in Hettwer et al. (2018). The procedure will be described in brief in the following. Lyophilized yeast was reactivated by incubation with diluted culture medium for 1 h at 31 °C and 500 rpm on an incubation shaker. 96-deep well plates were loaded with 400 μL of sample extracts in appropriate dilution levels (1, 1.6, 2.5 and 3.2 fold) and four replicates each, seven calibration levels as triplicates containing the 17β-estradiol standard in a concentration of 1, 2, 4, 8, 20, 40 and 80 ng L^{-1} as well as a negative control and blank samples. All wells were inoculated with 100 μL of yeast suspension except blanks containing pure medium only. The deep well plates were incubated for 20–21 h at 31 °C and shaken simultaneously at 850 rpm.

After incubation, 50 μL of clear supernatant was transferred to 96-well cell culture plates. 50 μL of substrate solution (1 mgmL^{-1} *p*-NPP in 0.5 M citric buffer) was added to the supernatant and left for incubation for 1 h at 37°C. For determination of the reporter gene expression, the optical density (OD) of phytase was measured at 405 nm with a microplate reader (Infinite M200, Tecan Trading AG, Maennedorf, Switzerland) before and after addition of 100 μL of 3 M NaOH to exclude the background signal. OD of yeast cell suspension diluted to 1:20 Milli-Q water was measured at 630 nm to ensure that observed reporter gene activity relies solely on the estrogenic activity of the sample but not on different yeast growth in the wells (Hettwer et al., 2018).

2.5. EEQ quantification and quality assurance

Evaluation of data, determination of method-specific detection and quantification limits (LOD_m, LOQ_m) as well as quantification of estrogen equivalent concentrations (EEQ_m) were calculated with the software BioVAL® provided by new_ diagnostics GmbH for each individual test (Tables S3 and S4). The BioVAL® report contained a statistical assessment for standard and sample outliers, the individual sigmoidal calibration curve for 17β-estradiol based on a four-parameter logistic function, LOD_m, LOQ_m and calculated EEQ_m. A more detailed description can be found in Hettwer et al. (2018).

The highest dilution was selected to quantify the EEQ_m and minimize the matrix effects of the SPE enrichment. EEQ_m below LOQ_m concentrations were set to the referring LOD_m, whereas concentrations below LOD_m were defined as "n.d.". As calibration curves and yeast growth differ between each biological test, method-specific detection and quantification limits were 4.35±0.92 ng L^{-1} and 6.15±1.61 ng L^{-1}. To obtain sample-specific LOD, LOQ and the final concentrations of EEQ, the enrichment factor (F) was taken into account (e.g., $EEQ_m/F = EEQ$). A comprehensive overview of the data is provided in Tables S3 and S4.

For recovery analysis, Milli-Q was spiked with the E2 standard to a final concentration of 0.2 ng L^{-1}. Milli-Q water without E2 standard was used for SPE blank to determine the possible contamination throughout the clean-up procedure. Standard samples were also acidified with 5 M HCl, followed by SPE and analyzed with the biological test system as described above for the environmental samples. For each test, two recovery samples were analyzed simultaneously.

Analyzed SPE blanks showed no estrogenic response in the bioassay or were below LOD. For the spiked Milli-Q water samples, the mean recovery rate was 73.27±25.20 %.

3. Results

3.1. Heiligendamm pier

During the first sampling period at Heiligendamm pier from November 2016 to October 2017, samples were taken every two weeks in triplicate and analyzed for estrogenic activity (Fig. 2A, S1A). Samples from November 2016 until the middle of May 2017 had EEQs below LOQ. However, from the end of May, EEQs suddenly increased to quantifiable levels of 0.13±0.06 to 0.20± 0.04 ng L^{-1}. In the end of July 2017, EEQs suddenly decreased again below the LOD.

Within the second sampling campaign from February 2018 until November 2018, in which samples were taken monthly in triplicate, a similar pattern as for the previous year was observed (Fig. 2C, S1B). EEQs between February and May 2018 were mostly below < LOD and increased suddenly from June 2018 to quantifiable EEQs ranging from 0.20±0.01 ng L^{-1} to 0.38±0.01 ng L^{-1}. Like in 2017, concentrations also decreased suddenly to 0.06±0.03 ng L^{-1} in September. Thereafter, EEQs were below LOD until November 2018.

The data from both years shows that throughout the year, EEQs rise and drop very suddenly with peak EEQs from May to August.

3.2. Baltic Sea

In investigated samples (n = 60) from six different cruises in the Baltic Sea collected from August 2017 to July 2018, EEQs were ranging from < LOD to 0.11 ng L^{-1}, with a mean of 0.03 ng L^{-1} and a median of 0.02 ng L^{-1} (Figs. 3 and 4).

Starting in August 2017, EEQs varied between < LOD – 0.11 ng L^{-1} with a mean of 0.06 ng L^{-1} and a median of 0.07 ng L^{-1}. Concentrations in November 2017 decreased to < LOD – 0.03 ng L^{-1} with a mean and median of 0.02 ng L^{-1} and did not

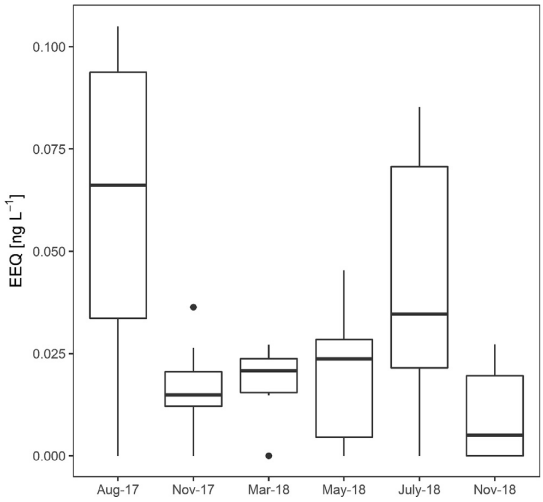

Fig. 3. Estradiol equivalent concentrations (EEQs) in ng L^{-1} for six different cruises (n = 10 for each cruise, • representing outliers) investigated in the western Baltic Sea from August 2017 to November 2018.

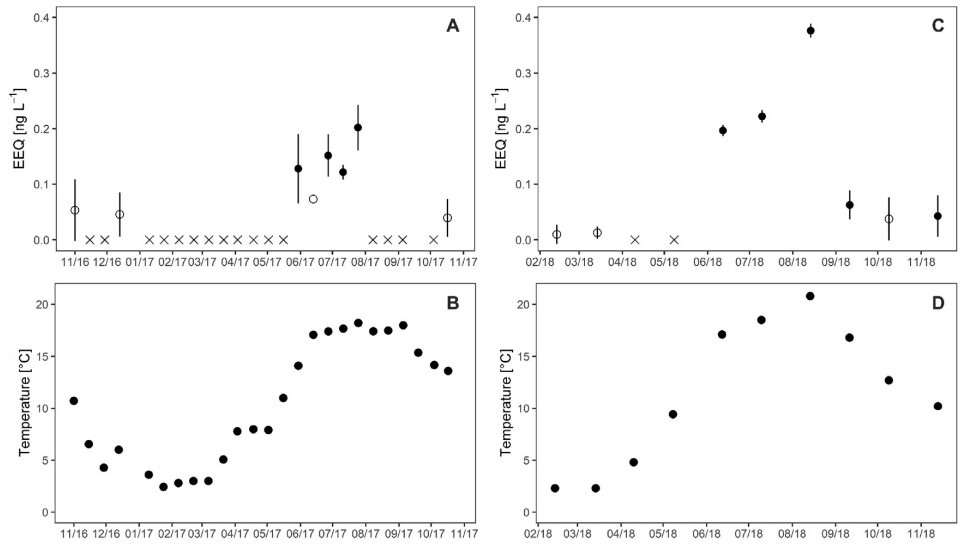

Fig. 2. Annual course for EEQs at Heiligendamm pier. Mean and standard deviation of estradiol equivalent concentrations (EEQs, × EEQ < LOD, ◦ LOD < EEQ < LOQ, • EEQ > LOQ) in ng L^{-1} of samples from Heiligendamm pier collected between November 2016 and October 2017 (**A**) and between February 2018 and November 2018 (**C**) with corresponding water temperature in °C (**B, D**).

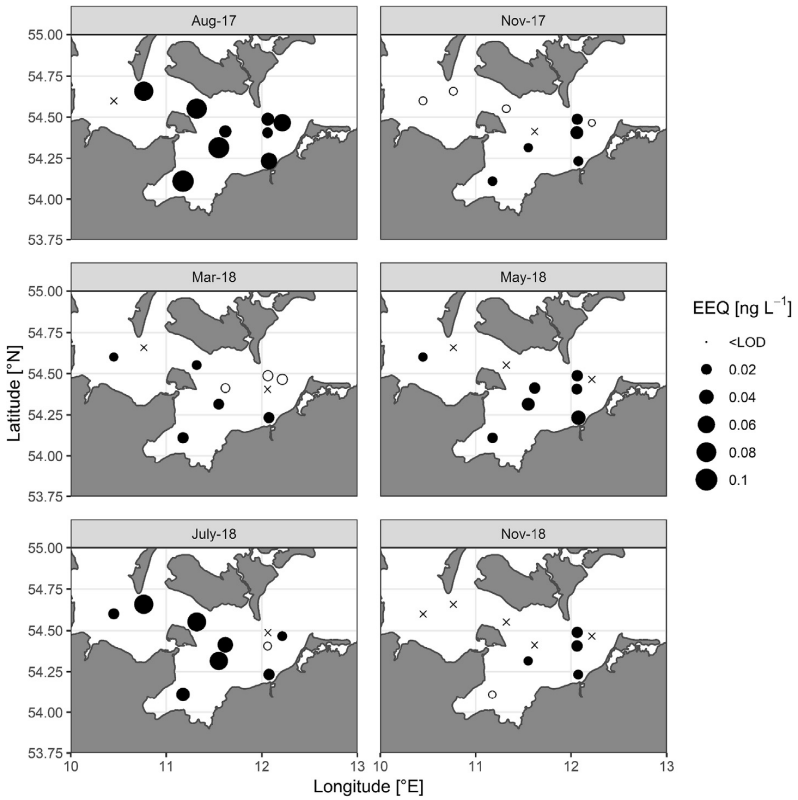

Fig. 4. Estradiol equivalent concentrations (EEQs) in ng L^{-1} (× EEQ < LOD, ◦ LOD < EEQ < LOQ, • EEQ > LOQ) displayed for six cruises in the Baltic Sea in August 2017, November 2017, March 2018, May 2018, July 2018 and November 2018 at ten different sampling sites (n = 1).

rise significantly in March 2018 with EEQs up to 0.03 ng L^{-1} with mean and median concentrations of 0.02 ng L^{-1}. In May 2018, EEQs increased slightly up to 0.05 ng L^{-1} with mean and median concentrations of 0.02 ng L^{-1}. Elevated concentrations were observed during the cruise in July 2018 displaying EEQs up to 0.09 ng L^{-1} with a mean and median concentration of 0.04 ng L^{-1}. Samples taken during November 2018 displayed concentrations in the range of < LOD – 0.03 ng L^{-1} with mean and median concentrations of 0.01 ng L^{-1}.

4. Discussion

In this study, we investigated samples taken at Heiligendamm pier and from the western part of the Baltic Sea during six sampling campaigns for estrogenic activity. For this, samples were enriched by solid phase extraction and were analyzed with the yeast-based test system sensitive to estrogen or estrogen-like substances.

4.1. Regional variability

Generally, observed EEQs within this study (< LOD – 0.38 ng L^{-1}) are in good agreement with concentrations found in literature summarized in Table 1 and are in the same order of magnitude when compared to studies conducted in coastal environments (0.01 – 0.82 ng L^{-1} (Beck et al., 2005)). However, concentrations were much lower than in inland water bodies such as rivers or in- and effluents of sewage treatment plants (11.81 – – 65.96 ng L^{-1} in the Rhine (Pawlowski et al., 2004)).

Elevated EEQs were found at times of high temperature as well as at sampling sites near the coast, i.e., Heiligendamm pier. As the study of Beck et al. (2006) indicates, high EEQs are much more likely to be observed in enclosed areas, such as the Wismar Bay, where dilution of riverine input may occur less than in the open

Table 1
Comparison of estradiol equivalent concentrations (EEQs) of surface water samples investigated in different regions.

Sampling Area	Method	EEQ [ng L^{-1}]	Reference
River Rhine, Germany	YES	11.81 – 65.96	Pawlowski et al. (2004)
Chinese Streams, China	YES	0.01 – 40.27	Yao et al. (2018)
Yundang Lagoon, China	EEQ_{calc}	4.56 – 13.79	Zhang et al. (2011)
Baltic Sea, Germany	YES	0.01 – 0.82	Beck et al. (2005)
Pearl River Delta, China	EEQ_{calc}	0.2 – 4.5	Xu et al. (2014)
South China Sea, China	EEQ_{calc}	0.01 – 4.66	Zhang et al. (2014)
Florida Keys, USA	YES	0.04 – 0.26	Singh et al. (2010)
Suruga Bay, Japan	Y2H	n.d. – 0.53	Hashimoto et al. (2007)
Tokyo Bay, Japan	MVLN	0.34 – 2.52	Hashimoto et al. (2005)
Baltic Sea, Germany	A-YES	n.d. – 0.37	this study

A-YES: Yeast estrogen screen based on *Arxula adeninivorans* (Hettwer et al., 2018). MVLN: Bioassay using MCF-7 human breast carcinoma cells (Demirpence et al., 1993). Y2H: Yeast two-hybrid system using rat liver S9 (Shiraishi et al., 2000). YES: Yeast estrogen screen based on *Saccharomyces cerevisiae* (Routledge and Sumpter, 1996). EEQ_{calc}: EEQs were calculated by multiplying single compound concentrations with respective Estradiol Equivalent Factors (EEF) (Jarošová et al., 2014). n.d. not detectable.

Baltic Sea. In close distance to Heiligendamm pier, the small tributary Mühlenfließ introduces riverine water including agricultural waste to the coast and might cause the rise in estrogenic activity in summer. Additionally, waters around the Heiligendamm pier are influenced by tourism especially in summer, which potentially affects estrogen concentrations as well (Statistisches Amt Mecklenburg-Vorpommern, 2018). Interestingly, the sampling site TFO5, although located close to the estuary of the Warnow River flowing through Rostock, shows no permanently higher values when compared to the other stations located further away from the coast. There may be other mechanisms influencing estrogenic activity in the Baltic Sea besides the indirect introduction of estrogenic substances by terrestrial wastewater effluents (Gong et al., 2014; Kolodziej et al., 2004; Ying et al., 2002).

4.2. Seasonal and interannual variability

Data obtained from sampling campaigns located in the Baltic Sea as well as at Heiligendamm pier show a seasonal trend which has already been observed in other studies, e.g., elevated estrogenic activity occurred in summer in the Northern Mediterranean Sea (Pinto et al., 2005). In this study, higher EEQs were most frequently detected in months with higher temperature (May to September), whereas comparably lower concentrations are more likely to be observed in colder months (October to April). These findings are comparable to a study of Krein et al. (2012) in Luxembourg Rivers. Additionally, differences have been found in between sampling years. In 2018 at Heiligendamm pier, a maximum EEQ of 0.38 ng L^{-1} was observed in August, while in 2017, the maximum EEQ of 0.20 ng L^{-1} was determined in July. In contrast, highest concentrations in the Baltic Sea were found in August 2017 and in July 2018, presumably indicating that the annual increase of EEQ is driven by different factors at the investigated sites. However, even in comparably warmer months, not all samples showed quantifiable EEQ concentrations. Advection of different water bodies might lead to changes in estrogenic activity levels, which might be the result of biological activity or anthropogenic sources.

We observed that EEQs correlate with the water temperature ($R^2 = 0.41$, $p < 0.001$ (Fig. S2)). As the temperature rises, on the one hand, the vegetation period begins, agricultural use increases and livestock is brought to the fields. On the other hand, enhanced summer tourism and the associated direct input of estrogens through recreational activities might be a reason for elevated EEQs. Furthermore, slightly increased rainfall during this season might have led to enhanced river discharge potentially transporting estrogenic compounds into the Baltic Sea (Fig. S2). In addition, the study of Beck et al. (2005) showed that estrogenic activity in this part of the Baltic Sea was mainly caused by the endogenous E1 and the synthetic EE2. Other studies demonstrated that phytoestrogens produced by phytoplankton species might be a reason for elevated estrogenic activity (Gong et al., 2014). However, no significant correlation between EEQ and chlorophyll *a* has been observed in this study.

During our investigations, we observed increased EEQs during sample dilution in particular for samples with comparably high EEQs (Fig. 5). We assume that estrogen activity might be suppressed at lower sample dilutions which became dissolved upon higher dilution, thus, leading to non-proportional EEQs during sample dilution. Frische et al. (2009) and Jarošová et al. (2014) emphasized possible masking effects which might arise upon the presence of antiestrogenic compounds for *in vitro* assays which could lead to underestimation of the estrogen activity in a sample. In particular, when using enrichment methods, such as SPE used within this study, fortification of the matrix might enhance antiestrogenic effects as well. Therefore, and based on our data, we strongly believe that the *de facto* estrogen activity in a sample is only accessible through analysis of a series of sample dilutions to overcome eventual inhibitory effects due to antiestrogenic compounds.

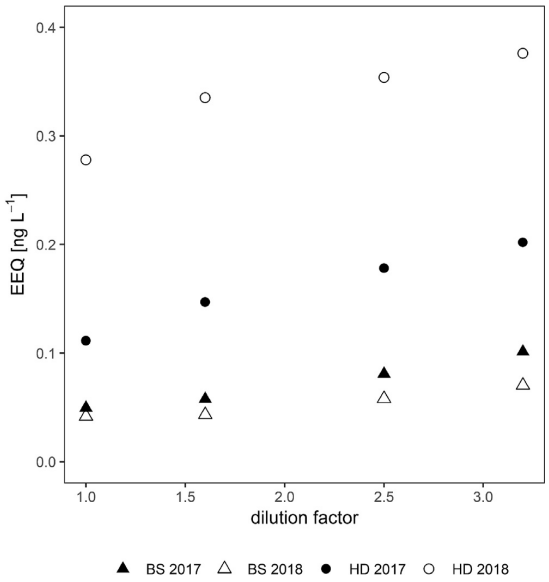

Fig. 5. Dilution dependent EEQ in ng L^{-1}. Non-proportional EEQ during sample dilution for selected Heiligendamm pier samples (circle) and Baltic Sea samples (triangle) in summer 2017 (black) and 2018 (white). Sample dilution factors were 1, 1.6, 2.5 and 3.2.

4.3. Risk assessment

As estrogens are released into the environment not only naturally, their enhanced occurrence especially in marine waters may be considered as a potential threat to aquatic organisms and serve as an indicator for anthropogenic pressure (Adeel et al., 2017). To assess the possible harm, risk quotients (RQ) were calculated using the measured environmental concentration (MEC) divided by a predicted-no-effect-concentration (PNEC). The RQs are categorized in RQ < 0.1 low risk, $0.1 < RQ < 1$ medium risk and $RQ > 1$ high risk for aquatic organisms (European Commission, 2003). For E2, Caldwell et al. (2012) suggested a PNEC of 2 ng L^{-1} based on a species sensitivity distribution where fish were the most sensitive species. However, in this current study, the EEQ was determined and, consequently, the use of a PNEC based solely on E2 might underestimate the toxicological risk for aquatic organisms arising from estrogenic effects. In this regard, Caldwell et al. (2012) suggested a PNEC of only 0.1 ng L^{-1} for EE2, as this synthetic estrogen has strong adverse effects to aquatic organisms. As a result of the different PNEC of the individual substances, a PNEC for EEQ of not more than 1 ng L^{-1} for biological tests is often considered (Jarošová et al., 2014). In this view, RQ of the investigated sites indicate an intermediate ecotoxical risk for the coastal sampling sites, when regarded for the temporarily elevated concentrations in summer (Table 2, RQ_{EEQ}). Baltic Sea samples show low to intermediate risk level.

As the EEQ summarizes total estrogenic activity, the specific composition is unknown. Therefore, assuming that total EEQ arises solely from EE2 leads to high risk for the organisms with RQ up to

Table 2
Risk quotients (RQ) based on maximum EEQs in all analyzed sampling systems calculated with different predicted-no-effect-concentrations (PNEC) for 17β-estradiol (E2), 17α-ethinylestradiol (EE2) and an estimated PNEC for EEQ. RQ < 0.1 low risk, 0.1 < RQ < 1 medium risk and RQ > 1 high risk.

	EEQ_{max} [ng L^{-1}]	RQ_{E2} [a]	RQ_{EE2} [a]	RQ_{EEQ} [b]
HD 2017	0.20	0.11	2.0	0.20
HD 2018	0.38	0.19	3.8	0.38
BS 2017	0.11	0.06	1.1	0.11
BS 2018	0.09	0.05	0.9	0.09

[a] $PNEC_{E2}$ = 2 ng L^{-1}, $PNEC_{EE2}$ = 0.1 ng L^{-1} (Caldwell et al., 2012).
[b] $PNEC_{EEQ}$ = 1 ng L^{-1} (Jarošová et al., 2014).

3.8. If the whole sample is composed only of E2, low and medium risk levels must be considered (Table 2, $RQ_{EE2/E2}$).

However, the chosen PNEC concentrations were obtained by regarding certain aquatic species which are most likely not the ones present in waters of the Baltic Sea at the investigated sampling sites and, thus, the risks can only be roughly estimated. For a better understanding of the mechanisms which might be induced by means of estrogens in an organism, biological studies with present species, in particular those living in the Baltic Sea, are necessary to evaluate the potential risk of estrogenic activity properly. Moreover, comprehensive information about the estrogenic composition is important.

Finally, natural and synthetic estrogens exhibit high log K_{OW} (17β-estradiol: 3.94, 17 β -ethinylestradiol: 4.15, estriol: 2.81 and estrone: 3.43 (Ying et al., 2002)), which highlights their potential to enrich on solid matter, such as particles and sediments (Duong et al., 2010). In this light, even temporarily elevated EEQs might lead to endocrine impacts for aquatic organisms in the long-term perspective, which cannot be specified at the current stage.

5. Conclusion

The used A-YES test in combination with SPE was successfully applied and gave a first overview of the estrogenic activity in the Baltic Sea. EEQs rose to quantifiable levels in late spring and declined abruptly in autumn. A first risk assessment shows that surface waters of the Baltic Sea may display an estrogenic effect at an intermediate level during summer months, especially close to the coast. Nevertheless, additional investigations are necessary to further assess the estrogenic activity, especially for differentiation between natural and anthropogenic origin. Therefore, single compound analysis, e.g., through HPLC-MS/MS, combined with bioassays, such as the A-YES system used in this study, is needed to give a comprehensive overview.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2019.124870.

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Natural and synthetic estrogenic compounds in the Pearl River Estuary and northern shelf of the South China Sea

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Abstract Endocrine disrupting compounds and in particular estrogenic substances have the ability to interact with the hormone system of organisms. Among them are not only synthetic but also natural substances that potentially stress the aquatic ecosystem. High human population densities such as around the Pearl River Estuary (PRE) are suspected of exerting significant anthropogenic pressure onto coastal areas. In this study, natural and synthetic estrogens as well as estrogen-like substances derived from plants and fungi were investigated in the PRE and at the adjacent northern shelf of the South China Sea. Maximum concentration of 3.6 ng L^{-1} for estrone (E1), 0.7 ng L^{-1} for 17α-ethinylestradiol (EE2), 12.9 ng L^{-1} for genistein (GEN), 11.9 ng L^{-1} for daidzein (DAI) and 1.9 ng L^{-1} for zearalenone (ZEN) were observed. While E1 and EE2 were detected in both fresh- and saltwater samples, GEN, DAI and ZEN were observed only at freshwater sampling sites. During the investigations, the analysis of 17β-estradiol (E2) and EE2 indicated a strong matrix dependence. Additionally, an estrogen screen observation showed estrogenic activity in form of estradiol equivalent quotients up to 0.17 ng L^{-1}.

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Natural and synthetic estrogenic compounds in the Pearl River Estuary and northern shelf of the South China Sea

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Abstract Endocrine disrupting compounds and in particular estrogenic substances have the ability to interact with the hormone system of organisms. Among them are not only synthetic but also natural substances that potentially stress the aquatic ecosystem. High human population densities such as around the Pearl River Estuary (PRE) are suspected of exerting significant anthropogenic pressure onto coastal areas. In this study, natural and synthetic estrogens as well as estrogen-like substances derived from plants and fungi were investigated in the PRE and at the adjacent northern shelf of the South China Sea. Maximum concentration of 3.6 ng L^{-1} for estrone (E1), 0.7 ng L^{-1} for 17α-ethinylestradiol (EE2), 12.9 ng L^{-1} for genistein (GEN), 11.9 ng L^{-1} for daidzein (DAI) and 1.9 ng L^{-1} for zearalenone (ZEN) were observed. While E1 and EE2 were detected in fresh and saltwater samples, GEN, DAI and ZEN were observed only at freshwater sampling sites. During the investigations, the analysis of 17β-estradiol (E2) and EE2 indicated a strong matrix dependence. Additionally, an estrogen screen observation showed estrogenic activity in form of estradiol equivalent quotients up to 0.18 ng L^{-1}.

1. Introduction

In general, estrogenic activity in the environment is mainly caused by natural and synthetic steroids, e.g., estrone (E1), 17β-estradiol (E2) and 17α-ethinylestradiol (EE2) (Adeel et al., 2017). E1 and E2 are the natural regulators of the female reproductive mechanism. However, estrogen active substances have been linked to adverse health effects. For example, abnormal changes in embryos of the viviparus eelpouts (*Zoarces viviparus*) arose from exposure to 5.7 ng

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L^{-1} of E2 (Morthorst et al., 2014). The synthetic EE2, which is the active ingredient in contraceptive pills, has been observed to act at doses as low as 0.1 ng L^{-1} to 1 ng L^{-1} (Purdom et al., 1994) and induces a rise in the vitellogenin concentration in the three-spined stickleback (*Gasterosteus aculeatus*; Andersson et al., 2007) as well as deformities and increased mortality rate in Chinese rare minnow (*Gobiocypris rarus*) and zebrafish (*Danio rerio*; Zha et al., 2008; Xu et al., 2008).

In addition to steroidal estrogens, natural substances produced by plants and fungi (phyto- and mycoestrogens) were observed to induce adverse health effects as well (Benassayag et al., 2002; Jarošová et al., 2014; Sarasquete et al., 2020). These include, for instance, isoflavones such as daidzein (DAI) and genistein (GEN), which are mostly found in connection to soy products but also in algae and some cyanobacteria species (Jarošová et al., 2015). In addition, there is evidence that mycotoxins like zearalenone (ZEN), which can be found on contaminated cereal crops, can be associated with estrogenic effects such as impaired gonadal development, intersex observations and decreased sperm production in fish (Bakos et al., 2013; Hartmann et al., 2007; Woźny et al., 2020). Next to steroidal estrogens and those derived from plants and fungi, chemicals such as bisphenol-A (BPA), nonylphenols and octylphenols were shown to elicit estrogenic effects on organisms as well (Campbell et al., 2006; Hotchkiss et al., 2008).

Insufficient removal rates of wastewater treatment plants and the improper disposal of domestic and industrial wastewater are considered to serve as the main pathway for natural and anthropogenic estrogenic compounds to enter the aquatic environment (Xu et al., 2014; Jiang et al., 2020). Furthermore, agriculture runoff, e.g., from rangeland grazing or the application of manure as fertilizers (Kolodziej & Sedlak, 2007), and the usage of estrogens as growth promoters in aquaculture can contribute to their release (Kolodziej et al., 2004). Once estrogenic compounds have entered riverine systems, they may end up in the oceans and potentially stress marine organisms.

In order to analyze natural and synthetic estrogenic substances, pre-concentration and analysis with extremely sensitive instruments, such as liquid chromatography with tandem mass spectrometry (LC-MS/MS), are required to decipher the complex composition of environmental samples. Complementary to instrumental analysis, *in vitro* biological assays, such as the *Arxula* yeast estrogen screen (A-YES), are used as they consider synergistic or antagonistic effects possibly occurring through the variety of different compounds in the environmental samples. The *Arxula adeninivorans* yeast strain is genetically modified with an integrated human estrogen receptor sensitive to estrogens and estrogen-like substances. Compounds that fit to the non-specific receptor binding site can elicit a response in the test by either inducing or inhibiting reporter gene expression. This response is measured in relation to the response of E2 and is therefore expressed in estradiol equivalent quotients (EEQ). As a result, the overall estrogenic effect exerted by the substance mixture of a water sample is determined with this *in vitro* bioassay. In this regard, bioassays capture both the estrogens known by instrumental analysis and the unknown substances in the environmental samples with affinity to the estrogen receptor (Hettwer et al., 2018; Kunz et al., 2015). Thus, the combination of instrumental analysis with bioassays may provide further insights into potential endocrine effects on organisms based on estrogenic compound compositions and their cumulative activity.

The Pearl River Estuary (PRE) is known for harboring some of the largest cities in the world. Cities such as Guangzhou, Hong Kong and Shenzhen are densely populated, which results in high discharge rates of domestic and industrial waste. Xu et al. (2014) reported estrogen concentrations of 0.72 ng L^{-1} for E1 and 0.45 ng L^{-1} for EE2 in the PRE, where altered fish reproduction effects have already been observed (Chen et al., 2016; Gong et al., 2009; Xie et al., 2019). Estrogens were also detected in the adjacent South China Sea (SCS) with concentrations of steroids up to 11.38 ng L^{-1},3.7 ng $L^{-1}$and 3.99 ng L^{-1} for E1, E2 and EE2, respectively (Deich et al., 2021; Zhang et al., 2014).

Previous studies in the Pearl River have investigated primarily the freshwater influents of the estuary as well as the recipient river water of wastewater treatment plants mainly for steroidal estrogens (Peng et al., 2008; Zhao et al., 2011; Yu et al., 2011). Moreover, the systems PRE and SCS were only regarded in separate studies, and, to our knowledge, data on estrogenic compounds in coastal marine environments are still limited. Hence, in this study, we aim to investigate both systems simultaneously for naturally occurring E1 and E2, the anthropogenically derived EE2 and plant- and fungi-derived estrogens, namely DAI, GEN and ZEN. For this, we obtained water samples from the PRE and the northern shelf of the SCS and conducted a chemical analysis of the estrogenic compounds. In addition, the samples were tested for their cumulative estrogenic activity. Based on these data, a complementary risk assessment for both systems was performed to evaluate the potential risk arising from current estrogen concentrations at the investigated sites.

2. Material and methods

2.1. Study site and sampling procedure

Samples were taken in the Pearl River Estuary and the coastal South China Sea during a research cruise on the Chinese R/V Hai Yang Di Zhi Shi Hao in September 2018 (Figure 1) within the framework of the Sino-German project MEGAPOL financed by the State Oceanic Administration (SOA, P.R. China) and the Federal Ministry of Education and Research (BMBF, Germany, grand numbers 03F0786A and 03G0269), respectively. Additional samples were collected during a second cruise a year later in the same season (August 2019) on the German r/v SONNE to assess the interannual variability on the seasonal scale. This study presented here focuses only on the shallow shelf stations. Furthermore, eight samples were taken at station SCS-29 within 26 h to investigate the influence of tides on the distribution pattern. The Pearl River Estuary is one of the largest and most complex river systems in the world. In recent years, the area near the mouth of the Pearl River has developed rapidly and harbors now high numbers of industries, factories and animal farming together with a high population density (e.g., Guangzhou >1,700/km^2; UN, 2018). About

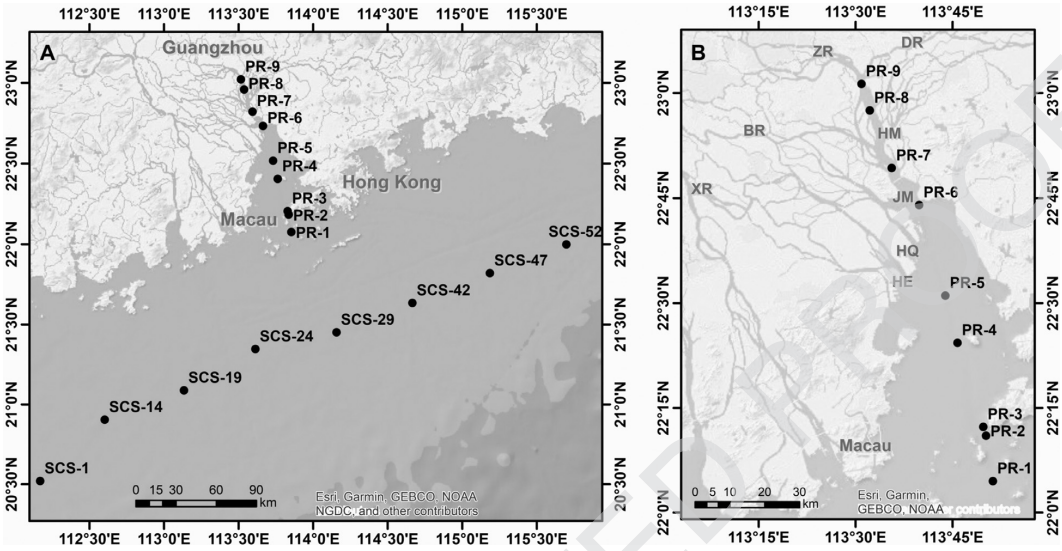

Figure 1 **A** Sampling stations along the Pearl River Estuary and the northern shelf of the South China Sea collected in September 2018 and August 2019. In 2018, surface water samples were taken during a research cruise with the R/V Hai Yang Di Zhi Shi Hao and in 2019 during a research cruise with the R/V SONNE (SO269). **B** Dongjiang River (DR), Zhujiang River (ZR), Beijiang River (BR), Xijiang River (XR) and Honqimen (HQ), Jiaomen (JM), Humen (HM) and Hengmen (HE) waterways, created with ArcMap® 10.7.1 (Copyright© 1995–2014 Esri, CA, USA).

3.4×10^{11} m^3 of river runoff are discharged annually into the adjacent South China Sea (Ni et al., 2008; Gong et al., 2009). In addition, this region is under the influence of the seasonally varying monsoon, leading to elevated discharge rates during the wet season (April-September; He et al., 2016).

Surface water of the Pearl River was collected through a towed CTD and pumping system called "towed fish" (Zhang et al., 2019) and was filled into 1 L amber glass bottles (Table 1). Along the PRE, the samples were taken at intervals of defined salinity levels (25, 20, 15, 10, 5, 0.5, 0) to examine estrogen concentration in freshwater and saltwater. At the coastal sampling sites, surface water was collected with a CTD SBE911 rosette system (Table 1). All samples were acidified with 1 mL of 5 M HCl to prevent potential biodegradation (Havens et al., 2010). Additionally, 1 mL of internal standard (E1-*d*, E2-*d*, EE2-*d*) was added to samples examined by LC-MS/MS to counteract the possible loss during the subsequent sample preparation (Deich et al., 2021).

Salinity and temperature were measured on board with a Seabird CTD SBE911 system. Samples for dissolved organic carbon (DOC), particulate organic carbon (POC), suspended particulate matter (SPM) and chlorophyll *a* (Chl *a*) were obtained simultaneously to water samples taken for estrogen analysis. DOC, POC, SPM and Chl *a* samples were frozen at −20°C and transported frozen to the Leibniz Institute for Baltic Sea Research Warnemünde (IOW) in Germany and were analyzed there using standardized procedures (see Supplementary following Grasshoff & Almgren, 1983).

2.2. Chemicals

Standards and internal standards for estrone (E1), 17β-estradiol (E2), 17α-ethinylestradiol (EE2), estrone-2,4,16,16-d_4 (E1-*d*), 17β-estradiol-2,4,16,16,17-d_5 (E2-*d*), 17α-ethinylestradiol-2,4,16,16-d_4 (EE2-*d*) were purchased from Neochema GmbH (Bodenheim, Germany). Genistein (GEN), daidzein (DAI) and zearalenone (ZEN) were purchased from Sigma Aldrich (Taufkirchen, Germany). Internal standard working solution (IS) was prepared in analytical grade water with a concentration of 20 ng L^{-1} E1-*d*, E2-*d* and EE2-*d* each (in 2019 1 ng L^{-1} of E1-*d* and 2 ng L^{-1} of E2-*d* and EE2-*d*). Recombinant yeast *Arxula adeninivorans* and culture medium were purchased from new diagnostics GmbH (Berlin/Dresden, Germany). Analytical grade water was obtained from Merck KGaA (Darmstadt, Germany), and Milli-Q water was prepared with Milli-Q® reference water purification system from Merck Millipore (Darmstadt, Germany). Acetonitrile, ethyl acetate and methanol LC-MS/MS grade were purchased from Promochem (Wesel, Germany). NH_4F was purchased from Sigma Aldrich (Taufkirchen, Germany). HCl was obtained from VWR International GmbH (Hannover, Germany) (Deich et al., 2021).

2.3. Sample extraction

Sample extraction was conducted as described in Deich et al. (2020, 2021). Basically, 1 L of sampled water was loaded on pre-conditioned 1000 mg Chromabond® C18 ec cartridges (Macherey-Nagel, Düren, Germany) after passing through a GF/F filter (0.7 μm, GE Healthcare Europe GmbH, Freiburg, Germany). After sample loading with a flow rate of approximately 10 mL min^{-1}, cartridges were washed with methanol and subsequently dried under nitrogen. Loaded solid-phase extraction (SPE) columns were frozen on board at −20°C; elution was conducted in the laboratory at IOW. For elution of analytes and direct pu-

Table 1 Pearl River and South China Sea sampling stations occupied during the cruise in September 2018.

Station No.	Lat. [N]	Long. [E]	Date	$Time_{UTC}$	S
PR-1	22.08	113.85	26-Sep-2018	20:15[a]	25.1
PR-2	22.18	113.84	26-Sep-2018	21:32[b]	19.9
PR-3	22.21	113.83	26-Sep-2018	21:40[b]	14.8
PR-4	22.41	113.76	26-Sep-2018	23:06[b]	9.6
PR-5	22.52	113.73	26-Sep-2018	23:55[b]	4.5
PR-6	22.73	113.66	27-Sep-2018	01:06[b]	0.5
PR-7	22.82	113.59	27-Sep-2018	01:46[b]	0.1
PR-8	22.96	113.54	27-Sep-2018	02:55[c]	0.1
PR-9	23.02	113.52	27-Sep-2018	03:27[d]	0.1
SCS-1	20.52	112.17	2-Sep-2018	01:39	32.5
SCS-14	20.90	112.60	13-Sep-2018	16:44	33.0
SCS-19	21.09	113.13	10-Sep-2018	15:21	32.6
SCS-24	21.35	113.61	13-Sep-2018	11:09	33.2
SCS-29	21.45	114.16	21-Sep-2018	20:21	33.8
SCS-42	21.63	114.67	8-Sep-2018	17:49	32.6
SCS-47	21.82	115.19	8-Sep-2018	11:47	32.6
SCS-52	22.00	115.70	7-Sep-2018	17:11	32.5

[a] low water
[b] flood tide
[c] high water
[d] ebb tide

rification from the matrix, 10 mL of EtOAc:MeOH (30:70, v:v) was passed through the SPE cartridge to which a preconditioned Sep-Pak Aminopropyl (NH2) Plus cartridge (Waters, Eschborn, Germany) was previously attached (Kumar et al., 2009). Eluates for the yeast estrogen screen were not treated with NH2 cartridges. The eluate was evaporated to dryness in a 40°C water bath under a gentle stream of compressed air. For LC-MS/MS analysis, the residue was redissolved in 1 mL of analytical grade water:ACN (70:30, v:v) and subsequently filtered through a syringe filter (RC 0.45 μm, Phenomenex). Samples processed with the yeast estrogen screen were redissolved in 6 mL of analytical grade water only. For more details, see Deich et al. (2020, 2021).

2.4. Instrumental analysis

Analysis was conducted on an LC-MS/MS system consisting of the LC Nexera-i and a triple mass spectrometer MS8060 (Shimadzu, Berlin, Germany) as described in Deich et al. (2021). A volume of 50 μL was separated on a Kinetex-Biphenyl column (150 mm x 4.6 mm x 2.6 μm, Phenomenex, Aschaffenburg, Germany) with analytical grade water as mobile phase A, ACN as mobile phase B and 15 mM NH_4F as mobile phase C, with an oven temperature of 30°C and a flow rate of 0.2 mL min^{-1}. The elution gradient was as follows: 69.3% A, 30% B and 0.7% C for 1 minute, reduced to 9.9% A, 90% B and 0.1% C within 7 minutes, to 100% B in 15 minutes, was held for 2 minutes and back to 69.3% A, 30% B and 0.7% C within 2 minutes with subsequent equilibration for 2 minutes.

The compounds were ionized by heated electrospray ionization (HESI) in the negative polarity and were detected in the multiple reaction monitoring (MRM) mode. For each estrogenic compound, four to seven specific mass transitions were acquired, of which the mass transition with the highest intensity was used for quantification, while the others were utilized for qualitative analysis (Deich et al., 2021). Instrument operation and data evaluation were done with Lab-Solutions 5.9® and LabSolutions Insight Library Screening 3.10® (Shimadzu, Berlin, Germany).

2.5. Yeast estrogen screen

The *Arxula* yeast estrogen screen (A-YES) was used as previously described in Hettwer et al. (2018) and Deich et al. (2020). In general, the 96-deep-well plates contained calibration levels of the 17β-estradiol, sample dilutions (1, 1.6, 2.5, 3.2, 5 and 8-fold) and negative controls, blank samples as well as recovery samples. The wells were inoculated with a mixture of the reactivated yeast and medium, whilst blank samples were treated only with pure medium. After incubation (20—21 h, 31°C), enzymatic substrate cleavage was conducted in microtiter plates containing the clear supernatant and substrate solution (1 mg mL^{-1} *p*-NPP in 0.5 M citric buffer). The plates were incubated for 1 h at 37°C. Subsequently, reporter gene activity was determined before and after adding 100 μL of 3 M NaOH at 405 nm on a microplate reader (Infinite M200, Tecan Trading AG, Männedorf, Switzerland). Optical density of the yeast cell suspension (1:20 with Milli-Q water) was measured at 630 nm to examine the samples for different yeast growth between each test.

2.6. Quantification and quality assurance

2.6.1. Instrumental Analysis

E1-*d*, E2-*d* and EE2-*d* were utilized as internal standards for E1, E2 and EE2, respectively. For GEN, DAI and ZEN quantification, E1-*d* was used as internal standard compound (IS; see Deich et al., 2021). Prior to sample preparation, internal standards were added to the water sample to a final

concentration of 20 ng L^{-1} of each IS (in 2019 1 ng L^{-1} of E1-*d*, 2 ng $L^{-1}$of E2-*d* and EE2-*d*). For the internal standard calibration, Milli-Q was spiked with estrogen standards to final concentrations ranging from 0 ng L^{-1} to 0.8 ng L^{-1}, and the calibration samples were subsequently processed with SPE before detection with LC-MS/MS. Each sample was measured twice and the mean was used for quantification. Deviation between both values ranged from <1% to 30%, with an average of 9%.

It was found previously that analysis of the target analytes is largely sensitive to the matrix and that the analytical performance reduces particularly for riverine sample water comparing to marine sample water (Petrovic, 2014). To account for this effect, assessment of LOD and LOQ were conducted using an environmental water sample (S: 15.9, T: 4.8°C) instead of using Milli-Q water for preparing a calibration. This better reflects the detection capabilities in environmental water samples. These are 0.1, 0.2, 0.3, 0.4, 0.3 and 0.1 ng L^{-1} for LOD and 0.3, 0.3, 0.6, 0.7, 0.5 and 0.2 ng L^{-1} for LOQ of E1, E2, EE2, GEN, DAI and ZEN, respectively.

The procedure and calibration were validated using Milli-Q water and environmental water samples. Aliquots were spiked with 0.4 ng L^{-1} and 0.8 ng L^{-1} of the target analytes (Deich et al., 2021). The evaluation of the validation data shows high accuracy for the analysis of estrogens and phytoestrogens in environmental samples, and deviations (RE%) of up to 22% must be considered. Very high precision could be obtained for the analysis of estrogens (RSD<7%). The procedure is less precise for the analysis of phytoestrogens with RSD% of up to 39%, which might be a result of utilizing E1-*d* as internal standard as it might not be ideal for the quantification of phytoestrogens. Regularly, blank samples were analyzed during sample preparation, and none of the target analytes were detected.

2.6.2. Yeast estrogen screen

A detailed description of estradiol equivalent quotient ($EEQ_{A\text{-}YES}$) quantification and quality assurance of the bioassay can be found in Hettwer et al. (2018) and Deich et al. (2020). During the bioassay, non-proportional EEQ may arise from matrix interference in the form of inhibitory or masking effects and competitive inhibition of the estrogen receptor due to co-eluting compounds (Frische et al., 2009; Neale et al., 2015). The effects are mitigated by using different dilution levels. Quantification was conducted using the highest quantifiable dilution to minimize dilution-dependent effects. Recovery analysis was conducted three times with Milli-Q water spiked with E2 to a final concentration of 0.2 ng L^{-1}. One blank sample was set up in Milli-Q water without E2 to examine possible contamination effects along the enrichment and clean-up procedure. Recovery analysis showed a mean recovery rate of 79% (n=3), whereas the blank sample showed no detectable reporter gene activity in the biological assay.

3. Results and discussion

3.1. Distribution of estrogenic compounds

Surface water samples from the Pearl River Estuary and the northern shelf of the South China Sea were analyzed for the target estrogenic compounds (dissolved E1, E2, EE2, DAI, GEN, ZEN). Along the estuary, E1 was observed at all nine stations (Figure 2A-C) with concentrations of 0.3 ng L^{-1} to 3.6 ng L^{-1}. In general, E1 concentration decreased towards the South China Sea. The decrease between station PR-9 and PR-7 may be attributed to mixing with river waters from different distributaries of lower E1 content. From station PR-6 to PR-1, the salinity increases towards the mouth of the estuary and mixing with seawater reduces the E1 concentration here. However, at PR-4 and PR-6, concentrations slightly increased. At these points, the water from the rivers Dongjiang, Beijiang, Xijiang is channeled via the Honqimen, Jiaomen, Humen and Hengmen waterways into the estuary (Figure 1B), which could explain the different non-linear concentration patterns. Interestingly, the synthetic estrogen EE2 was only detected at the station located closest to the sea, PR-1 (0.7 ng L^{-1}) and the natural estrogen E2 was not detected at any station.

During this study, strong matrix interference in the form of signal suppression drastically reduced the the internal standard signal, especially in the upstream PRE water samples. Therefore, it is assumed that if E2 and EE2 are present in the surface water samples, the detection of these compounds is strongly interfered due to high matrix loads of the sample in combination with environmental concentrations close to detection limits. Matrix components could interfere during SPE enrichment and during ionization for mass spectrometric analysis, particularly if electrospray-ionization is used (Busetti et al., 2012; Cotrim et al., 2016; Magi and Di Carro, 2018). Thus, consequences may include inefficient extraction during sample preparation and ion amplification or suppression in MS analysis of the target analytes. Those effects might derive from the dissolved organic matter as already stated by Trufelli et al. (2011), Petrovic (2014) and Białk-Bielińska et al. (2016).

The measured concentrations are in good agreement with the reported data. For example, Gong et al. (2009) investigated water from PRE for estrogenic compounds and found concentrations up to 8.2 ng L^{-1} for E1. The study of Xu et al. (2014) in the PRE revealed estrogen concentrations in the riverine outlets during the wet season of up to 1.2 ng L^{-1} for E1 and EE2. In contrast, sampling sites at the Mondego River estuary's mouth (Portugal) showed highest E1 concentration of 15 ng L^{-1}, 13 ng L^{-1} for E2 and 9 ng L^{-1} for EE2 (Rocha et al., 2014).

In samples from the northern shelf of the SCS, only the estrogens E1 and EE2 were detected (Figure 3). E1 was present at all stations (0.2—1.0 ng L^{-1}), while EE2 was detected only at five stations (0.4—0.6 ng L^{-1}, Figure 2). In contrast to the maximum values found by Zhang et al. (2014) in the coastal South China Sea for E1 and EE2 (11.16 ng L^{-1} and 3.99 ng L^{-1}, respectively), concentrations of E1 and EE2 observed in this study are comparably low. Interestingly, highest concentrations of E1 were found at the easternmost and westernmost stations, and the lowest concentration was found at SCS-29, which can be regarded as an extension of the estuary. In addition, this station shows comparably high salinity and low water temperature (Figure 3A,D), most likely because of horizontal advection of different water bodies. In general, seawater samples showed estrogen concentration in the same order of magnitude as at PR-1.

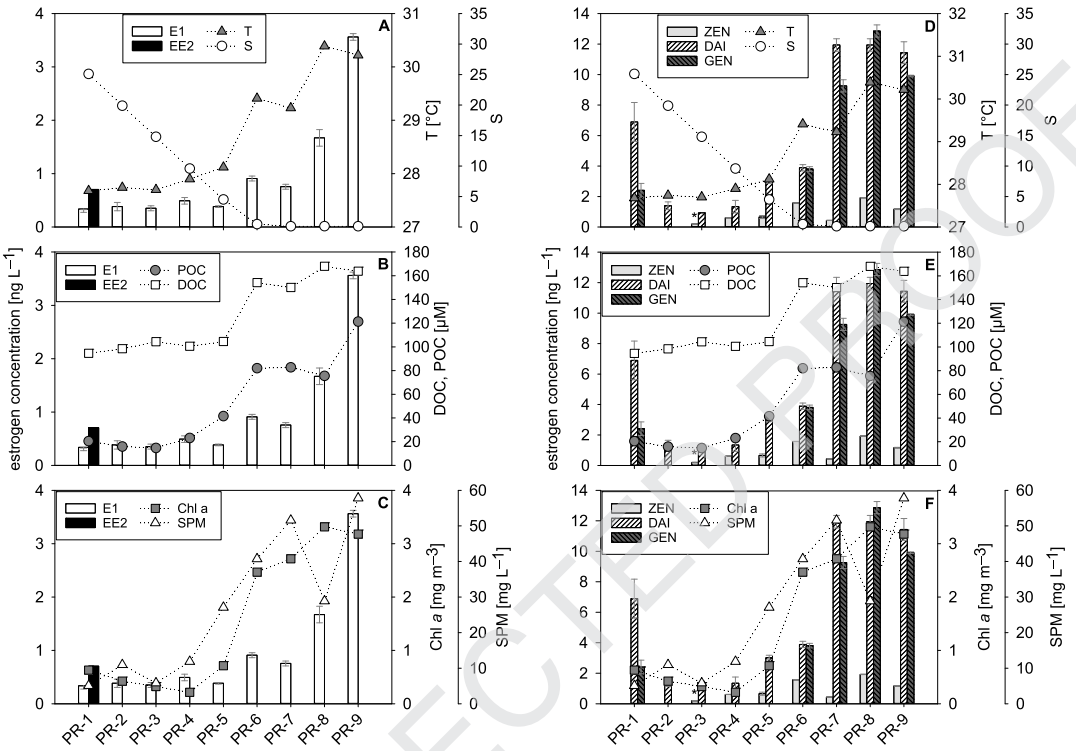

Figure 2 Estrogenic compound concentration observed at the Pearl River sampling stations. **A**, **D** salinity (S) and temperature (T). **B**, **E** dissolved organic carbon (DOC) and particulate organic carbon (POC). **C**, **F** chlorophyll *a* (Chl *a*) and suspended particulate matter (SPM). Error bars represent the results of the duplicate measurement, *LOD < concentration < LOQ.

The mycoestrogen ZEN was detected at six out of nine sampling stations (n.d.—1.9 ng L^{-1}), and highest concentrations were observed upstream at PR-8 and PR-6 (Figure 2D-F). This suggests that riverine inflow from tributaries into the estuary are loaded with ZEN to varying degrees, thereby locally increasing the concentration at these stations.

The phytoestrogen DAI was detected in all nine water samples within the estuary (1.0—11.9 ng L^{-1}, Figure 2D-F), whereas GEN was only detected at four stations close to the city of Guangzhou (PR-6 to PR-9) and at PR-1 (n.d.—12.9 ng L^{-1}). Both phytoestrogens decrease with increasing salinity. However, they show comparably high concentrations at station PR-1, which was the only station where EE2 was observed as well. In comparison, the study of Rocha et al. (2016) showed concentrations of DAI and GEN in the Mira River (Portugal) of up to 20 ng L^{-1} and 69.2 ng L^{-1}, respectively, whilst the Mondego River estuary (Portugal) showed DAI and GEN concentrations of 11.9 μg L^{-1} and 5.1 μg L^{-1} (Rocha et al., 2014).

Based on the fact that the phytohormones were only found in PRE, we assume that they either result from land-based point sources such as food industries or were only produced by freshwater algae or cyanobacteria species or both (Prochazkova et al., 2018). Also, surface waters of the SCS are oligotrophic, and most of the primary production occurs at the deep chlorophyll maxima. Therefore, phytoestrogens during the study are most likely not detected in the nutrient-depleted surface waters of low productivity.

The exact transport pathway of the estrogenic substances from the PRE to the marine system is unclear. However, based on the herein determined estrogen compound concentrations at PR-6 and the annual discharge of 3.4×10^{14} L from the PRE into the SCS adds the annual mass transport of hormones up to 0.3 t, 1.3 t, 1.3 t and 0.5 t for E1, GEN, DAI and ZEN, respectively. Nevertheless, these mass transports must be viewed critically since the individual measurements are merely snapshots. However, the measurements from the campaign in 2019 show that the variability on the seasonal scale is relatively low (Figure S1), as the concentration measured during both campaigns are in the same order of magnitude. Measurements over a tidal cycle (26 h) at a position close to shore did not indicate any significant changes in concentrations (Figure S2). This suggests that there is a continuous supply of estrogen-containing waters into the SCS.

Furthermore, only the dissolved phase of the water sample was examined in this study. Estrogenic compounds associated to the suspended particulate matter were not regarded but might contribute to estrogen input into the marine environment. This highlights the importance of regularly monitoring such hormonal substances in order to draw more precise conclusions.

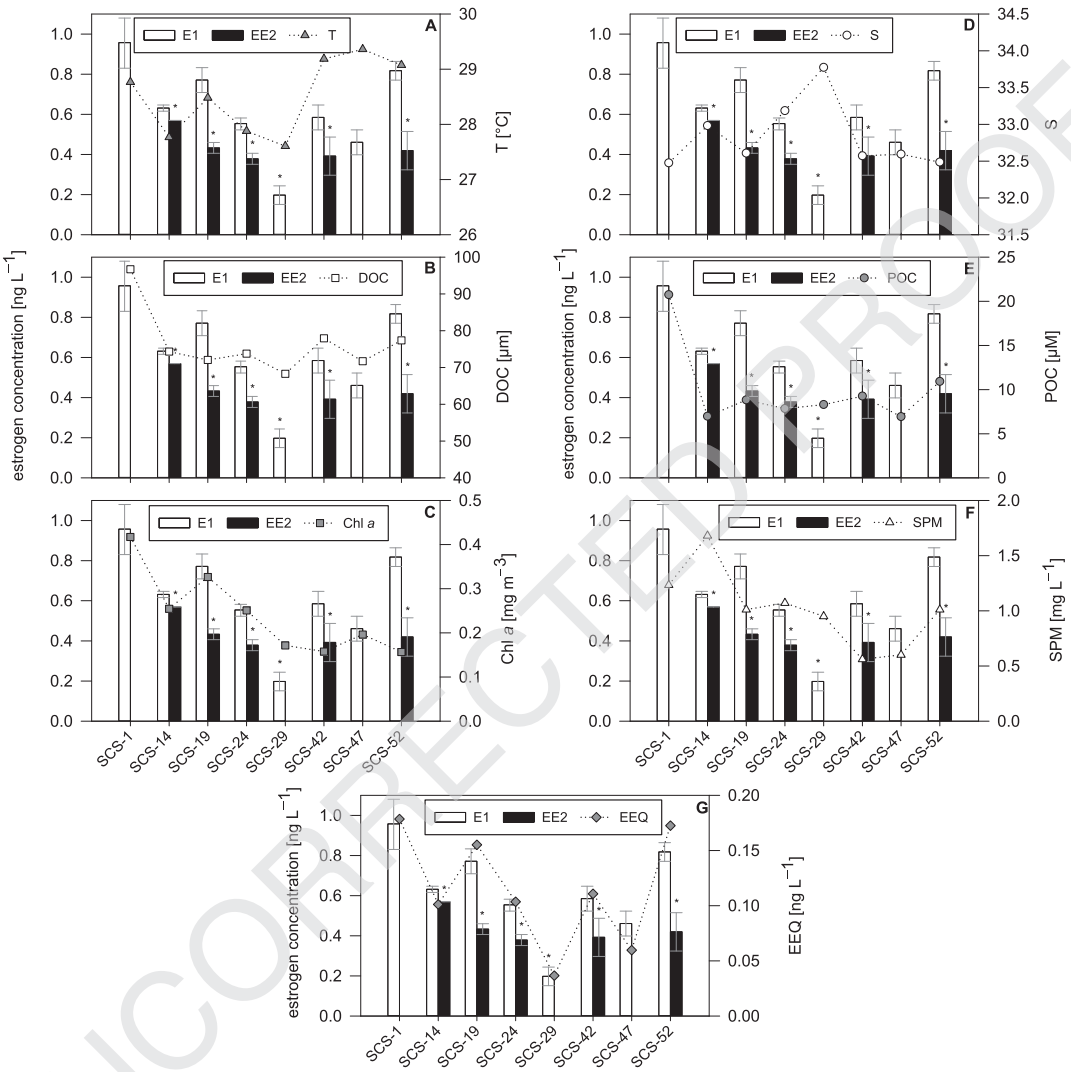

Figure 3 Estrogenic compound concentration observed at the stations within the northern shelf of the SCS during September 2018. **A** temperature (T), **B** dissolved organic carbon (DOC), **C** chlorophyll a (Chl a), **D** salinity (S), **E** particulate organic carbon (POC), **F** suspended particulate matter (SPM) and **G** estradiol equivalent concentrations ($EEQ_{A\text{-}YES}$). Error bars represent the results of the duplicate measurement, *LOD < concentration < LOQ.

3.2. Correlation with environmental parameters

Dissolved organic carbon (DOC), particulate organic carbon (POC), suspended particle matter (SPM) and chlorophyll *a* (Chl *a*) samples were collected along the Pearl River and from the northern shelf of the South China Sea (Figure 2, 3) In PRE, higher concentrations were observed upstream, while lowest concentrations were observed downstream where mixing with seawater occurred. However, linear regression revealed non-conservative mixing behavior of estrogenic substances (Figure S4). Consequently, the concentration gradient in the PRE is not only influenced by mixing with seawater but also through permanent mixing of freshwater with varying degrees of contamination merging into the estuary through the numerous outlets and waterways. Also, adsorption to suspended particulate matter and the resulting elimination from the water column should be considered.

Chl *a* concentrations rose from station PR-3 towards PR-1 (Figure S7), correlating with GEN ($R^2 = 0.72$, $p = 0.07$) and DAI concentrations ($R^2 = 0.74$, $p = 0.003$), which could be attributed to a local source as well as to advection, patchiness of the sample or both. High contaminant concentrations are often associated with high DOC concentrations

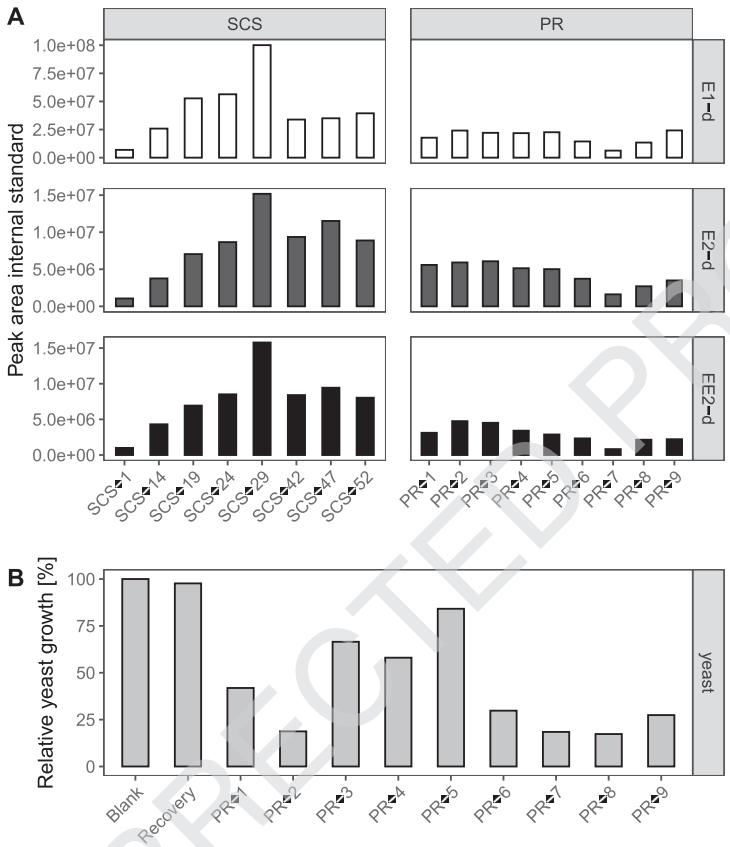

Figure 4 **A** Peak areas of internal standards E1-*d*, E2-*d* and EE2-*d* at PRE and SCS stations. **B** Yeast growth of A-YES samples relative to blank samples along the PRE (undiluted extracts).

(Gong et al., 2016). Our study shows significant correlations between DAI and E1 with DOC (Fig. S5, $R^2 = 0.61$, $p = 0.01$ and $R^2 = 0.42$, $p < 0.01$, respectively).

During our analysis of samples taken along the PRE, we observed declining peak areas of internal standards from the estuary's mouth towards the head of the estuary (Figure 4A, Figure 5, Figures S9-S10). At the same time, peak areas of internal standards correlate significantly with measured environmental parameters DOC, POC, SPM, Chl *a* and S. Reduced signals of the internal standards might arise through loss of analyte during SPE as well as adsorption to suspended particulate matter and thus elimination from the water column. Additionally, hampered ionization in the ESI-MS/MS by compounds with similar chemical behavior might lead to signal suppression (Petrovic, 2014). Here, especially the presence of DOC seems to influence the determination of internal standards in the MS, the SPE procedure or both. Lowest relative peak areas of all three internal standards are observed at PR-9 to PR-6, i.e., at stations with highest DOC concentrations. This supports the hypothesis that estrogenic substances are complexed by dissolved organic matter (Figure 5).

The relative decrease in the signals of the internal standards in the MS is similar for E1-*d*, E2-*d*, and EE2-*d*. Nevertheless, the analysis of E1-*d* is more sensitive than for E2-*d* and EE2-*d*, which allowed quantification of E1 even if peak areas were significantly reduced due to the influence of matrix components. This supports our previous assumption that matrix components in the sample water impede detection of particularly E2 and EE2.

3.3. Biological response to estrogenic compounds

To derive a cumulative estrogenic potential of a water sample, equivalent concentrations to the most estrogenic estradiol are often determined (e.g., Hettwer et al., 2018; Deich et al., 2020), which allows to assess potential adverse effects to marine organisms based on estrogenic activity. Herein, the Arxula yeast estrogen screen was utilized to obtain estradiol equivalent concentrations for the stations in the PRE and SCS (EEQA-YES, Table 2, Figure 3).

Estrogenic activity was observed at PR-1, PR-3, PR-7, PR-9 and all SCS stations. Quantitative estradiol equivalent concentrations in the PRE were only obtained for PR-

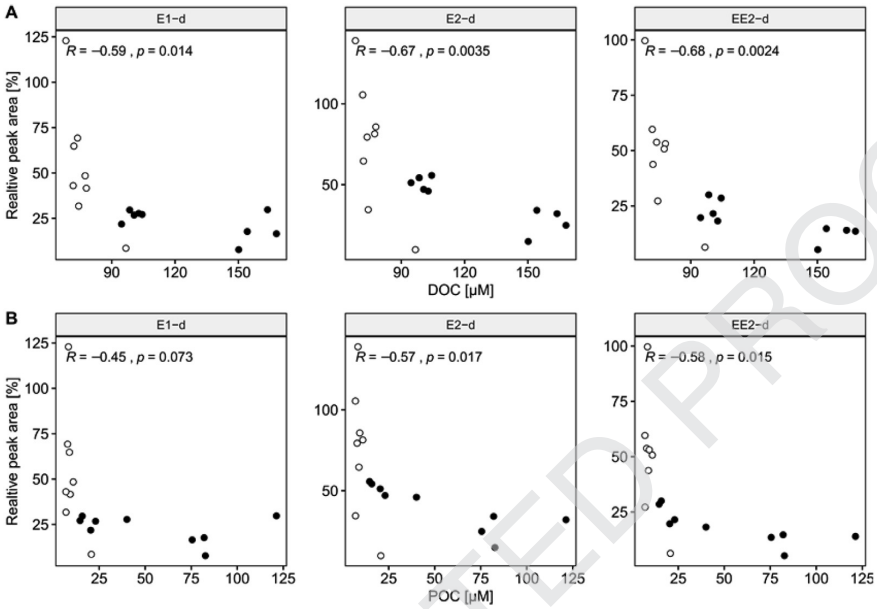

Figure 5 Correlation of relative peak areas of internal standards and **A** dissolved organic carbon (DOC) and **B** particulate organic carbon (POC). R and p-values are indicated, ● PR sampling stations, ○ SCS sampling stations. Relative peak areas were calculated by the ratio of peak areas of samples to peak areas of calibration samples.

Table 2 Contribution of estrogenic substances to the calculated estrogenic activity (EEQ_{calc}) as well as the determined estrogenic activity by yeast estrogen screen ($EEQ_{A\text{-}YES}$), n.d. = not detected, n.a. = not applicable.

Station	estrogen concentration [ng L^{-1}]					EEQ_{calc} [ng L^{-1}]	contribution to EEQ_{calc} [%]					$EEQ_{A\text{-}YES}$ [ng L^{-1}]
	E1	EE2	GEN	DAI	ZEN		E1	EE2	GEN	DAI	ZEN	
PR-1	0.3	0.7	2.4	6.8	n.d.	0.7	4	96	<1	<1	n.a.	0.15
PR-2	0.4	n.d.	n.d.	1.4	n.d.	<0.1	100	n.a.	n.a.	<1	n.a.	n.d.
PR-3	0.4	n.d.	n.d.	0.9	0.2	<0.1	99	n.a.	n.a.	<1	1	0.17
PR-4	0.5	n.d.	n.d.	1.3	0.6	<0.1	98	n.a.	n.a.	<1	2	n.d.
PR-5	0.4	n.d.	n.d.	3.0	0.7	<0.1	97	n.a.	n.a.	<1	3	n.d.
PR-6	0.9	n.d.	3.8	3.9	1.6	0.1	96	n.a.	1	<1	3	n.d.
PR-7	0.8	n.d.	9.3	11.9	0.4	0.1	96	n.a.	3	<1	1	qual.[a]
PR-8	1.7	n.d.	12.9	11.9	1.9	0.2	96	n.a.	2	<1	2	n.d.
PR-9	3.6	n.d.	9.9	11.4	1.2	0.3	99	n.a.	1	<1	<1	qual.[a]
SCS-1	1	n.d.	n.d.	n.d.	n.d.	0.1	100	n.a.	n.a.	n.a.	n.a.	0.18
SCS-14	0.6	0.6	n.d.	n.d.	n.d.	0.6	8	92	n.a.	n.a.	n.a.	0.10
SCS-19	0.8	0.4	n.d.	n.d.	n.d.	0.5	16	84	n.a.	n.a.	n.a.	0.16
SCS-24	0.6	0.4	n.d.	n.d.	n.d.	0.4	12	88	n.a.	n.a.	n.a.	0.10
SCS-29	0.2	n.d.	n.d.	n.d.	n.d.	<0.1	100	n.a.	n.a.	n.a.	n.a.	0.04
SCS-42	0.6	0.4	n.d.	n.d.	n.d.	0.4	12	88	n.a.	n.a.	n.a.	0.11
SCS-47	0.5	n.d.	n.d.	n.d.	n.d.	<0.1	100	n.a.	n.a.	n.a.	n.a.	0.06
SCS-52	0.8	0.4	n.d.	n.d.	n.d.	0.5	16	84	n.a.	n.a.	n.a.	0.17

[a] qualitatively detected, but not quantitatively

1 and PR-3 with $EEQ_{A\text{-}YES}$ of 0.15 ng L^{-1} and 0.17 ng L^{-1} (Table 2). In the SCS, $EEQ_{A\text{-}YES}$ ranged from 0.04 to 0.18 ng L^{-1} (Figure 3). Data analysis revealed non-proportional dilution effects especially for the PRE sampling sites, i.e., increasing $EEQ_{A\text{-}YES}$ concentrations with increased sample dilution within the biological test (Figure S11). This effect was previously discussed in connection with complex sample compositions and resulting matrix effects which become alleviated upon increasing sample dilution within the test (Hettwer et al., 2018; Deich et al., 2020). In view of the sample enrichment on SPE within this current study, the obtained non-proportional dilution effects might also re-

sult from matrix enrichment during the sample preparation. Strongly reduced growth of the yeast cells in the undiluted samples compared to the control samples indicates the impact of the matrix on the test (Figure 4B). Therefore, it cannot be excluded that the final $EEQ_{A\text{-}YES}$ are above the herein presented data. For those sites at which no estrogenic activity was observed, matrix interference may have inhibited detection.

The estradiol equivalent concentration of a water sample can also be obtained based on distinct relative potency of an estrogenic compound to estradiol and the observed environmental concentration (Eq. 1) with EEQ_{calc} = summarized estradiol equivalent concentration from chemical analysis, EEF_i = estradiol equivalent factor of the individual estrogenic compound and C_i = obtained concentration of the individual estrogenic compound.

$$EEQ_{calc} = \sum EEF(i)x\,C(i) \quad (1)$$

Within this study, estradiol equivalent factors (EEF(i)) for the analytes E1, E2, EE2, DAI, GEN and ZEN were determined with the A-YES bioassay (Table S2). However, based on an estradiol equivalent factor of 0.97 (Table S2), EE2 largely contributes to the cumulative estrogenic activity of a water sample among the herein investigated analytes. In view of the conclusion that EE2 detection at PR-2 to PR-9 is impeded due to matrix interference, determination of EEQ_{calc} and a comparison to $EEQ_{A\text{-}YES}$ is not reasonable for these sites.

For the site PR-1, EEQ_{calc} was determined with 0.68 ng L^{-1} (Table 2), which exceeds the measured $EEQ_{A\text{-}YES}$ of 0.15 ng L^{-1}. In those SCS samples where EE2 was detected, the determined EEQ_{calc} is also higher than the EEQ obtained with the estrogen screen. This indicates that either derived EEQ_{calc} is too high or that $EEQ_{A\text{-}YES}$ is underestimated. Chen et al. (2016) also observed higher EEQ_{calc} than estrogenic activity and concluded on possible antagonizing effects of natural and synthetic compounds which interact with the receptor's binding site and inhibit a response within the bioassay. Furthermore, Neale et al. (2015) reported that co-eluted DOC could reduce the bioavailability of E2, which led to suppression of E2 agonist levels within the bioassay. *Vice versa*, EEQ obtained from an *in vitro* bioassay might be higher than the one based on chemical analysis. In such cases, the difference may arise due to the fact that the *in vitro* bioassay regards the cumulative estrogenic activity including the entire substance mixture of a sample, whereas chemical analysis focuses only on a number of target analytes. In this regard, substances like BPA or nonylphenols and octylphenols are known for eliciting estrogenic effects on organisms (Campbell et al., 2006; Hotchkiss et al., 2008). For example, Xu et al. (2014) detected BPA concentrations from 10 to 178 ng L^{-1} and 11 to 163 ng L^{-1} of nonylphenol in the PRE, which they suspected were associated with wastewater from nearby factories.

Also, interference of co-eluting compounds with the yeast cells might lead to a discrepancy between EEQ_{calc} and $EEQ_{A\text{-}YES}$ (Wang et al., 2011). This corresponds to the finding within this study, i.e., inhibition of yeast cell growth in all samples from PRE (Figure 4B) and the derived non-proportional $EEQ_{A\text{-}YES}$. Therefore, as stated above, the final $EEQ_{A\text{-}YES}$ at PR-1 is most likely higher than 0.15 ng L^{-1}. However, these test results rely only on one estrogenic activity test system. An extensive analysis with alternative bioassays could provide a more comprehensive picture and might be one way of resolving discrepancies between calculated and measured activity more thoroughly.

Table 3 Risk quotients (RQ) at Pearl River and South China Sea stations based on predicted no-effect concentrations (PNEC) for estrone (E1) and 17α-ethinylestradiol (EE2). RQ = environmental concentrations/PNEC, RQ<0.1 low risk, 0.1≤RQ<1 medium risk and RQ≥1 high risk. n.a. = not applicable.

Station	RQ_{E1}[a]	RQ_{EE2}[b]	$RQ_{EEQ\_A\text{-}YES}$[c]
PR-1	0.1	7.0	0.15
PR-2	0.1	n.a.	n.a.
PR-3	0.1	n.a.	0.17
PR-4	0.1	n.a.	n.a.
PR-5	0.1	n.a.	n.a.
PR-6	0.2	n.a.	n.a.
PR-7	0.1	n.a.	n.a.
PR-8	0.3	n.a.	n.a.
PR-9	0.6	n.a.	n.a.
SCS-1	0.2	n.a.	0.18
SCS-14	0.1	6.0	0.10
SCS-19	0.1	4.0	0.16
SCS-24	0.1	4.0	0.10
SCS-29	<0.1	n.a.	0.04
SCS-42	0.1	4.0	0.11
SCS-47	0.1	n.a.	0.06
SCS-52	0.1	4.0	0.17

[a] $PNEC_{E1}$ = 6 ng L^{-1} (Caldwell et al., 2012)
[b] $PNEC_{EE2}$ = 0.1 ng L^{-1} (Caldwell et al., 2012)
[c] $PNEC_{EEQ}$ = 1 ng L^{-1} (Jarošová et al., 2014)

The obtained data reflect previous findings that phytoestrogens only marginally contribute to the estrogenic potential especially in oligotrophic surface waters, and that the estrogenic activity is mainly determined by natural and synthetic steroids (Jarošová et al., 2014). Highest concentrations of analyzed phytoestrogens were found at the sites PR-7 to PR-9, of more than 10 ng L^{-1} for GEN and DAI and about 2 ng L^{-1} for ZEN. Based on their estradiol equivalent factors (Table S2), their contributions to the EEQ_{calc} were not more than 3% (Table 2). These might be even lower in view of the impeded detection of EE2 in the PRE. This observation agrees with Xu et al. (2014), who reported that the predominant contributors to $EEQ_{A\text{-}YES}$ in the PRE are E1 and EE2.

3.4. Risk assessment

To investigate if detected concentrations of endocrine substances may exert risk to aquatic organisms, a risk assessment was conducted based on risk quotients (RQs) and organism specific predicted no-effect concentrations (Table 3). RQs were estimated by relating the environmental concentration to the distinct predicted no-effect concentration (PNEC) of a substance. RQs above 1 represent a high risk for the organisms, between 0.1 and 1 a medium risk and less than 0.1 low risk. Here, RQs were determined

based on determined E1 and EE2 concentrations (Table 3). Among the herein analyzed compounds, they were reported to contribute the most to the estrogenic potential of the water samples. Also, RQs were calculated based on measured cumulative estrogenic activity $EEQ_{A\text{-}YES}$.

In the PRE, E1 concentrations increased towards the city of Guangzhou and reached a level at which medium risk to aquatic organisms might be considered for station PR-6 to PR-9 (Table 3). The determined E1 concentrations in the SCS likely pose a medium risk to organisms.

An EEQ concentration of not more than 1 ng L^{-1} is often considered as PNEC for biological tests (Jarošová et al., 2014). Risk quotients derived from $EEQ_{A\text{-}YES}$ indicate medium risk already at PR-1 and PR-3 and six of the eight analyzed samples from the SCS. In contrast, the environmental concentrations obtained for EE2 may pose a high risk to aquatic organisms throughout.

It must be noted that the herein applied PNEC concentration for E1 and EE2 were derived from a species sensitivity distribution method based on mostly freshwater fish species (Caldwell et al., 2012). Taking into account that marine species are often more sensitive to environmental pollution than freshwater species, even higher risks must be considered for the SCS sites than concluded herein.

3.5. Estrogenic substances in coastal areas

A comprehensive study conducted by Lu et al. (2020) showed the presence of estrogenic substances along the Chinese coast from the Bohai Sea to the South China Sea. In that study, E1 was the main steroidal estrogen with concentrations reaching 204.4 ng L^{-1} in winter (average: 87.2 ng L^{-1}) and 9.8 ng L^{-1} (average: 2.7 ng L^{-1}) in summer. It was concluded that the significant decrease of estrogen concentrations in summer arise mainly from the increased precipitation brought by the monsoon. In this regard, the E1 concentrations in the seawater of the South China Sea reported in this study were slightly lower compared to the findings of Lu et al. (2020).

In the Baltic Sea, Beck et al. (2005) showed that the hormones E1 and EE2 could be found with up to 0.53 ng L^{-1} and 17.2 ng L^{-1}, respectively. The phytoestrogens DAI and GEN could not be detected at the stations close to the coast, which corresponds to our findings. Estrogenic activity in the Baltic Sea was detected up to 0.82 ng L^{-1} (Beck et al., 2006; Deich et al., 2020). Pojana et al. (2007) investigated surface waters from the Venice Lagoon, Italy, and detected concentrations up to 10 ng L^{-1} for E1, 175 ng L^{-1} for E2 and 34 ng L^{-1} for EE2. It was concluded that the observed concentrations arise mainly from industrial and municipal wastewater, which is redistributed within the lagoon due to the tidal forces. Major contributors to the calculated estrogenic activity (1.1—191 ng L^{-1}) were E2 and EE2. In contrast, another study in the Mediterranean Sea conducted in the Thermaikos Gulf, Greece, reported estrogen concentrations below detection limits (Arditsoglou and Voutsa, 2012). Griffith et al. (2016) investigated seawater in the Massachusetts Bay, United States of America, for estrogenic substances and observed up to 0.5 ng L^{-1} for E1 as well as 0.09 ng L^{-1} for E2 and EE2.

4. Conclusion

Within this study, we measured natural and synthetic estrogenic substances in water samples along the Pearl River and the northern shelf of the South China Sea that potentially pressure marine organisms and act at high risk levels when considering EE2 concentrations. Whilst steroidal estrogens were found in the PRE and SCS, plant and fungi derived estrogens were observed only at PRE sampling sites.

The estimation of estrogenic activity is still problematic even with information about present compounds, as synergistic and antagonistic effects cannot be detected by instrumental analysis. Therefore, biological assays were used to complement the instrumental analysis in their statements and to present a more comprehensive picture of the potential harm exerted onto marine organisms.

In this study, matrix interference largely impeded chemical and estrogenic activity analysis in the samples from the Pearl River. However, the complementary risk assessment based on chemical data and estradiol equivalent concentrations depicts that medium risk must be considered for aquatic organisms along the Pearl River; even high risk for the SCS shelf region based on EE2 concentrations.

Overall, this underlines the importance of effective analysis tools to survey estrogenic substances. To estimate potential estrogenic sources, further research is needed to gather information about the seasonal and inter-annual distribution patterns of estrogenic substances as environmental conditions can change within weeks, and the measurements represent only results from a single quasi synoptic study. With regularly monitoring, it will be possible to obtain a holistic view of the anthropogenic fingerprint of estrogenic substances in the northern South China Sea.

Uncited References:

Schwartz et al., 2010

Declaration of Competing Interest

The authors have no conflicts of interest to declare.

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Author credit statement

Carina Deich: sampling, method development, analyses, draft writing and final paper; Marion Kanwischer: analyti-

cal support, method development and manuscript revisions; Ruifeng Zhang: technical support during the campaign, PRE discussion and draft revision; Joanna J. Waniek: project leader, general project and study idea, securing funding, supervision of paper progress and revision of all manuscript versions.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.oceano.2021.08.001.

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Publication III

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Occurrence and distribution of estrogenic substances in the northern South China Sea

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Abstract Estrogenic substances are today among the contaminants of emerging concern. Besides naturally occurring estrogens, other natural and synthetic substances can mimic a hormonal action due to their structural resemblance to hormones, possibly affecting the endocrine system of living organisms. Estrogens have been detected in inland water bodies such as influents and effluents of waste water treatment plants as well as in rivers, but data on their distribution and variability in the marine ecosystem are still limited. Surface water samples obtained during two research cruises on the northern shelf of the South China Sea (SCS) near the Pearl River Estuary, in September 2018 and in August 2019, were investigated for estrogenic substances, namely estrone (E1), 17β-estradiol (E2), 17α-ethinylestradiol (EE2), genistein (GEN), daidzein (DAI) and zearalenone (ZEN). Among the target analytes, the natural hormones E1 and E2, as well as the synthetic EE2, were the most abundant with maximum concentrations of 1.1 ng L^{-1}, 0.7 ng L^{-1} and 0.6 ng L^{-1}, respectively. Of substances produced by plants and fungi, GEN, DAI and ZEN, only GEN was detected (1.2 ng L^{-1}). High concentrations occurred predominantly close to the coast, which was also reflected in the calculated estradiol equivalent quotients (up to 1.4 ng L^{-1}). In general, the distribution of estrogenic substances observed in both years shows a regional and inter-annual variability consistent with the modeled surface current data for the SCS. Regarding single estrogenic compounds and estradiol equivalents, marine organisms in the northern SCS might be exposed to high potential risk.

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Occurrence and distribution of estrogenic substances in the northern South China Sea

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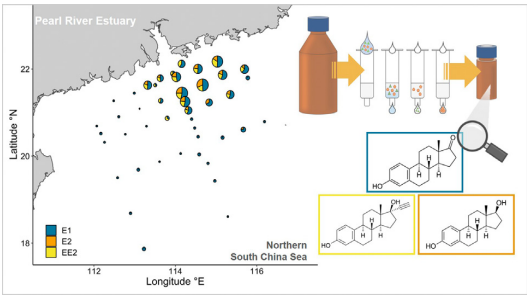
HIGHLIGHTS

- Analysis of estrogenic substances in surface waters from the northern South China Sea
- E1, E2 and EE2 were detected with concentrations up to 1.1 ng L^{-1}, 0.7 ng L^{-1} and 0.6 ng L^{-1}.
- Near-shore stations show the highest estrogen concentrations.
- EE2 concentrations might pose a high potential risk to marine organisms.

GRAPHICAL ABSTRACT



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ABSTRACT

Estrogenic substances are today among the contaminants of emerging concern. Besides naturally occurring estrogens, other natural and synthetic substances can mimic a hormonal action due to their structural resemblance to hormones, possibly affecting the endocrine system of living organisms. Estrogens have been detected in inland water bodies such as influents and effluents of waste water treatment plants as well as in rivers, but data on their distribution and variability in the marine ecosystem are still limited. Surface water samples obtained during two research cruises on the northern shelf of the South China Sea (SCS) near the Pearl River Estuary, in September 2018 and in August 2019, were investigated for estrogenic substances, namely estrone (E1), 17β-estradiol (E2), 17α-ethinylestradiol (EE2), genistein (GEN), daidzein (DAI) and zearalenone (ZEN). Among the target analytes, the natural hormones E1 and E2, as well as the synthetic EE2, were the most abundant with maximum concentrations of 1.1 ng L^{-1}, 0.7 ng L^{-1} and 0.6 ng L^{-1}, respectively. Of substances produced by plants and fungi, GEN, DAI and ZEN, only GEN was detected (1.2 ng L^{-1}). High concentrations occurred predominantly close to the coast, which was also reflected in the calculated estradiol equivalent quotients (up to 1.4 ng L^{-1}). In general, the distribution of estrogenic substances observed in both years shows a regional and inter-annual variability consistent with the modeled surface current data for the SCS. Regarding single estrogenic compounds and estradiol equivalents, marine organisms in the northern SCS might be exposed to high potential risk.

1. Introduction

Endocrine disrupting compounds (EDCs) can adversely affect the hormone system of humans and animals due to their structural resemblance to endogenous hormones. EDCs comprise several natural substances as well as industrial chemicals or pharmaceuticals. The female

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sex hormones estrone (E1) and 17β-estradiol (E2) are endogenous substances that regulate essential functions in the endocrine system of organisms. Despite their natural occurrence and regulatory functions, increased concentrations of E1 and E2 can lead to negative effects on living organisms (Sumpter and Johnson, 2005). Hence, they are considered as a subclass of EDCs by now (Campbell et al., 2006). For example, Lu et al. (2010) observed feminization effects such as elevated E2 plasma concentration and vitellogenin induction in adult male goldfish exposed to river water from the Yangtze River, China, in which E1 and E2 were present (3.8 ng L^{-1} and 0.97 ng L^{-1}, respectively). In Danish streams, male brown trout also showed high vitellogenin levels (Bjerregaard et al., 2006; Lu et al., 2010).

Next to E1 and E2, 17α-ethinylestradiol (EE2) is a particularly strong estrogen, specifically designed for and used as the active ingredient in contraceptive pills. Purdom et al. (1994) showed that 0.1 ng L^{-1} of EE2 are sufficient to cause a rise in vitellogenin levels in male trout. Kidd et al. (2007) showed that a whole lake population of fathead minnow experienced induced vitellogenin levels in male species as well as impacts on gonadal development after chronic exposure to EE2 levels of 5–6 ng L^{-1}, which ultimately led to the near extinction of this species.

Steroidal hormones mainly enter the river system via sewage treatment plants and are consequently transported into the oceans (Xu et al., 2014). For example, Jiang et al. (2020) have reported removal efficiencies of less than 30% of wastewater treatment plants for E1, E2 and EE2 in China. Additionally, waste from livestock farming, the application of liquid manure and thus, field leaching are also considered as a source of hormones (Kolodziej et al., 2004; Kolodziej and Sedlak, 2007). Moreover, estrogens are known for being used in aquaculture as growth promoters (Hoga et al., 2018).

In addition to steroidal hormones, other natural compounds in the environment have a similar structure to hormones and hence, can mimic their action. These are produced by plants or fungi, i.e., phyto- and mycoestrogens (Benassayag et al., 2002). Phytoestrogens such as genistein (GEN) and daidzein (DAI) are isoflavones and are associated with soy products and clover. In addition, some freshwater and saltwater cyanobacteria species and algae also produce phytoestrogens (Gong et al., 2014; Jarošová et al., 2015; Procházková et al., 2017; Sychrová et al., 2012). The mycotoxin zearalenone (ZEN) is produced by the *Fusarium* fungus, which infests maize, corn and soy (Hartmann et al., 2007; Schenzel et al., 2012). Although their estrogenic effect is minor compared to the steroidal hormones E1, E2 and EE2, they often occur in the aquatic environment in concentrations up to 5.6 μg L^{-1} (Rocha et al., 2014), and they have already been linked with adverse health effects as well. For example, a study of Shao et al. (2019) demonstrated the influence of GEN on the embryo mortality of zebrafish. ZEN and its metabolites were shown to induce a rise in vitellogenin levels (Schwartz et al., 2010) and potentially cause intersex and decreased sperm production in fish (Bakos et al., 2013). Woźny et al. (2020) also observed abnormal development of rainbow trout gonads after feedborne exposure to ZEN.

For millennia now, people have been drawn to the seaside. As a result, large cities and megacities are spread along the coasts nowadays. Such high population densities often lead to increased discharge of waste, which ends up in the marine ecosystem and might disturb the sensitive organisms there. However, there are still few studies on estrogenic substances in the marine environment (Atkinson et al., 2002; Beck et al., 2005; Deich et al., 2020; Singh et al., 2010; Zhang et al., 2014). The Pearl River Estuary (PRE), located at the northern South China Sea (SCS) coast, comprises a population of more than 42 Mio. people (Gong et al., 2012). It has already been shown that the Pearl River Estuary transports estrogenic substances into the ocean (e.g., Chen et al., 2016; Gong et al., 2016; Xu et al., 2014). Studies in the South China Sea revealed concentrations of up to 11.4 ng L^{-1} for E1, 3.7 ng L^{-1} for E2 and 4.0 ng L^{-1} for EE2 (Deich et al., submitted; Zhang et al., 2014).

The aim of this study is to detect estrogens in the marine environment prone to be influenced by the seasonally varying monsoon and the hereof resulting drastic change in river discharge. Furthermore, this study aims to relate the observed distribution pattern to the prevailing hydrographic conditions in the area spanning from the PRE over the shelf and continental slope towards the deep SCS. Surface water samples from two research cruises, in September 2018 and in August 2019, were analyzed for the substances E1, E2, EE2, DAI, GEN and ZEN. The analytes concentrations and distribution patterns were compared between both years and related to the modeled surface currents in the SCS. In addition, the estrogenic potential was calculated, and the potential risk that might act on marine organisms was assessed.

2. Material and methods

2.1. Study site

This study was conducted as part of the Sino-German project MEGAPOL funded by the German Federal Ministry of Education and Research (BMBF, Germany) and the State Oceanic Administration (SOA, China). The sampling area stretches from the northern continental shelf of the South China Sea across the continental slope to the deep SCS (17–23°N, 112–117°E, Fig. 1A).

The South China Sea is a large marginal sea in the western Pacific Ocean, with a size of approximately 3.5×10^6 km^2 (Su, 2004). High riverine outflows influence the coast of the northern South China Sea (NSCS). The coastal area is home of a dense population, and its industrialization has undergone rapid development in recent years. This consequentially leads to high quantities of domestic as well as industrial waste water draining into rivers and thereby into the marine ecosystem. Midway along the coast of the sampling area lies the Pearl River, known as one of China's largest rivers with a highly urbanized catchment area, that drains into the SCS through the Pearl River Estuary. For example, discharge rates of the PRE are up to 42,430 m^3 s^{-1} with significant hourly and daily changes (He et al., 2016), which add up to 3.4×10^{11} m^3 of discharge per year (Gong et al., 2009; Ni et al., 2008). As a consequence of the high riverine discharges, the coast of the NSCS is nutrient-rich, whereas the wide basin of the northern South China Sea remains oligotrophic (He et al., 2016).

The prevailing East Asia monsoon winds influence the SCS and its circulation (Hu et al., 2000). In summer, the southwesterly monsoon causes an anticyclonic surface circulation, while in winter a cyclonic surface circulation is observed. In the study area, large-scale surface currents in the NSCS are the Guangdong Coastal Current (GCC), the South China Sea Warm Current (SCSWC) and the South China Sea Branch of Kuroshio (SCSBK) (Fig. 1B).

The GCC is observed at the coast of the Chinese Guangdong province and is affected by the strength of Pearl River discharge. The GCC itself is divided by the PRE into the eastern GCC (EGCC) and western GCC (WGCC; Fig. 1B). The EGCC flows with the monsoons and enters the East China Sea through the Taiwan Strait in summer. The WGCC flows southwesterly all year round along the coast and enters the Gulf of Tonkin via the Qiongzhou Strait (Chen et al., 2019; Huang et al., 1994). However, a strong prevailing summer monsoon might reverse the WGCC into a northeastward direction (Huang et al., 1994). The Pearl River Plume or Zhujiang River Diluted Water (ZRDW) might be directed eastwards during a strong summer monsoon due to the resulting anticyclonic surface circulation and the reversed, eastern directed WGCC (Bai et al., 2015). However, easterly seasonal and inter-annual variations are observed and are directly correlated to the water discharge of the Pearl River (Huang et al., 1994). For example, upon high riverine discharge during the wet season and an existing cyclonic cold eddy occurring near Dongsha Island (20.7°N, 116.7°E, Fig. 1B), the buoyant plume might be entrained across the shelf into the oligotrophic basin (He et al., 2016; Huang et al., 1994).

Further off-shore, the South China Sea Warm Current is observed at 100–200 m-isobath and is known as a northeastward bound current throughout the year, which intensifies in summer and weakens during

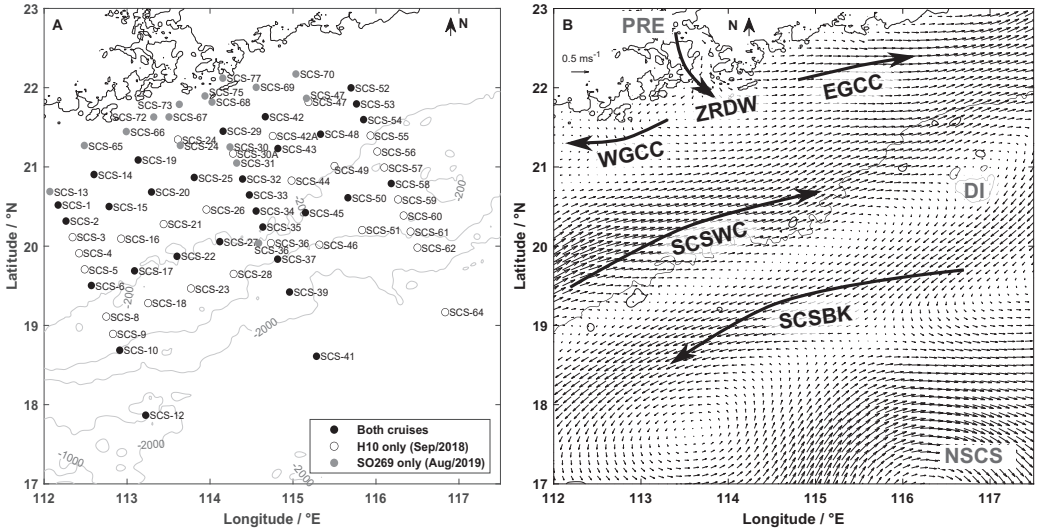

Fig. 1. A Sampling stations in the northern South China Sea (NSCS). Stations sampled in September 2018 and August 2019 (●), only in September 2018 (○) and only in August 2019 (◉). Bathymetry was obtained from ETOPO1 (solid lines, Amante and Eakins, 2009). B Main surface circulation during the summer monsoon in August 2019 (→, modeled with HAMSOM), 200 m-isobath (solid line) and the location of the Pearl River Estuary (PRE) and Dongsha Island (DI). Main surface currents in the study area: Zhujiang River Diluted Water (ZRDW), eastern and western Guangdong Coastal Current (EGCC, WGCC), South China Sea Warm Current (SCSWC) and South China Sea Branch of Kuroshio (SCSBK), adapted from Huang et al. (1994).

the winter monsoon (Huang et al., 1994; Wang et al., 2014). South of the SCSWC flows the South China Sea Branch of Kuroshio, which can be regarded as a compensatory current (SCSBK, Fig. 1B). It is known as a relatively stable (south-)westerly current that originates from the Kuroshio Current, a north-flowing surface current in the northern Pacific Ocean that flows east of the Philippines along the coast of Luzon and Taiwan. It weakens during the winter monsoon and part of the Kuroshio Current intrudes the SCS through the Luzon Strait (Huang et al., 1994; Hu et al., 2000).

2.2. Chemicals

Target analyte standards of GEN, DAI and ZEN were obtained from Sigma Aldrich (Taufkirchen, Germany). E1, E2, EE2 and the respective internal standards E1-*d*, E2-*d*, EE2-*d* were obtained from Neochema GmbH (Bodenheim, Germany). Working solutions of analytes and internal standards were prepared in analytical grade water suitable for liquid chromatography-mass spectrometry (LC-MS grade; Merck KGaA, Darmstadt, Germany). Further, LC-MS grade solvents ACN and MeOH were obtained from Promochem (Wesel, Germany), and EtOAc was obtained from Merck KGaA (Darmstadt, Germany). Calibration samples were prepared with Milli-Q water (Milli-Q® water purification system, Merck Millipore, Darmstadt, Germany). HCl and NH_4F were purchased from VWR International GmbH (Hannover, Germany).

2.3. Sample treatment

Water samples were taken in the wet season during a cruise on board of the Chinese R/V Hai Yang Di Zhi Shi Hao in September 2018 and on board of the German R/V SONNE in August 2019 (Fig. 1A). Surface water was collected with a CTD SBE 911 rosette system and stored in 1 L amber glass bottles. The samples were acidified with 5 M HCl to a pH of 2–3. 1 mL of internal standard stock solution (IS) was added to the samples. In 2018, the IS contained 20 ng mL^{-1} of E1-*d*, E2-*d* and EE2-*d*, whereas it contained 1 ng mL^{-1} of E1-*d* and 2 ng mL^{-1} of E2-*d* and EE2-*d* in 2019. Subsequently, samples were enriched over a filtration unit with a glass fiber filter (Whatman™, 0.7 μm) attached to a pre-conditioned C18 ec cartridge (Chromabond®, Macherey-Nagel, Düren, Germany). After the enrichment step, the columns were washed with 2.5 mL of 5% aqueous MeOH solution and analytical grade water each. The columns were then shortly dried under gentle vacuum. The samples were kept at −20 °C until further analysis in the home laboratory at Leibniz Institute for Baltic Sea Research in Warnemünde, Germany.

There, the analytes were eluted from the columns with 10 mL of EtOAc:MeOH (30:70, v:v) over an attached Aminopropyl Sep-Pak Plus cartridge (Waters, Eschborn, Germany) (Kumar et al., 2009). The extract was blown until dryness under a gentle stream of clean air in a 40 °C water bath, subsequently reconstituted in 1 mL of ACN:analytical grade water (30:70, v:v) and passed through a syringe filter.

2.4. Instrumental analysis

Chromatographic separation of analytes was conducted on a Nexera-i Shimadzu system (Shimadzu, Berlin, Germany) equipped with a Kinetex Biphenyl column (150 mm × 4.6 mm × 2.6 μm, Phenomenex, Aschaffenburg, Germany). Gradient separation was performed with analytical grade water, ACN and 15 mM NH_4F at 0.2 mL min^{-1}. Detection of analyte masses was conducted on a heated electro spray ionization coupled to a MS8060 tandem mass spectrometer (Shimadzu, Berlin, Germany). Intensive mass transitions were selected for each analyte. The most intensive mass transition was used for quantification (quantifier), while the other transitions were used for analyte qualification (qualifier, for more details see Supplementary material). Samples were measured twice, and the mean was taken for quantification.

Quantification was conducted using calibrations with internal standards in the range of 0–0.8 ng L^{-1} in 2018 and 0–1.0 ng L^{-1} in 2019. Generally, calibration samples were prepared with Milli-Q water and were subsequently filtered, enriched by solid phase extraction and then analyzed with LC-MS/MS. Limits of detection (LOD) and quantification (LOQ) were determined using a calibration containing all targeted analytes, which was set up with an environmental water sample (for more details see Supplementary material). LOD and LOQ were 0.1 ng L^{-1} and 0.3 ng L^{-1} for E1, 0.2 ng L^{-1} and 0.3 ng L^{-1} for E2, 0.3 ng L^{-1} and 0.6 ng L^{-1} for EE2, 0.4 ng L^{-1} and 0.7 ng L^{-1} for GEN,

Table 1
Estrogen concentration in surface water samples from cruises in September 2018 and August 2019, with detection frequency and number of samples with detectable estrogen concentrations versus the number of analyzed samples (in parentheses), n.d. = not detected, n.a. = not applicable.

Compound	Cruise	Min	Max	Mean	Detection frequency [%]	
		[ng L^{-1}]				
E1	Sep 18	0.2	1.0	0.5	98	(56/57)
	Aug 19	0.2	1.1	0.6	100	(44/44)
E2	Sep 18	n.d.	n.d.	n.a.	0	(0/57)
	Aug 19	0.2	0.7	0.3	32	(14/44)
EE2	Sep 18	0.4	0.6	0.4	12	(7/57)
	Aug 19	0.3	0.6	0.4	39	(17/44)
GEN	Sep 18	n.d.	n.d.	n.a.	0	(0/57)
	Aug 19	1.2	1.2	n.a.	2	(1/44)
DAI	Sep 18	n.d.	n.d.	n.a.	0	(0/57)
	Aug 19	n.d.	n.d.	n.a.	0	(0/44)
ZEN	Sep 18	n.d.	n.d.	n.a.	0	(0/57)
	Aug 19	n.d.	n.d.	n.a.	0	(0/44)

0.3 ng L^{-1} and 0.5 ng L^{-1} for DAI and 0.1 ng L^{-1} and 0.2 ng L^{-1} for ZEN. If the concentration was below the LOD, the sample was not considered for further evaluation and is referred to as not detected (n.d.). One sample from 2018 (SCS-6) and two from 2019 (SCS-35, SCS-54) were excluded from further evaluation due to complete loss of internal standard during the clean-up procedure.

2.5. Ocean model

In this study, we applied the regional 3D hydrodynamic Hamburg Shelf Ocean Model, HAMSOM (Backhaus, 1985; Pohlmann, 2006) to the northern South China Sea (13–24°N, 105–121°E). The horizontal spatial resolution is 2′ (approximately 3 km) with 22 layers in vertical direction. The numerical simulation was performed from 2017 to 2019 with a time step of 90 s, using the first year to spin-up the model. We saved daily averaged values of sea surface height and current velocity for the years 2018 and 2019 to subsequently drive the tracer model (Hainbucher et al., 1987). The bathymetry data were derived from marine navigational charts.

Regarding the driving force of the ocean model, tides, atmospheric forcing and river input were considered. The atmospheric forcing was obtained from hourly ERA5 data (Copernicus Climate Change Service (C3S), 2017) with a spatial resolution of 0.25°, and it comprises wind, sea level pressure, air temperature, cloudiness, precipitation and relative humidity. The river input was extracted from the monthly climatological WaterGAP model data (Müller Schmied et al., 2014), with a horizontal resolution of 0.5°. The only exception was made for the Pearl River, for which monthly observational data from the "Ministry of Water Resources of the P.R. China" were utilized. Tides were added to the open boundary sea surface height, using 13 tidal components extracted from the TPXO8-atlas_v1 dataset (Egbert and Erofeeva, 2002). The open boundary and initial temperature and salinity fields were obtained from the Mercator-Ocean dataset (https://resources.marine.copernicus.eu/?option=com_csw&view=details&product_id=GLOBAL_ANALYSIS_FORECAST_PHY_001_024). The open boundary sea surface heights were derived from the same data set. However, in order to obtain a reasonable Kuroshio intrusion, dynamic heights had to be replaced by the ones calculated by HAMSOM.

The model results were validated using CTD profiles of temperature and salinity at different positions within the working area and model domain. An example of the modeled vertical distribution of temperature and salinity at station SCS-39 is shown in the Supplementary material (Fig. S2) for 2018 and 2019. Taking into account the limitations of the simulation, the model results show a reasonable agreement with the observations.

3. Results and discussion

3.1. Estrogen concentration in September 2018

Within the SCS, E1 is the predominant estrogen and was detected in all surface water samples except for SCS-5 (Table 1, Fig. 2). Both high and low E1 concentrations spread from the shelf to the deep sea (0.2–1.0 ng L^{-1}, Fig. 2A). Moderately higher E1 concentrations are located primarily at the western sampling sites as well as at the nearshore stations (up to 1.0 ng L^{-1}). Besides E1, only EE2 was observed as another estrogenic substance during this cruise in September 2018. Highest EE2 concentrations were also present in proximity to the coast and reached up to 0.6 ng L^{-1} (Fig. 2B). The observed estrogen concentrations are of the same order of magnitude as those observed by Zhang et al. (2014), who as well determined estrogenic compounds in the South China Sea during the wet season (Table 2).

Modeled monthly mean surface currents for September 2018 (summer monsoon) show a comparably strong WGCC, whereas the EGCC is comparatively weak. Also, a weak northwards directed SCSWC is

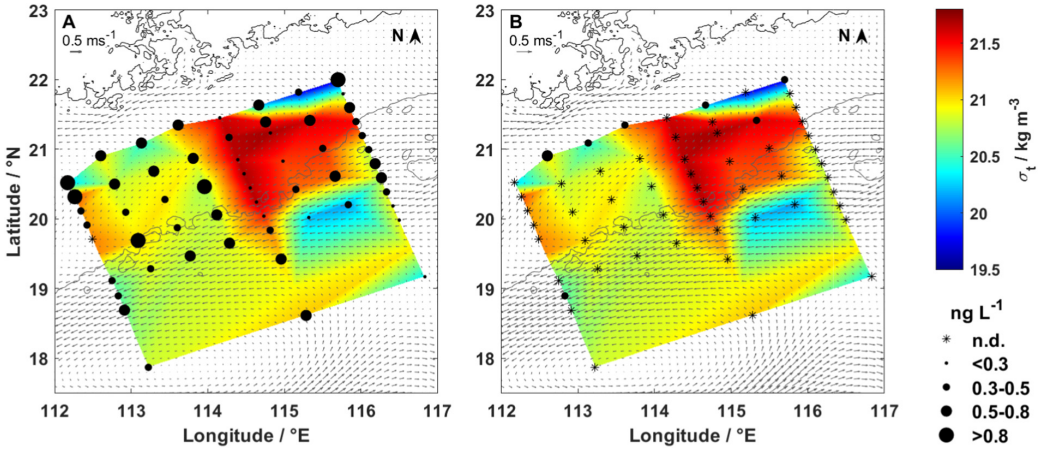

Fig. 2. Concentration of A E1 and B EE2 with modeled surface currents (→, HAMSOM), bathymetry (solid lines, from ETOPO1, Amante and Eakins, 2009), 200 m-isobath and sea surface density from the cruise in September 2018 (color coded, calculated using temperature and salinity CTD data at surface, UNESCO, 1983). The size of ● is related to the detected concentrations, n.d. = not detected (*). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
Estrogen concentration in surface water samples determined in other studies, n.d. = not detected, n.a. = not applicable.

Region	E1	E2	EE2	GEN	DAI	ZEN	Time	Reference
	[ng L^{-1}]							
South China Sea	n.d.–1.0	n.d.	n.d.–0.6	n.d.	n.d.	n.d.	Sep 2018 (wet season)	This study
	0.2–1.1	n.d.–0.7	n.d.–0.7	n.d.–1.2	n.d.	n.d.	Aug 2019 (wet season)	
South China Sea	n.d.–11.16	n.d.–3.71	n.d.–3.99	n.a.	n.a.	n.a.	Aug 2012 (wet season)	Zhang et al. (2014)
Pearl River Estuary, China	n.d.–3.6	n.d.	n.d.–0.7	n.d.–12.9	n.d.–11.9	n.d.–1.9	Sep 2018 (wet season)	Deich et al., submitted
PRE, receiving water, China	3.2–14	<LOQ–0.42	1.3–4.2	n.a.	n.a.	n.a.	June 2011 (wet season)	Xu et al. (2014)
PRE, river outlets, China	<LOQ–1.2	<LOQ	<LOQ–1.2	n.a.	n.a.	n.a.	June 2009 (wet season)	
	<LOQ–3.1	<LOQ	<LOQ–1.56	n.a.	n.a.	n.a.	Jan 2010 (dry season)	
Maluan Bay, China	0.56–3.8	0.55–1.62	1.14–2.39	n.a.	n.a.	n.a.	Nov 2015 (dry season)	Wang et al. (2017)
Halifax Harbour, Canada	n.a.	n.d.–0.47	<0.14	n.a.	n.a.	n.a.	July 2005	Robinson et al. (2009)
Coastal areas, Kuwait	n.d.	n.d.–6.6	n.d.–25.6	n.a.	n.a.	n.a.	Oct 2015–Aug 2016	Saeed et al. (2017)
Mira River, Portugal	4.1±0.4	32.0±12.0	42.2±5.7	40.3±3.2	17.4±3.0	n.a.	June/Sep 2011	Rocha et al. (2016)
Baltic Sea, Germany	0.1–0.53	n.d.	n.d.–17.2	n.d.	n.d.	n.a.	July 2003, 2004	Beck et al. (2005)

observed at the shelf, and a southwestwards directed SCSBK south of the 200 m-isobath. The modeled surface currents show a southwestwards driven Pearl River plume that only partially affects the nearshore sampling stations. It should be mentioned that we present monthly mean currents together with quasi-synoptic discrete measurements, and therefore the temporal current variability is suppressed.

Generally, it is assumed that the targeted hormones enter the sea mainly through freshwater inputs, which would result in a concentration gradient from the continental shelf towards the wide basin of the South China Sea. However, changes in this concentration gradient might arise through adsorption onto particles and subsequent sedimentation as well as degradation during the transport. For example, E1 is a major breakdown product of E2 and EE2 (Adeel et al., 2017), which could account for the high abundance and high concentrations of E1.

In addition, horizontal and vertical mixing (e.g. cross-shelf transport, eddies) should also be considered as a possible way of estrogen transport into the open sea. In 2018, E1 concentrations increased towards off-shore stations along the middle transect (SCS-29 to SCS-41, 0.2–0.7 ng L^{-1}). Measured temperature and salinity, as well as the calculated density at surface, indicate different water bodies in the study area (Fig. 2A). Especially along the middle transect, the stations with low concentrations are influenced by comparatively denser seawater ($\sigma_t \geq 21$ kg m^{-3}). The stations SCS-29–41, 43–46, 49–51 were sampled after the passage of the super-typhoon "Mangkhut", which intensified the mixing within the upper 20–30 m of the water column and subsequently led to density changes (Liu et al., 2020). However, we cannot determine the exact influence of the typhoon on the estrogen concentrations and spatial pattern, as data from stations sampled before and after are not available.

For this cruise, Pearson correlation coefficients were determined for estrogen concentrations, temperature, salinity and chlorophyll *a* (Table S3). A negative correlation was observed for E1 concentrations and salinity ($R = -0.48$, $p < 0.01$), which could indicate freshwater input of E1. Furthermore, a positive correlation with chlorophyll *a* ($R = 0.33$, $p < 0.05$) was observed for E1 concentrations.

3.2. Estrogen concentration in August 2019

In 2019, 44 stations were sampled and analyzed for estrogenic compounds. As in 2018, E1 was dominant at all stations with concentrations ranging from 0.2 to 1.1 ng L^{-1} (Fig. 3A). In addition, the hormones EE2 and E2 were detected more frequently during this cruise (Table 1), with concentrations of 0.3–0.6 ng L^{-1} and 0.2–0.7 ng L^{-1}, respectively (Fig. 3B, C).

In contrast to 2018, comparably high estrogen concentrations of E1, EE2 and E2 were detected at eastern sampling sites, particularly at those stations in proximity to the coast (Fig. 3). Less dense water ($\sigma_t \leq 18$ kg m^{-3}) observed at western stations close to the coast (SCS-19, 24–25, 65–68) is attributed to the Pearl River plume. Interestingly, even though potentially influenced by riverine water, those stations show low estrogen concentrations. Less dense water (18 kg $m^{-3} \leq \sigma_t \leq 21$ kg m^{-3}) also occurred at eastern stations close to the coast (SCS-29–30, 42–43, 46–50, 69–70), which is attributed to the Pearl River plume as well, that has already drained into SCS and mixed with the surface seawater brought by the SCSWC.

In 2019, the samples were also taken in summer, i.e., the sample area was under the influence of the southwest monsoon as in 2018. The modeled surface currents show a comparatively weak WGCC, while the EGCC is comparatively strong. Also, the northeastwards SCSWC is strong during this sampling period compared to the previous year. Consequently, the Pearl River plume is deflected eastwards and was present west of Dongsha Island (20.7°N, 116.7°E). The comparatively less dense water and the direction of the flow suggest that the high concentrations of hormones are likely to be carried into the SCS via the Pearl River outflow. However, there is no significant correlation between estrogen concentrations and salinity in 2019 (Table S4). The significant positive correlations between E1 and E2 ($R = 0.66$, $p < 0.01$) as well as E1 and EE2 ($R = 0.62$, $p < 0.01$) might imply a similar source of these estrogens and a similar distribution within the northern South China Sea.

Both sampling campaigns were conducted in the wet season, but the spatial distribution of estrogen varied between both years. The modeled surface current data indicated inter-annual differences of surface currents. Also, the sea surface density showed different water bodies in both years. In contrast to 2018, a buoyant Pearl River plume was observed in 2019, which affects the coast near stations. The pronounced influence of the Pearl River plume and its direction could cause the varying estrogen concentrations in the northern South China Sea and could explain the abundance of E2 in 2019. To the best of our knowledge, little is known about the residence time of estrogenic substances in marine systems. Therefore, the differences between 2018 and 2019 may arise through the sampling of water bodies of different ages. In addition, it is possible that a fresh input of estrogens was measured in 2019 and E2 was not yet degraded to E1. In this regard, ratios of E1/E2 and E1/EE2 were calculated. For example, the ratios at station SCS-68 show an E1/E2 ratio of 1.9 and at station SCS-52 4.8. The ratio of E1/EE2 also increases from SCS-68 to SCS-52 from 1.6 to 2.4. However, a clear distribution pattern was not derived (data not shown). Thus, it cannot be concluded that the share of E1 increases due to the degradation of E2 and EE2, or if rather horizontal and vertical mixing, adsorption and sedimentation are the major factors causing the observed distribution patterns.

For comparison, Zhang et al. (2014) reported elevated estrogen concentrations in the SCS also at stations which are presumably influenced by the Pearl River plume (Table 2). In that study, high concentrations of EE2 (3.99 ng L^{-1}) were detected at the estuary's mouth and decreased towards the wide basin of the SCS. In a study of Xu et al. (2014) conducted in the Pearl River Estuary, E1, E2 and EE2 were detected in the receiving river water (Table 2). However, concentrations decreased

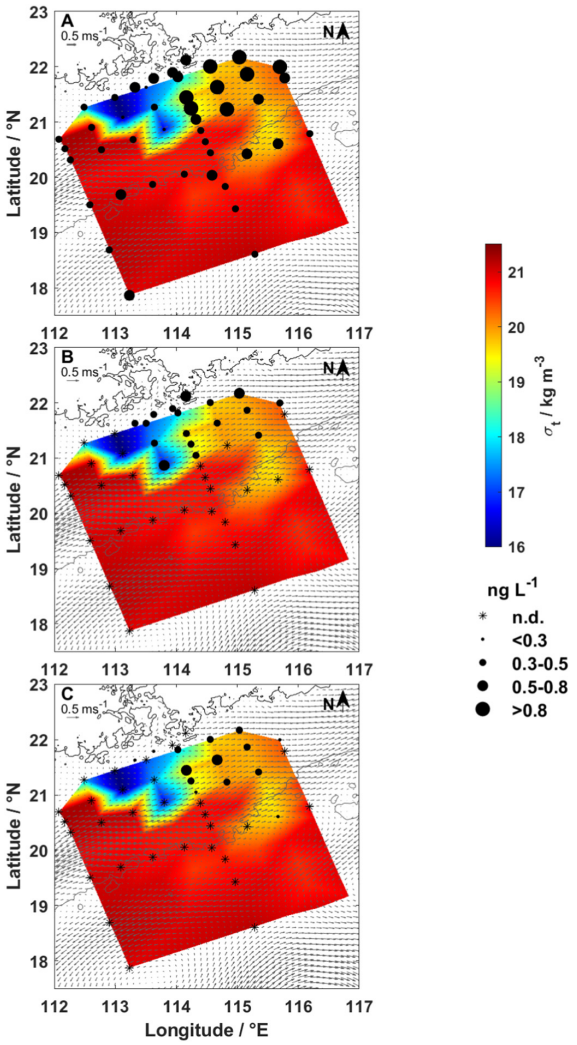

Fig. 3. Concentration of A E1, B EE2 and C E2 with modeled surface currents (→, HAMSOM), bathymetry (solid lines, from ETOPO1, Amante and Eakins, 2009), 200 m isobath and sea surface density from the cruise in August 2019 (color coded, calculated using temperature and salinity CTD data at surface, UNESCO, 1983). The size of ● is related to the detected concentrations, n.d. = not detected (*). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

towards the estuary's mouth, where only traces of E1 and EE2 (up to 1.2 ng L^{-1}) were detected. Also, only E1 (up to 3.6 ng L^{-1}) and EE2 (up to 0.7 ng L^{-1}) were reported by Deich et al. (submitted) in the Pearl River Estuary. Nevertheless, the existing studies reported variability of the distribution of estrogenic substances of different degree. Hence, the understanding of the distribution pattern is still limited and does not allow a final conclusion regarding the occurrence or absence of individual estrogenic substances.

3.3. Plant- and fungi derived estrogens

Estrogen-like substances produced by plants or fungi are known to elicit an estrogenic effect on organisms' endocrine systems. However, only limited data are available regarding their presence in the marine environment. Hence, their investigation is of increasing relevance. In this study, we analyzed all samples taken in the NSCS for GEN, DAI and ZEN. Among them, only GEN was detected with a concentration of 1.2 ng L^{-1} at a single station close to the coast (SCS-14, 2019).

On the one hand, phytoestrogens and mycoestrogens are produced on land by different plant or fungi species and can be introduced into the marine system via river waters (Jarošová et al., 2015; Schenzel et al., 2012). On the other hand, there is evidence of phytoestrogens in cells of algae and cyanobacteria species, which can be released upon cell death, as well as in their exudates (Gong et al., 2014; Sychrová et al., 2012). Rocha et al. (2013) and Ribeiro et al. (2016) reported that phytoestrogens occur more frequently in the Douro River (Portugal) with the beginning of the vegetation period in the spring and summer. In addition, Ribeiro et al. (2009) showed that the concentration of GEN and DAI in the Douro River increased due to a prolonged drought and became diluted upon rainfall. GEN, DAI as well as ZEN were investigated in the Pearl River Estuary in a previous study (September 2018, wet season, Deich et al., submitted), and GEN and DAI were detected at the estuary's mouth with concentrations of 2.4 ng L^{-1} and 6.8 ng L^{-1}, respectively (Table 2). Hence, it is possible that the substances, which were transported via the Pearl River water, were strongly diluted within the marine system, or that they might have degraded. Moreover, this study investigated only filtrated surface water samples. Thereby, any estrogen-like substances adsorbed onto particles were disregarded. Apart from the shelf, the NSCS is an oligotrophic system with limited primary production and a chlorophyll *a* maximum at depths of 50–60 m. Thus, the maximum of phytoestrogens produced within the marine system may not have been considered.

3.4. Estrogenic activity

Estradiol equivalent quotients (EEQ) were calculated according to Eq. (1) to estimate the estrogenic activity of each water sample (Beck et al., 2006). The cumulative quotient indicates the estrogenic potential, which can act on organisms in the aquatic environment in relation to the natural hormone E2. The EEQ takes into account the distinct equivalent factor of each estrogen (EEF_i) as well as the measured environmental concentration (C_i). Equivalence factors, i.e., the relative potency of the target analyte compared to E2, of E1, E2 and EE2 are 0.25, 1 and 1.25, respectively (Beck et al., 2006) indicating that E1 is less potent than E2, whereas EE2 has a stronger estrogenic effect on organisms compared to E2.

$$EEQ = \sum EEF(i) \times C(i) \quad (1)$$

In 2018, calculated EEQ in the SCS based on E1, E2 and EE2 concentrations ranged from <0.1–0.9 ng L^{-1}. Comparatively high EEQs were observed at sampling sites near the coast (Fig. 4). Although E1 was detected ubiquitously in the SCS, elevated EEQs were mainly observed at sampling sites where EE2 was detected as well. Because of its strong estrogenic potency, EE2 accounts for up to 82% of estrogenic activity at its occurrence. During the cruise in August 2019, EEQs were in the range of 0.1–1.4 ng L^{-1}. High EEQs were detected east of the PRE at near-shore sampling sites mirroring the total amount of the estrogens detected in this area. Where present, E2 and EE2 contributed up to 66% and 92% to the estrogenic activity, respectively.

Overall, the observed EEQs in both years are in good agreement with those of Zhang et al. (2014) who detected elevated EEQ (up to 4.7 ng L^{-1}) in proximity to the coast or estuaries of the SCS. Other studies have shown calculated EEQ in the range of 4.2–10.6 ng L^{-1} in the Maluan Coastal Bay, China (Wang et al., 2017) and 5–13.8 ng L^{-1} in the Yundang Lagoon, China (Zhang et al., 2011). However, when estimating the estrogenic potential based on chemical analysis, it is important to note that the calculations rely solely on the herein targeted hormones. Thereby, other estrogenic substances are not included into consideration and interactions such as synergistic or antagonistic effects

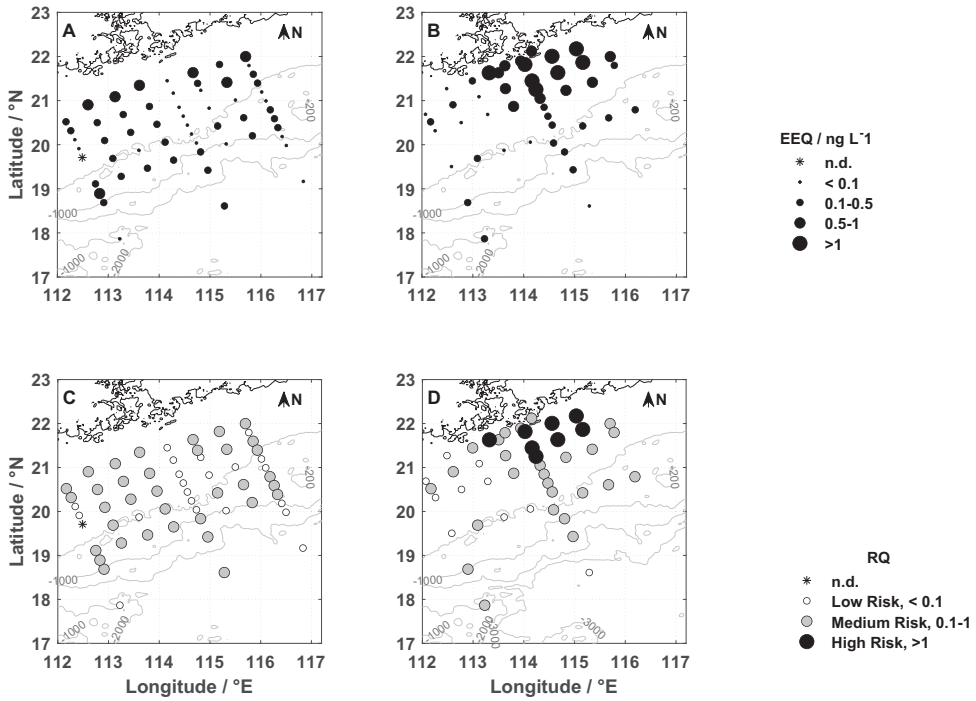

Fig. 4. Calculated estradiol equivalent concentrations (EEQ) in the SCS A in September 2018 and B August 2019. The size of ● is related to the detected concentrations, n.d. = not detected (*). Bathymetry (solid lines) was obtained from ETOPO1 (Amante and Eakins, 2009). Calculated risk quotients (RQ) in the SCS C in September 2018 and D August 2019. The color and size of ● is related to risk level (○ low risk, ● medium risk, ● high risk).

among the substances are not considered as well. The results emphasize the relevance of studies under the whole spectrum of environmental conditions, taking into account environmental concentrations, but also the full range of natural and synthetic substances in the water sample that could potentially affect aquatic organisms.

3.5. Risk assessment

In order to estimate the potential risk arising from the observed estrogen compound concentration, a risk assessment was performed. This considers observed environmental concentrations in relation to the predicted no-effect concentration of a particular estrogen (PNEC). If the obtained risk quotient (RQ) is less than 0.1, low risk to marine organisms is considered. RQ between 0.1 and 1 is considered as medium risk, while RQ above 1 depicts a high potential risk for the organisms in the environment (Hernando et al., 2006).

Observed RQ based on E1 concentration and a PNEC of 6 ng L^{-1} (Caldwell et al., 2012) show a low to moderate risk to marine organisms in both years (Fig. 5A, B). In 2018, no concise distribution pattern can be identified for RQs based on E1 concentrations. A moderate risk is observed sporadically at stations near the coast as well as at stations further off-shore (Fig. 5A). In contrast to 2018, moderate risk based on E1 predominantly occurs at near-shore stations under the influence of the Pearl River plume in 2019 (Fig. 5B). However, moderate RQs also occur sporadically at stations further off-shore as well. Among the studied and detected estrogens, EE2 is the most potent and potentially harmful estrogen, which is reflected in the comparably low PNEC of 0.1 ng L^{-1} (Caldwell et al., 2012). EE2 concentrations were present in both years in surface waters mainly close to the coast, and the resulting RQs could already pose a high potential risk to the marine organisms at these stations (Fig. 5C, D). In 2018, E2 was not detected, and therefore a RQ could not be derived. In 2019, E2 was detected at stations close to the coast, and the derived RQs indicate a medium risk to marine organisms for this estrogen (Fig. 5E).

Overall, both years show that comparatively high RQs occur mainly at near-shore stations under the influence of the Pearl River plume. This leads to the conclusion that marine organisms at the coast are at higher risk than those further off-shore. However, due to the seasonal variability, the inter-annual variability and the prevailing complex surface currents system in the SCS, estrogens might be transported off-shore, where they might pose a potential threat as well.

In addition, RQs can also be assessed for the estrogenic potential instead of considering separate RQs for individual substances. For this, a PNEC of 1 ng L^{-1} is suggested for EEQ (Jarošová et al., 2015). In this case, EEQ levels observed in 2018 could result in a low to moderate risk, while in 2019, EEQ might pose a high potential threat to marine organisms (Fig. 4C, D). This is mainly dominated by the occurrence of EE2 in 2019 compared to 2018. In both years, comparatively high RQs were present at stations near the coast, which are frequently influenced by riverine waters that might contain estrogenic substances.

The risk quotients obtained from individual estrogenic compounds differ from those related to the cumulative estrogenic potential. The differences presumably derive from the cumulative PNEC value that is considered for the EEQ risk assessment. However, this value does not take into account the different composition of the samples. Although the relative estrogenic equivalent factors of individual substances are included in the calculated EEQ, it does not correspond to the cumulative PNEC. Using station SCS-10 (2018) as an example, the RQ is <0.1 considering E1 concentrations (Fig. 5A), while it is >0.1 considering the EEQ, despite E1 being the only contributor to the calculated estrogenic potential at that station (Fig. 4C). Thus, regarding only the risk quotient of EEQ could lead to overestimation of the risk. However, the risk can be underestimated as well, e.g., station SCS-52 (2019) reveals a moderate

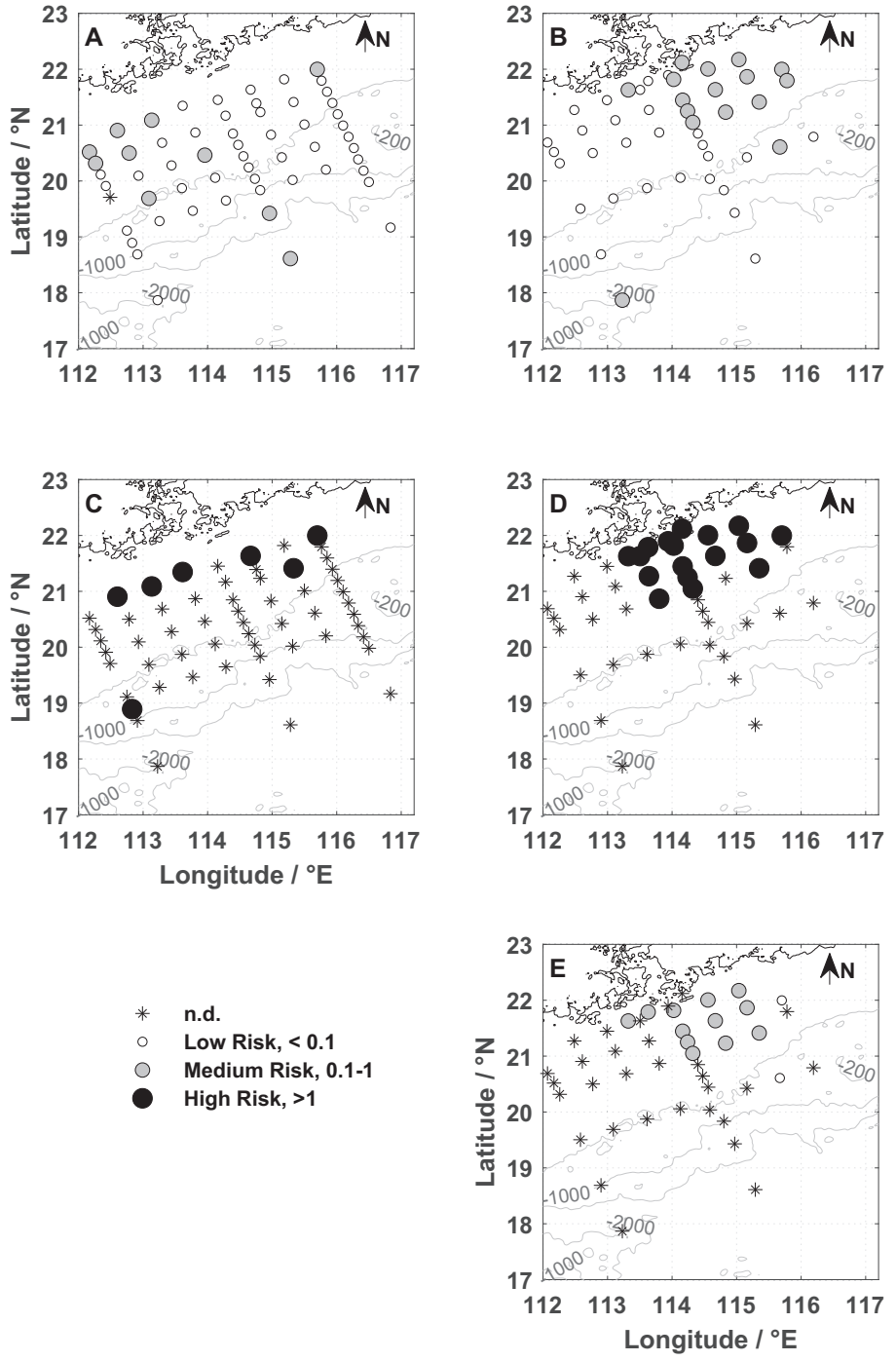

Fig. 5. Calculated risk quotients (●) for individual estrogenic compounds in the SCS for E1 in September 2018 and August 2019 (A, B), for EE2 in September 2018 and August 2019 (C, D) and for E2 in August 2019 (E). Bathymetry (solid lines) was obtained from ETOPO1 (Amante and Eakins, 2009). The color and size of ● is related to risk level (○ low risk, • medium risk, ● high risk), n.d. = not detected (*).

potential risk regarding RQ based on EEQ (Fig. 4D), while the EE2 concentration suggests that a high potential risk should be considered (Fig. 5D).

In this regard, a cumulative RQ can be derived (Fig. 6), which results from the addition of RQs obtained from individual substances (Gustavsson et al., 2017; Posthuma et al., 2019). At station SCS-72

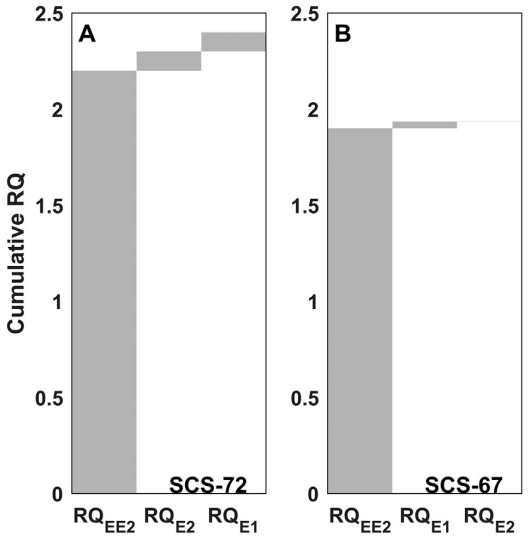

Fig. 6. Calculated cumulative risk quotients for estrogenic compounds in August 2019 at A SCS-72 and B SCS-67. RQ_{EE2} = risk quotient for EE2, RQ_{E2} = risk quotient for E2 and RQ_{E1} = risk quotient for E1.

(August 2019, Fig. 6A), the cumulative RQ shows a high risk as already observed for the RQ based on EEQ values (Fig. 4D). In contrast, station SCS-67 (August 2019, Fig. 6B) shows a high potential risk based on the cumulative RQ as well, however the RQ based on EEQ indicates only a moderate risk.

Overall, the risk assessment and the calculated EEQ must be viewed critically, as they only consider the targeted estrogenic compounds, which ultimately might not reflect the actual situation, i.e., combinatory effects between substances, to which marine organisms in this area might be exposed. Furthermore, long-term exposure effects cannot be assessed, as this would have to include comprehensive monitoring of the sampling area as well as *in vitro* and *in vivo* tests with the marine organisms from the respective region.

4. Conclusion

In this study, surface water samples obtained from the northern South China Sea were investigated for selected estrogenic substances in September 2018 and August 2019. It was shown that high estrogenic concentrations were mostly detected at stations under the influence of the Pearl River plume which drained differently into the NSCS during both cruises, i.e., comparably high concentrations for all estrogenic compounds were detected at the northern shelf of SCS at the stations close to the coast. The observed distribution patterns reflect the transport pathway based on modeled monthly mean surface currents.

Although naturally occurring E1 was the most frequently detected estrogen in this study, the NSCS is also anthropogenically pressured by the synthetic EE2, which itself is supposedly the most potent steroidal estrogen. In contrast to steroidal estrogens, both phytoestrogens DAI, GEN and the mycoestrogen ZEN were hardly detected in the surface waters of the NSCS, leading to the assumption that those transported via rivers into the marine environment have been removed from the water column, and that the production of phytoestrogens might occur at different depths in the water column. Furthermore, there is a need for studies on the occurrence of plant- and fungi-derived estrogens and their ecotoxicological effects on the marine environment, which also require improved methods for their analysis.

Calculated estrogenic activity in the South China Sea arises mainly from steroidal estrogens and could pose a medium to high potential threat to marine organisms at coastal areas based on calculated risk quotients. The risk assessment performed in this study provides a useful tool for a first overview of the SCS's status. Nevertheless, combined test systems, e.g., *in vitro* and *in vivo* biological test in combination with single compound analysis, are indispensable in order to assess the potential risk to the marine organisms accurately.

CRediT authorship contribution statement

CD Conceptualization, Methodology, Chemical analysis, Visualization, Writing - Original draft, **HF** Visualization, Writing - Review & editing, **JA** Chemical analysis, Writing - Review & editing, **WL** Modeling, Writing - Review & editing, **TP** Modeling, Writing - Review & editing, **JW** Conceptualization, Supervision, Project coordination, Funding acquisition, Writing - Review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.145239.

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A. Appendix

Table A.1.: Literature referred to in Fig. 1.2, n.d. = not detected, n.a. = not analyzed.

Region	Lon	Lat	E1	E2	EE2	Reference
	[°E]	[°N]		[ng L^{-1}]		
Antarctic	-60.76	-62.96	0.05	0.04	0.14	Esteban et al., 2016
Antarctic	166.35	-77.64	<7	n.d.	<1.4	Emnet et al., 2015
Argentina	-58.01	-35.64	n.d.	369	43	Valdés et al., 2015
Australia	152.83	-27.47	5.08	1.54	0.22	Ying et al., 2009
Brazil	-47.58	-22.02	14.7	14.8	0.16	Campanha et al., 2015
Canada	-72.86	46.19	6	38	n.a.	Cathum & Sabik, 2001
Canada	-63.58	45.70	n.a.	0.57	0.14	Robinson et al., 2009
China	117.49	39.18	49.8	21.2	24.4	Lei et al., 2009
China	114	20	11.38	3.7	3.99	Zhang et al., 2014
China	103.38	35	15.61	2.3	n.a.	Wang et al., 2012
France	2.35	48.84	3	3	2.9	Cargouet et al., 2004
Germany	11.36	54.02	0.53	n.d.	17.2	Beck et al., 2005
Germany	8.49	49.57	19	5.6	1.5	Pawlowski et al., 2004
Indonesia	106.91	-6.69	77.5	6.4	9.1	Duong et al., 2010
Iran	48.57	34.57	9	10	2	Jafari et al., 2009
Italy	12.56	41.91	9.3	1	0.45	Baronti et al., 2000
Japan	140.1	35.94	6.6	1	n.d.	Isobe et al., 2003
Kuwait	47.77	29.31	n.d.	6.6	25.6	Saeed et al., 2017
Laos	102.24	19.1	4.5	6.4	22.4	Duong et al., 2010
Malaysia	116.18	6.05	6.5	2.3	8.6	Duong et al., 2010
Mexico	-99.16	19.54	0.17	0.02	0.06	Gibson et al., 2007
Netherlands	5.29	52.58	7.2	1	0.4	Vethaak et al., 2005
Norway	2.88	63.12	50	0.25	0	Tollefsen et al., 2007
Portugal	-8.78	37.72	25.1	58.8	42.2	Rocha et al., 2016
South Africa	23.62	-33.95	1.06	0.135	n.a.	Farounbi & Ngqwala, 2020
South Korea	126.77	34.96	12.2	5.6	4.5	Duong et al., 2010
Spain	1.88	41.58	8.1	7.6	5	Petrovic et al., 2002
Taiwan	121.04	25	31.1	14.5	14.1	Chen et al., 2007
Thailand	105.47	15.28	26.1	7.5	10.4	Duong et al., 2010
Uruguay	-54.28	-34.68	n.d.	2.35	11.6	Griffero et al., 2019
USA	-70.99	42.33	0.5	0.09	0.09	Griffith et al., 2016
USA	-156.36	21.80	1.7	n.a.	n.a.	Atkinson et al., 2002
USA	-80.39	25.11	5.2	1.8	n.d.	Singh et al., 2010
USA	-90.23	29.82	4.7	4.5	n.d.	Zhang et al., 2007

Table A.2.: Correlation matrix of estrogens targeted in this study with pharmaceutical and personal care products analyzed in Fisch et al., 2021.

		GEN	ZEN	DAI	EE2	SMZ	SMX	SMT	SMP	MET	CBM	CAF	S
Estrone	E1	0.57	0.49	0.65	-0.67	0.60	0.09	0.66	-0.11	0.97*	0.78**	0.89*	-0.48
Genistein	GEN		0.09	0.88	0.00	0.39	-0.87	0.66	-0.78	0.69	0.87	0.81	-0.68
Zearalenone	ZEN			0.46	–***	-0.68	0.21	0.59	0.11	0.54	0.61	0.59	-0.68
Daidzein	DAI				0.00	0.68	0.36	0.78**	0.26	0.70**	0.79**	0.80*	-0.52
17α-ethinylestradiol	EE2					–***	–***	–***	–***	-0.63	–***	0.93*	-0.85**
Sulfamerazine	SMZ						-0.25	0.72	-0.33	0.56	0.67	0.61	-0.75
Sulfamethoxazole	SMX							0.71**	0.96*	0.01	0.48	0.19	-0.67
Sulfamethazine	SMT								0.70	0.65	0.94*	0.80*	-0.82*
Sulfamethoxypyridazine	SMP									-0.18	0.36	-0.02	-0.61
Metoprolol	MET										0.80*	0.96*	-0.56*
Carbamazepine	CBM											0.90*	-0.77**
Caffeine	CAF												-0.75*

* significant correlations with $p \leq 0.01$
** significant correlations with $p \leq 0.05$
*** not enough data points

Lebenslauf

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