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Original Article

Analysis of selected steroid hormones in sea of Marmara sediment samples by LC-ESI/MS-MS

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ABSTRACT

Background and Aims: Sediment is the general name given to the muddy structure located at the bottom of aquatic environments such as the sea. In our study, the amounts of steroid hormones were investigated in the sediment samples taken from the Marmara Sea. According to other studies, it has been determined that the excess of the hormone load in the sediments may be an indicator of human/animal sourced pollution, as well as the negative effects of the hormones mixed in the seas with the ecological cycle on the health of humans and animals.

Methods: In our study, 31 selected human/animal, plant, natural and synthetic hormone-steroids were studied using Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry (LC-ESI/MS-MS). Methanol and QuEChERS were used as extraction methods. Sediment samples were taken from a total of 27 points selected for sampling at the Marmara Sea.

Results: According to the results we found, the androgens: androsterone (24.50-1718.18 ng g^{-1}), testosterone (86.30-1600.32 ng g^{-1}); the estrogens: mestranol (33.73-228.32 ng g^{-1}), equilin (53.44-1232.53 ng g^{-1}); the progestagens; pregnenolone (37.50-374.76 ng g^{-1}), progesterone (39.96-405.60 ng g^{-1}); levonorgestrel (325.25 and 937.93 ng $g^{-}1$); the fecal sterols: cholestanone (57.57-1726.32 ng g^{-1}), coprostanol + epicoprostanol (51.43-1370.33 ng g^{-1}); and the plant sterol; campesterol (35.30-1859.90 ng g^{-1}) were the compounds detected.

Conclusion: Estrogens and progestogens are active components of birth control pills, and cholestanone and coprostanol + epicoprostanol are steroids that are indicative of human/animal pollution. Coprostanol + epicoprostanol and cholestanone, which are indicators of fecal pollution, were detected in all sediment samples. In our study, steroid hormones were detected for the first time in Sea of Marmara sediments and possible environmental risks were evaluated.

Keywords: Marmara Sea, sediment, LC-ESI/MS-MS, steroids, fecal sterols

INTRODUCTION

The Sea of Marmara is a channel between the Black Sea and the Mediterranean, along with the Bosphorus and Dardanelles Straits. The polluting materials are fed into the Sea of Marmara via water by a surface current from the Black Sea and a deep current from the Mediterranean (Kut, Topcuoglu, Esen, Küçükcezzar, & Güven, 2000). The Sea of Marmara forms a link between two large semi-enclosed basins, the Mediterranean and the Black Sea (Erel, 1992). The coastal area of the Sea of Marmara contains 87% of Turkey's entire coastal settlement population (Erel, 1997). Increasing industrial and domestic activities in the Marmara Region mainly affect the coastal and shelf areas of the Marmara Sea. The northern part of the Sea of Marmara is subject to increased human interventions compared to the southern part in the form of industrial (metal, medicine, food, chemical, textile) waste disposal, fishing, dredging, recreation, and port activities. It receives pollution not only from a variety of local land-related sources but also from the densely populated and industrialized Istanbul Metropolitan and maritime transport. Istanbul is the metropolitan region with the densest population and the highest industrialization rate in Turkey. It covers about 15% of Turkey's total population and 40% of its industrial activity (Orhon, Uslu, Meriç, Salihoğlu, & Filibeli 1994). For this reason, it makes the biggest contribution to various pollutions in the Sea of Marmara. In addition to industrial and domestic waste from Istanbul Metropolitan, dissolved and particulate impurities from the Danube are transported to the Bosphorus by coastal currents (Sur, Özsoy, & Ünlüata, 1994; Tuğrul & Polat, 1995). In the coastal areas of densely populated big cities, the anthropogenic component of the sediments predominates. Surface sediments become a source of nourishment

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for biological life, a transport agent for pollutants, and a sink for organic and inorganic sediments. At the same time, the Sea of Marmara is exposed to a very high level of pollution due to the spillage of not only domestic but also industrial wastes (Topçuoğlu, Kırbaşoğlu, & Yılmaz, 2004).

Nowadays, one of the most common environmental issues is water quality (Zhang & Chen 2014). Sediment analysis is also used to understand water quality and detect water pollution. Sediments play an important role in the fate of xenobiotics in aquatic environments. They reflect the existing water system and are used to detect the presence of insoluble contaminants after mixing with surface waters (Chapman, Wang, Janssen, Persoone, & Allen, 1998). One of the substance groups that cause the most pollution in the aquatic environment and are analyzed in sediments is endocrine-disrupting compounds (EDC). At the same time, EDCs are among the most important substances affecting the quality of water. Natural and synthetic hormones (estrogens, progestogens and androgens), phytosterols and some industrial chemical compounds form a group of pollutants called endocrine disruptors. The presence of EDCs in the environment poses a pollution threat due to their effects on ecology and human health (Gutendorf & Westendorf, 2001). Steroid hormones and sterols can cause pollution that affects not only aquatic organisms but also the entire ecosystem and humans through the food chain. Chemical compounds of anthropogenic origin are important factors of contamination in both water and sediments. This causes a potential ecotoxicological risk (Vargas et al., 2001).

Steroid hormones have lipophilic properties. Therefore, they tend to accumulate in solid formations such as sediment (Praveena, Kwan, & Aris, 2012). Aquatic steroids have become a public issue in recent years (Ying, Kookana, & Ru, 2002). Steroids have endocrine-disrupting effects on aquatic organisms, such as adversely affecting fertility, feminization and hermaphroditism, even at low concentrations (1 ng L-1) in target tissues (Fick, Lindberg, Tysklind, & Larsson 2010; Mills & Chichester, 2005; Zeilinger et al., 2009). In one study, it was shown that the presence of ethinylestradiol (5ng L-1) in water seriously affects the reproductive ability of zebrafish (Ryan & Vandenbergh, 2006). Natural and synthetic steroids have been widely detected in a variety of environmental matrices, including surface and groundwater, soil, and sediments (Bradley et al., 2009; Chang, Wan, & Hu, 2009; Liu et al., 2012).

Steroid hormones can be divided into five subgroups depending on their structural features: estrogens, androgens, progestagens, glucocorticoids and mineralocorticoids (Refsdal, 2000). Estrogens and progestogens are widely used as contraceptives and drugs due to their protective properties against various diseases. They are applied in hormone replacement therapy to be used in the treatment of hormonal disorders (Álvarez Sánchez, Capote, Jiménez, & Luque de Castro, 2008; Flor, Lucangioli, Contin, & Tripodi, 2010). Estrogens are primarily used as growth promoters and enhancers in contraception, management of menopausal and postmenopausal syndrome, physiological replacement, and the treatment of prostate cancer (Cleve et al., 2012). For this reason, it is also detected in treated sewage wastewater (Chang & Huang, 2010). Testosterone, androsterone, and many analogs of dihydrotestosterone are used as therapeutic and anabolic agents that promote muscle growth; however, they can cause growth retardation and precocious puberty in children (Lastair, Ood, Arrie, Agatell, & Remner, 1996). Androgens such as testosterone and trenbolone acetate are often preferred in cattle breeding to accelerate growth (Galbraith, 2002). Androgens are thought to be responsible for the masculinization of fish found in rivers where waste from paper mills is dumped (Drysdale & Bortone, 1989; Bortone & Cody, 1999). In studies of androgens, female mosquitofish's anal fin morphometrics modify with androstenedione (Jenkins et al., 2001).

Phytosterols are naturally found in oils, grains, vegetables, and fruits (Froehner, MacEno, & Martins, 2010). They are widely used in the human diet due to their hypocholesterolemic properties, so they have protective properties for cardiovascular diseases (Miettinen, Strandberg, & Gylling, 2000; Sullivan, Brooks, Tindale, Chapman, & Ahmed, 2010; Furtula et al., 2012). In addition, in vitro analyses have shown that phytosterol-rich macroalgae extracts have anti-inflammatory, antibacterial, antifungal, antiulcerative, and antitumor properties (Lopes, Sousa, Valentão, & Andrade, 2013). Furthermore, wastewater from the paper industry often contains high concentrations of phytosterols. One of these plant sterols, β-sitosterol, is considered to be one of the causes of reproductive dysfunction in fish (Maclatchy, Peters, Nickle, & Van Der Kraak, 1997; Orrego, Guchardi, Krause, & Holdway, 2010).

Corticosteroids are divided into glucocorticoids and mineralocorticoids. Drugs in both these corticosteroid groups are used in humans because they reduce inflammation, and suppress allergic reactions and immune system activity (Charman & Williams, 2003).

Fecal sterols, such as coprostanol and epicoprostanol are biomarkers of pollution of coastal areas and urban centers in temperate and tropical regions and result from the anaerobic microbial conversion of cholesterol in the gut of humans and animals (Martins, Fillmann, & Montone, 2007; Bull, Lockheart, Elhmmali, Roberts, & Evershed, 2002). Studies of cholesterol and its metabolites in human feces show cholesterol accounts for approximately 20% of the neutral sterol concentration in feces, coprostanol 65%, coprostanone 10%, and cholestanol + cholestanone + epicoprostanol approximately 5% (Jing, Grebenok, & Behmer, 2013).

A meticulous extraction technique followed by sensitive and selective analysis is required to understand the effect of steroids in the water-sediment system (Sadílek et al., 2016). Analysis of steroid hormones and sterols in sediment samples is usu-

ally performed by gas chromatography (GC-MS) tandem mass spectrometry (Biache & Philp, 2013; Sojinu, Sonibare, Ekundayo, & Zeng, 2012; Pisani et al., 2013; Birk, Dippold, Wiesenberg, & Glaser, 2012). However, very few papers are available using the LC-MS method. These studies were also carried out in river sediments (Matić, Grujić, Jauković, & Laušević, 2014, Matić Bujagić, Grujić, Jauković, & Laušević, 2016). There is also a study on the Golden Horn (Sea of Marmara, Turkey) Estuary sediment (Aydoğan & Yurdun, 2021).

Although up to 90% of solids are removed in wastewater treatment plants, some chemical compounds such as nitrogen, phosphorus, lead, EDCs, and steroid hormones, which are hydrophobic and resistant to biodegradation, are also found in effluent because they accumulate on small particles (Gutendorf & Westendorf, 2001; Dartan et al., 2022). The fact that steroid hormones found in wastewater treatment plant effluent waters are also found in drinking water inlet waters at the same rate indicates the necessity of advanced technology treatment systems for these endocrine disruptors (Yarahmadi et al., 2018).

The aim of this study is to analyze 31 selected human, animal and plant sterols and hormones in marine sediment samples using LC-MS/MS with the electrospray ionization technique.

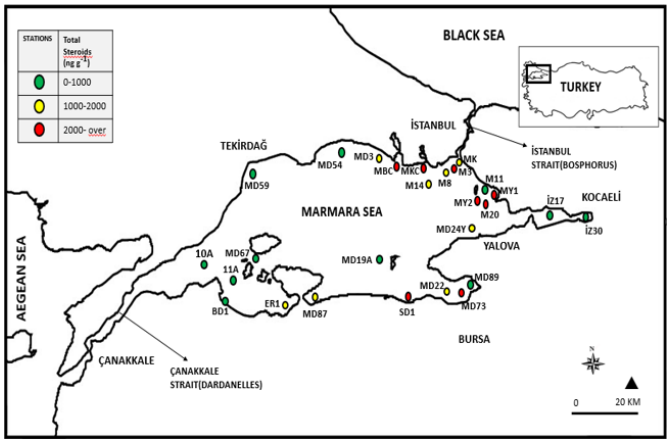

Figure 1. Sampling points in the Marmara Sea and steroid concentrations in sediment samples (ng g^{-1} dw)

MATERIALS AND METHODS

Chemicals and reagents

Depending on the frequency of use and detection in environmental samples, the hormones to be analyzed in the sediments were determined. In this study, a total of 31 steroid hormones and sterols were selected. Human and animal sterols: 17α– ethinylestradiol (Dr. Ehrenstorfer GmbH), estriol (Dr. Ehrenstorfer GmbH), estrone (Dr. Ehrenstorfer GmbH), levonorgestrel (Dr. Ehrenstorfer GmbH), mestranol (Cayman Chemical Company), norethindrone, equilin (Dr. Ehrenstorfer GmbH), 11-deoxycorticosterone, 11-deoxycortisol, 17α-OH-progesterone, 4-androstenedione, 17α-pregnenolone, aldosterone, androsterone, corticosterone, cortisol, dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEAs), dihydrotestosterone, 17β-estradiol, pregnenolone, progesterone, testosterone, cholesterol (Cayman Chemical Company), 5α-cholestan-3-one (cholestanone) (Alfa Aesar), 5β-cholestan-3β-ol (coprostanol) (Sigma-Aldrich), 5β-cholestan-3α-ol (epicoprostanol) (Sigma-Aldrich), 5α-cholestan-3β-ol (β-cholestanol) (Alfa Aesar); Plant sterols: desmosterol (Cayman Chemical Company), campesterol (Cayman Chemical Company), stigmasterol (Supelco). Standard materials, whose company names are not given, were included in a kit (JSM-CL-6500, Sem Laboratory Equipment Marketing Industry and Trade Inc., Turkey). HPLC grade methanol from Riedel-de-Haen and formic acid from Lachema cat.nr. 30587 (Czech Republic) were obtained.

The QuEChERS extract tubes were provided by Agilent Technologies (Massy, France). The extraction kit (QuEChERS extract salt packet 5982-6755 AOAC method, 2007) contained 6 g magnesium sulfate and 1.5 g sodium acetate. The clean-up kit (dispersive SPE 5982-5158 15 mL fatty samples AOAC) contained 1.2 g magnesium sulfate, 400 mg PSA, and 400 mg c18E. The steroid hormone and sterols' standard solutions were prepared at 1 mg mL^{-1} and 100 µg mL^{-1}. Methanol was used as a dilution solvent to prepare working standards and they were diluted to 1 µg mL^{-1} by mixing the appropriate amounts of the standard solutions. All samples were stored at -20 °C. The standard curves of the steroid hormones and sterols were linear in concentration ranges of 50, 100, 250 and 500 ng mL^{-1}.

Sample collection

Figure 1 shows the sampling points in the Sea of Marmara. Marmara Sea sediment samples were obtained from the Istanbul University Institute of Marine Sciences and Management.

Sample extraction

In our study, two different extraction methods were tried. The first is the extraction method with Methanol, the second is the extraction method with QuEChERS. Additionally, in our study, internal standards (IS) with deuterium were used to eliminate the Matrix effect.

Methanol extraction

Sample extraction was performed using 1 g of dry sediment samples. Five mL of methanol with 0.1% formic acid was added to the sediment, vortexed (LMS VTX-3000L 20W Harmony Mixer Uzusio) for 1 min. and sonicated (Elma Ultrasonic LC30) for 10 min. It was then centrifuged (Hettich Zentrifugen D-78532 Tuttlingen) at 2,000 x g for 10 min. The supernatant

was transferred to a conical glass tube. This process was repeated 2 more times and repeated three times in total and the extracts were combined. The clear solution collected in the glass tube was evaporated just to dryness in a 40 °C heater (Stuart SBH130D) under a gentle stream of nitrogen. The dry residues were dissolved using 50 µL acetone and vortexed for 1 min. Then, 950 µL of methanol was added and vortexed for 1 min again. It was centrifuged for 10 min at 2,000 x g. Then, the supernatant was transferred to the vial for injection into the LC-ESI-MS/MS.

QuEChERS extraction

The QuEChERS extraction method was performed according to the Phenomenex Applications note (TN-0096) (Estil et al.2016). In the QuEChERS extraction method, 1 g of dried sediment sample was taken, 10 mL of deionized water was placed on it and vortexed for 1 min. Then, 10 mL of 1% Acetic acid in Acetonitrile was added and vortexed for 1 min. Three point five grams of QuEChERS 5982-0755 salt was weighed and added to the falcon tube and vortexed again for 1 min. Since this extraction method was applied with 2 g of sediment, QuEChERS 5982-0755 salt was taken as 3.5 g instead of 7 g. After vortexing, it was centrifuged at 4,000 rpm for 5 minutes. It was left at -20 °C for 1 night. Approximately 9 mL of supernatant was taken from each sample and added to the ready QuEChERS 5982-5158 tube, vortexed for 1 min. It was then centrifuged at 3000 rpm for 10 minutes. Approximately 5 mL of supernatant was taken and placed in a tube, and the solvent was evaporated until dry under nitrogen flow at 35 °C using a hot heater. Then, 50 µL of acetone was added and vortexed for 1 min. Finally, 950 µL of methanol: water (1:1) was added, and vortexed for 1 min. It was centrifuged at 4,000 rpm for 10 min. After centrifugation, the supernatants were transferred to clean tubes, centrifuged again with Quickspin, transferred to vials and made ready for the LC-MS/MS analysis.

LC-ESI-MS/MS analysis

This method was made according to the work of Aydoğan & Yurdun, 2021. Analyses of sediments were performed on the Agilent Infinity 1290 HPLC system (Agilent Technologies, Santa Clara, CA, USA). The mass spectrometer settings of the kit (Sem Laboratory Equipment Marketing Industry and Trade Inc., Istanbul, Turkey) were as follows: drying gas flow 11 L min^{-1}, drying gas temperature 350 °C, sheath gas flow 11 L min^{-1}, sheath gas temperature 400 °C, nebulizer pressure 30 psi, capillary voltages were 5,500 and 3,000 V for positive and negative respectively with 500 V nozzle voltage for both of the polarities. Values of compound steroid mass spectrometer parameters and method performance parameters are the same as Aydoğan & Yurdun, 2021's work. Recovery results are shared in Table 2. Limits of detection (LODs) and quantification (LOQs) are shown in Table 3.

RESULTS

In the study, 27 of the Marmara Sea sediment samples were studied by methanol extraction and 21 of them were studied using QuEChERS Extraction, and both were analyzed by LC-MS/MS. The chromatogram of the steroid mix standard solutions is shown in Figure 2.

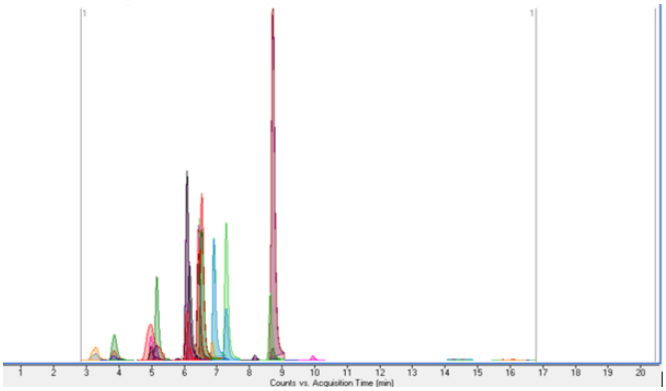

Figure 2. Chromatogram of steroid mix standards (for retention time and details, see Aydoğan & Yurdun, 2021).

In sediment samples taken from 27 points of the Marmara Sea, 31 thirty-one selected steroid hormones and sterols were analyzed with methanol extraction. The following compounds were detected: Androgens: androsterone (24.50-1718.18 ng g^{-1}), testosterone (86.30-1600.32 ng g^{-1}); estrogens: mestranol (33.73-228.32 ng g^{-1}), equilin (53.44-1232.53 ng g^{-1}); progestogens: pregnenolone (37.50-374.76 ng g^{-1}), progesterone (39.96-405.60 ng g^{-1}); levonorgestrel (325.25 and 937.93 ng g^{-1}); fecal sterols: cholestanone (57.57-1726.32 ng g^{-1}), coprostanol + epicoprostanol (51.43-1370.33 ng g^{-1}); plant sterol: campesterol (35.30-1859.90 ng g^{-1}). However, since cholesterol, cholestanol and stigmasterol could not be detected by ESI, analyses could not be made in the sediments. The percentage amounts of sterols in the sediments are shown in Figure 3.

The amounts of deoxycortisol, deoxycorticosterone, aldosterone, androstenedione, corticosterone, cortisol, desmosterol, DHEA, DHEAs, dihydrotestosterone, estradiol, estriol, estrone, norethindrone could not be determined because they were below the LOQ value. In the LC-MS/MS device, DHEAs was studied in the negative mode and all other steroid hormones and sterols were studied in the positive mode. The amounts of steroid hormones and sterols detected in sediment samples are shown in Table 1.

In methanol extraction, the recovery studies were prepared by adding 100 ng g^{-1} (62.5- 101.0) and 500 ng g^{-1} (58.3-

Table 1. Steroid concentrations in Marmara Sea sediment samples (extracting with QuEChERS) (ng g^{-1} dw)

Stations	Cholestanone	Androsterone	Equilin	Mestranol	Pregnenolone	Progesterone	Testosterone	Total Steroids
MD89	<LOQ	129.04	134.84	62.02	43.02	<LOQ	30.07	398.99
MD87	<LOQ	129.23	78.12	42.26	79.15	<LOQ	<LOQ	328.76
M11	78.32	111.59	<LOQ	<LOQ	<LOQ	<LOQ	502.81	692.72
MD73	106.11	504.81	87.82	<LOQ	<LOQ	78.41	375.99	1153.14
MD22	72.07	569.23	41.83	<LOQ	<LOQ	92.75	55.40	831.28
MD19A	67.13	108.72	<LOQ	<LOQ	<LOQ	25.83	280.52	482.20
MK	105.03	134.77	<LOQ	<LOQ	<LOQ	30.56	384.15	654.51
İZ-30	<LOQ	147.80	114.77	52.51	45.66	<LOQ	<LOQ	360.74
MD-3	84.75	365.21	<LOQ	<LOQ	<LOQ	59.81	207.43	717.20
SD1-3	<LOQ	134.55	115.98	133.85	42.18	<LOQ	31.13	457.69
M14	<LOQ	89.29	87.45	48.34	66.68	<LOQ	30.28	322.04
MD-8	120.95	113.52	<LOQ	<LOQ	<LOQ	61.55	174.27	470.29
MY1	<LOQ	102.15	414.45	84.15	845.65	<LOQ	32.83	1479.23
MD59	<LOQ	120.10	101.44	45.99	44.32	<LOQ	29.52	341.37
MKC-D	115.81	325.99	68.66	<LOQ	<LOQ	358.12	117.49	986.07
ER1	<LOQ	86.82	85.97	48.79	96.20	<LOQ	<LOQ	317.78
MD67	<LOQ	66.71	147.63	41.06	<LOQ	<LOQ	28.83	284.23
M3	<LOQ	147.14	82.33	61.05	<LOQ	<LOQ	32.41	322.93
MY2	<LOQ	99.61	202.07	52.32	<LOQ	<LOQ	29.91	383.91
MD20	86.38	136.96	65.46	<LOQ	<LOQ	51.91	187.43	528.14
MD72	<LOQ	79.02	83.22	45.56	<LOQ	<LOQ	<LOQ	207.80

112.4) of each standard solution to the sediment samples before extraction and they were left to dry at room temperature for one night. Then the extraction procedure was applied. For methanol extraction analyses, method performance parameters are the same as in the study by Aydoğan & Yurdun, 2021.

Recovery with QuEChERS extraction was studied by adding 100 and 500 ng g^{-1} steroids to the sediment with the above method, but the recovery results were low 31.8- 142.7 (100 ng g^{-1}), and 21.6- 155.1 (500 ng g^{-1}). Also, estriol, campesterol, DHEAs, coprostanol+epicoprostanol, desmosterol, and androsterone could not be detected (Table 3).

Results were found by using the parameters (steroids mass spectrometer parameters and method performance parameters) in our previous study on steroids (Aydoğan & Yurdun, 2021). Analyses of both studies were carried out at the same time.

DISCUSSION

In the published research, few studies have been found on the analysis of steroid hormones and steroids in the Golden Horn (Sea of Marmara, Turkey) Estuary sediment (Aydoğan & Yurdun, 2021; Lyons et al., 2015; Readman, Fillmann, Tolosa, Bartocci, & Mee, 2005; De Castro Martins, Montone, Carvalho Gamba, & Pellizari, 2005) and the studies are generally in river sediments (Chou & Liu, 2004; Matić et al., 2014; Matić Bujagić et al., 2016; Frena, Bataglion et al., 2016; Frena, Santos, et al., 2016; Hájková et al., 2007; Froehner, Martins, & Errera, 2009; López de Alda, Gil, Paz, & Barceló, 2002).

Overall, the general distribution of hormones in the Sea of Marmara sediment samples is as follows: Cholestanone> testosterone> androsterone> equilin> campesterol> coprastanol+ epicoprastanol> pregnenolone> progesterone> mestranol> levonorgestrel. The highest concentration of steroids was MY-1 (6479.34 ng g^{-1}), and the lowest concentration was 11A (310.80 ng g^{-1}).

Total steroid concentrations in the Sea of Marmara stations were determined in the range of 310.80-6479.34 ng g^{-1} (Table 1). The highest values were found at sediment sampling points MY-1 (6479.34 ng g^{-1}), M3 (4548.81 ng g^{-1}), SD1 (4273.99 ng g^{-1}), MKC-D (2368.53 ng g^{-1}), MD73 (2305.39 ng g^{-1}), M20 (2228.73 ng g^{-1}), MY2 (2051.00 ng g^{-1}) and MBC (2004.85 ng g^{-1}). Cholestanone, testosterone, androsterone, and equilin amounts were found high in the sediments. This is a strong indication that the pollution sources of Marmara Sea sediments are generally of human origin due to equilin, vegetable origin due to campesterol, and feces origin due to cholestanone (Figure 3).

Cholesterol, cholestanol, cholestanone, coprostanol, and epicoprostanol are sterols that are indicators of fecal contamina-

Table 2. Limits of detection (LODs) and quantification (LOQs) (Aydoğan& Yurdun, 2021) for QuEChERS extraction: recoveries at two concentration levels, method repeatability (relative standard deviations, RSDs)

Steroid Hormones/Sterols	Extracting with QuEChERS Recovery, % (RSD, %)		LOD ($ngmL^{-1}$)	LOQ ($ng\ mL^{-1}$)
	Spiking level 100 $ng\ g^{-1}$	Spiking level 500 $ng\ g^{-1}$		
Estrogens				
Estriol	n.d.	n.d.	10.91	36.35
17-α Ethinylestradiol	80.4 (4.4)	74.3 (11.1)	12.07	40.23
Estradiol	56.7 (10.9)	53.0 (12.2)	7.09	23.63
Estrone	33.1 (7.7)	47.8 (12.4)	12.23	40.77
Mestranol (Synthetic)	47.7 (4.9)	103.2 (2.3)	8.11	27.02
Equilin (Synthetic)	67.5 (7.8)	77.2 (5.9)	12.35	41.15
Androgens				
Androstenedione	54.5 (8.2)	52.7 (2.2)	11.91	39.72
Androsterone	n.d.	n.d.	6.35	21.16
Testosterone	53.5 (7.0)	51.3 (5.1)	8.61	28.71
DHEA	74.7 (14.8)	43.7 (7.8)	9.04	30.13
DHEAs	n.d.	n.d.	4.35	14.51
Dihydrotestosterone	142.7 (8.5)	153.6 (6.8)	4.67	15.55
Progestogens				
Pregnenolone	66.5 (5.3)	129.8 (7.7)	11.05	36.85
Progesterone	41.5 (3.6)	45.4 (9.9)	7.70	25.68
17-α-OH-pregnenolone	31.8 (4.9)	41.7 (12.1)	7.79	25.96
17-α-OH-progesterone	53.5 (8.9)	52.3 (7.6)	2.54	8.48
Levonorgestrel (Synthetic)	32.2 (1.7)	32.7 (6.5)	12.78	42.60
Norethindrone (Synthetic)	98.9 (4.5)	155.1 (3.2)	5.97	19.91
Fecal Sterols				
Coprostanol+ Epicoprostanol	n.d.	n.d.	9.41	31.37
Cholestanone	76.9 (3.3)	43.5 (1.9)	11.22	37.40
Plant sterols				
Campesterol	n.d.	n.d.	9.80	32.66
Desmosterol	n.d.	n.d.	9.24	30.80
Glucocorticoids				
Deoxycortisol	56.4 (7.0)	44.9 (15.2)	6.40	21.32
Cortisol	39.8 (11.3)	21.6 (16.1)	4.80	15.99
Mineralocorticoids				
Corticosterone	71.3 (8.3)	55.2 (8.3)	4.27	14.24
Deoxycorticosterone	50.2 (5.1)	51.7 (2.3)	11.94	39.80
Aldosterone	53.2 (9.7)	36.5 (7.2)	6.49	21.62

Table 3. Steroid concentrations in Marmara Sea sediment samples (extracting with methanol) (ng g^{-1} dw)

Stations	Cholestanone	Androsterone	Campesterol	Coprostanol+ Epicoprostanol	Equilin	Levonorgestrel	Mestranol	Pregnenolone	Progesterone	Testosterone	Total Steroids
11A	57.57	53.04	<LOQ	88.42	78.04	<LOQ	33.73	<LOQ	<LOQ	<LOQ	310.80
MD-89	308.76	184.42	51.70	132.40	61.15	<LOQ	137.11	43.32	<LOQ	<LOQ	918.85
MD-87	780.11	296.89	104.86	72.40	175.39	<LOQ	121.25	151.63	<LOQ	<LOQ	1702.52
10A	111.14	95.22	36.26	131.53	119.19	<LOQ	58.92	53.41	<LOQ	<LOQ	605.65
MBC	741.30	340.07	228.34	220.11	262.48	<LOQ	175.05	37.50	<LOQ	<LOQ	2004.85
M11	86.28	112.03	81.07	106.82	119.19	<LOQ	90.75	59.72	<LOQ	<LOQ	655.86
MD73	670.84	377.28	126.43	85.71	538.67	<LOQ	181.77	324.71	<LOQ	<LOQ	2305.39
MD22	704.10	345.07	88.48	<LOQ	165.60	<LOQ	228.32	374.76	<LOQ	<LOQ	1906.33
MD19A	119.55	85.49	<LOQ	156.67	126.01	<LOQ	63.06	191.11	<LOQ	<LOQ	741.89
MK	105.90	75.69	<LOQ	74.67	1232.53	<LOQ	210.70	197.44	<LOQ	<LOQ	1896.93
İZ-30	127.29	123.80	86.50	303.67	179.76	<LOQ	<LOQ	77.57	<LOQ	<LOQ	898.59
MD-3	522.64	263.24	319.90	278.53	82.13	<LOQ	191.95	41.84	<LOQ	<LOQ	1700.23
BD1	67.79	144.89	<LOQ	<LOQ	184.73	<LOQ	85.66	136.87	<LOQ	<LOQ	619.93
MD-54	186.29	188.89	58.07	88.25	259.94	<LOQ	63.25	<LOQ	<LOQ	<LOQ	844.69
SD1	787.82	346.69	1859.90	328.25	594.91	<LOQ	109.49	246.93	<LOQ	<LOQ	4273.99
M14	175.68	162.82	<LOQ	75.28	179.19	325.25	<LOQ	<LOQ	194.06	86.30	1198.57
M8	98.33	412.65	76.16	57.74	<LOQ	<LOQ	<LOQ	<LOQ	145.38	217.32	1007.58
MY1	1726.32	1718.18	<LOQ	1370.33	990.88	<LOQ	<LOQ	<LOQ	134.09	539.56	6479.34
MD59	71.79	51.14	<LOQ	51.43	<LOQ	<LOQ	<LOQ	<LOQ	39.96	588.09	802.40
MKC-D	279.17	209.52	128.93	184.94	<LOQ	937.93	<LOQ	<LOQ	405.60	222.44	2368.53
ER-1	258.34	138.94	93.07	133.30	195.20	<LOQ	<LOQ	<LOQ	117.63	280.67	1217.16
MD-24Y	234.12	128.07	151.61	168.49	53.44	<LOQ	<LOQ	<LOQ	131.66	458.43	1325.82
M20	87.36	136.10	45.79	203.23	57.14	<LOQ	<LOQ	<LOQ	98.79	1600.32	2228.73
MD-67	128.64	24.50	<LOQ	122.68	115.12	<LOQ	<LOQ	<LOQ	54.15	494.55	939.65
M3	1055.99	252.34	1468.91	142.72	<LOQ	<LOQ	<LOQ	<LOQ	195.32	1433.54	4548.81
İZ-17	74.78	152.14	<LOQ	104.89	156.81	<LOQ	<LOQ	<LOQ	114.23	334.76	937.60
MY2	194.59	56.67	35.30	118.40	<LOQ	<LOQ	<LOQ	<LOQ	124.99	1521.05	2050.10

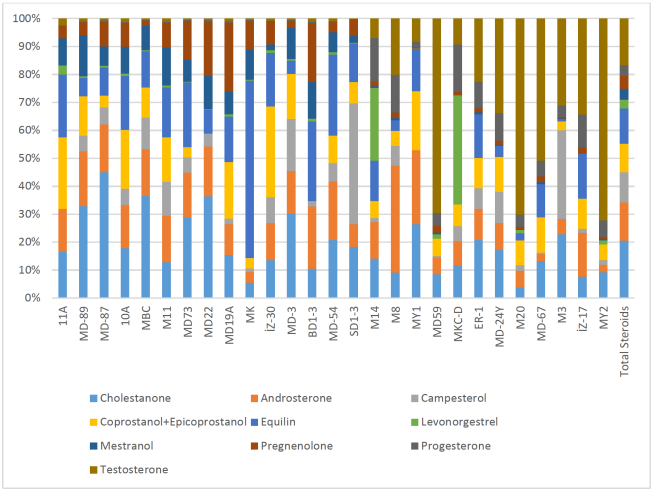

Figure 3. Distribution of steroid hormones in sediments of Sea of Marmara stations

tion. In our study, coprostanol and epicoprostanol results were given together because they could not be differentiated with the LC-ESI-MS/MS detector. Coprostanol + epicoprostanol 51.43-1370.33 ng g^{-1} and cholestanone 57.57-1726.32 ng g^{-1} were detected in sediment samples. Coprostanol + epicoprostanol concentrations in the sediment were found to be considerably higher in other studies (except Aydoğan &Yurdun, 2021's and Sojinu et al., 2012's) compared to our study. Results found by other researchers are as follows: 174- 4170 ng g^{-1} (Matić et al., 2014); 6.5- 1555 ng g^{-1}(Martins et al., 2007); 10- 2350 ng g-1 (Martins et al., 2011); 8.03- 465.54 ng g^{-1} (Sojinu et al., 2012); 34.37- 2603 ng g^{-1} (Lyons et al., 2015); and 42.82- 103.26 ng g^{-1} (Aydoğan &Yurdun,2021). Additionally, the cholestanone concentrations in our study (57.57-1726.32 ng g^{-1}) are higher than all of the other studies: Patos Lagoon sediments 6.9-172.2 ng g^{-1} (Martins et al., 2007), Danube River 79 and 899 ng g^{-1}(Matić et al., 2014), Niger Delta 2.55-771.58 ng g^{-1}(Sojinu et al., 2012) and the Golden Horn Estuary 157.57-1163.07 ng g^{-1} (Aydoğan &Yurdun, 2021). Also, the highest levels of cholesterol (37-16000 ng g^{-1}), coprostanol (12-440 ng g^{-1}) and cholestanol (37-1900 ng g^{-1}) were detected in sediment samples from the Bosphorus (Readman et al., 2005). Some authors emphasized that coprostanol levels between 10-100 ng g^{-1} are an indicator of uncontaminated environments, values greater than 100 ng g^{-1} are an indicator of sewage pollution in determining the pollution levels in the sediment, and they stated that 500 ng g^{-1} indicates meaningful sewage pollution. (Gonzalez-Oreja & Saiz-Salinas, 1998; Lyons et al., 2015; Tolosa, Mesa, & Alonso- Hernandez, 2014). In our study, the amount of coprostanol + epicoprostanol was found to be above 100 ng g^{-1} at 17 points (İZ17, 104.89 ng g^{-1}; M11, 106.82 ng g^{-1}; MY2, 118.40 ng g^{-1}; MD67, 122.68 ng g^{-1}; 10A, 131.53 ng g^{-1}; MD89, 132.40 ng g^{-1}; ER1, 133.30 ng g^{-1}; M3, 142.72 ng g^{-1}; MD19A, 156.67 ng g^{-1}; MD-24Y, 168.49 ng g^{-1}; MKC-D, 184.94 ng g-1; M20, 203.23 ng g^{-1}; MBC, 220.11 ng g^{-1}; MD3, 278.53 ng g-1; İZ30, 303.67 ng gCholesterol, cholestanol, cholestanone, coprostanol, and epicoprostanol are sterols that are indicators of fecal contamination. In our study, coprostanol and epicoprostanol results were given together be-

cause they could not be differentiated with the LC-ESI-MS/MS detector. Coprostanol + epicoprostanol 51.43- 1370.33 ng g^{-1} and cholestanone 57.57-1726.32 ng g^{-1} were detected in sediment samples. Coprostanol + epicoprostanol concentrations in the sediment were found to be considerably higher in other studies (except Aydoğan &Yurdun, 2021's and Sojinu et al., 2012's) compared to our study. Results found by other researchers are as follows: 174- 4170 ng g^{-1} (Matić et al., 2014); 6.5- 1555 ng g^{-1}(Martins et al., 2007); 10- 2350 ng g-1 (Martins et al., 2011); 8.03- 465.54 ng g^{-1} (Sojinu et al., 2012); 34.37- 2603 ng g^{-1} (Lyons et al., 2015); and 42.82- 103.26 ng g^{-1} (Aydoğan &Yurdun,2021). Additionally, the cholestanone concentrations in our study (57.57-1726.32 ng g^{-1}) are higher than all of the other studies: Patos Lagoon sediments 6.9-172.2 ng g^{-1} (Martins et al., 2007), Danube River 79 and 899 ng g^{-1}(Matić et al., 2014), Niger Delta 2.55-771.58 ng g^{-1}(Sojinu et al., 2012) and the Golden Horn Estuary 157.57-1163.07 ng g^{-1} (Aydoğan &Yurdun, 2021). Also, the highest levels of cholesterol (37-16000 ng g^{-1}), coprostanol (12-440 ng g^{-1}) and cholestanol (37-1900 ng g^{-1}) were detected in sediment samples from the Bosphorus (Readman et al., 2005). Some authors emphasized that coprostanol levels between 10-100 ng g^{-1} are an indicator of uncontaminated environments, values greater than 100 ng g^{-1} are an indicator of sewage pollution in determining the pollution levels in the sediment, and they stated that 500 ng g^{-1} indicates meaningful sewage pollution. (Gonzalez-Oreja & Saiz-Salinas, 1998; Lyons et al., 2015; Tolosa, Mesa, & Alonso- Hernandez, 2014). In our study, the amount of coprostanol + epicoprostanol was found to be above 100 ng g^{-1} at 17 points (İZ17, 104.89 ng g^{-1}; M11, 106.82 ng g^{-1}; MY2, 118.40 ng g^{-1}; MD67, 122.68 ng g^{-1}; 10A, 131.53 ng g^{-1}; MD89, 132.40 ng g^{-1}; ER1, 133.30 ng g^{-1}; M3, 142.72 ng g^{-1}; MD19A, 156.67 ng g^{-1}; MD-24Y, 168.49 ng g^{-1}; MKC-D, 184.94 ng g-1; M20, 203.23 ng g^{-1}; MBC, 220.11 ng g^{-1}; MD3, 278.53 ng g-1; İZ30, 303.67 ng g^{-1}1; SD1-3, 328.25 ng g^{-1}; MY1, 1370.33 ng g^{-1}1) and above 500 ng g^{-1} at only one point (MY1, 1370.33 ng g-1). According to these results, we can think that pollution is starting at the 16 points mentioned. At the highest point (MY1), maybe we can say that there is pollution.; SD1-3, 328.25 ng g^{-1}; MY1, 1370.33 ng g^{-1}1) and above 500 ng g^{-1} at only one point (MY1, 1370.33 ng g^{-1}). According to these results, we can think that pollution is starting at the 16 points mentioned. At the highest point (MY1), maybe we can say that there is pollution.

Another finding was that amounts of pregnenolone (37.50-374.76 ng g^{-1}) were found in all sediments. As far as we have researched, only one study (Aydoğan & Yurdun, 2021) of pregnenolone analysis has been conducted in sediment samples, and the result was 44.19-418.00 ng g^{-1}. For the first time in marine sediment research, sediment analysis was performed with this study and the result was obtained. Pregnenolone is the main steroid from which all other steroid hormones are formed. Pregnenolone is consideredto be a strong indicator of human-induced pollution because it is used as the main metabolite of cholesterol and cholesterol to pregnenolone conversion with cytochrome P-450 side chain cleavage enzyme, and therefore, it is recommended to perform pregnenolone analysis in sediment in similar studies to be carried out from now on. Since an ESI ion source is used in our study but an APCI ion source is required for cholesterol analysis, we think that pregnenolone analysis is meaningful, especially in cases where an ESI ion source is used.

Hormones are the most potent endocrine disruptors even at ng L^{-1} levels. The presence of progesterone in aquatic environments even at low levels (0.1-10 ng L^{-1}) has been linked with different steroidal effects in aquatic species (Díaz-Cruz et al., 2009). This is because they are able to interact with the endocrine system. As such, they interfere with reproductive, growth and development systems in both humans and animals. Some associated changes that have been slowly creeping into the wild fish populations include a reduction in fertility, changes in sex ratio (alteration of sexual development) incidence and inducing feminization. In the study by Mulabagal, Wilson, & Hayworth, 2017, the amount of progesterone found in the sediment was 2.91-22.3 pg g^{-1}. In another study conducted by Omar, Aris, Yusoff, & Mustafa, 2018, it was found to be between 0.7-5.34 ng g^{-1}. Lastly, in another study by Aydoğan & Yurdun, 2021, it was found to be between 1.59-6.03 ng g^{-1}. Considering these results, we can say that the amount of progesterone in our study was significantly higher than in other studies (39.96-405.60 ng g^{-1}). According to the values we found, we predict that some sea creatures in the Marmara Sea and its surroundings may experience negative effects such as feminization, masculinization, and damage to growth and development systems.

The levonorgestrel values in our study (325.25 and 937.93 ng g^{-1}) were found to be significantly higher than the results of previous analyses. The study performed by López de Alda et al., 2002 found it to be 0.05-2.18 ng g^{-1}, and Aydoğan & Yurdun, 2021 found it to be 1.55-7.78 ng g^{-1}. We think that the use of oral contraceptives may be more due to the dense population, a correlation exists between a dense population and the concentration of oral contraceptives released into the environment, and therefore the amount of levonorgestrel may have been found to be high.

To our knowledge, there are only three studies that have analyzed and detected mestranol (Aydoğan & Yurdun, 2021; Matić et al., 2014; Matić Bujagić et al., 2016). In the study by Matić et al., 2014, (Danube River), only one of six sediments (10 ng g^{-1}), and in the study by Matić Bujagić et al., 2016, only 2 of 11 sediments (Danube River and Topčiderka River) contain small amounts (11 ng g^{-1}, 19 ng g^{-1}). Also, mestranol was found in the study by Aydoğan & Yurdun, 2021 (82.34-335.82 ng g^{-1}). In our study, mestranol was found in all 27 marine sediments (33.73-228.32 ng g^{-1}). Mestranol is a synthetic steroid hor-

mone, a prodrug of ethinylestradiol, which enters the body as a result of its use as a contraceptive drug and is then excreted. Therefore, it is considered to be an indicator of estrogenic pollution.

As we researched, there is only one study detecting equilin in river or marine sediment (Aydoğan & Yurdun, 2021). In that study, it was determined as 54.46- 2201.00 ng^{-1}. In our study, 53.44-1232.53 ng g^{-1} of equilin was found in very high amounts in all 27 sediments. Equilin is a substance obtained from mares and produced synthetically and used for contraceptive purposes. It is thought that the amount of oral contraceptive use is high in this region, and therefore the high amount of equilin is a very strong indicator of the presence of human-induced pollution. The fact that the amount of equilin is high at MK, SD1, and MY1 points may make us think that estrogen-induced pollution, that is, human-induced pollution, is high at these points.

Androgenic steroids' excretion from the human body is via the urinary system. For this reason, they mix with the seas through the sewers and cause negative effects on the reproduction-development systems of sea creatures. At the same time, as a result of microbial degradation of paper mill wastes, progesterone and androstenedione are synthesized over converted to phytosterols. The most common phytosterols that undergo this conversion in the paper mill are sitosterol (72%), stigmastanol (11%) and campesterol (8%). According to one study (Jenkins, Wilson, Angus, Howell, & Kirk, 2003), the amount of androstenedione in the Fenholloway River sediment is 0.7±0.2 µg/L. In another study (Aydoğan & Yurdun, 2021), it was found to be 19.91-22.71 ng g^{-1}.

No previous androsterone and testosterone analyses have been found in marine sediments except in one study. In the study conducted by Aydoğan & Yurdun, 2021 in Haliç-Istanbul-Turkey, 72.66-467.56 ng g^{-1} androsterone, and 12.54-16.1 ng g^{-1} testosterone were detected. We also analyzed the amounts of androsterone, which are extremely high in our study (24.50-1718.18 ng g^{-1}). The amount of testosterone we found was extremely high too (86.30-1600.32 ng g^{-1}).

According to the study by Matić et al., 2014, campesterol amounts were 97-733 ng g^{-1}; in Matić Bujagić et al., 2016's study 52-1106 ng g^{-1} campesterol was detected; according to the study by Ali, Humrawali, & Latif, 2009, campesterol amounts were 0.98-14.70 µg g^{-1}; Aydoğan & Yurdun, 2021 found campesterol levels to be 143.90-1423.90 ng g^{-1}. In our study, similar results (35.30-1859,90 ng g^{-1}) were obtained.

In the QuEChERS extraction, sediment samples were studied with fewer samples than the samples studied with methanol extraction. As the recovery results were low using QuEChERS, some steroids could not be detected and the results obtained with methanol extraction are more significant. The results obtained using methanol extraction were taken into account in this study, as can be seen in Tables 1 and 2.

CONCLUSION

The amount of coprostanol + epicoprostanol was found to be above 100 ng g^{-1} at 17 points and above 500 ng g^{-1} at only one point. Based on this data, it can be considered that there is fecal pollution in the Marmara Sea. This is an indication that there is sewage pollution in the area. Moreover, we can say that there is no pollution between 0-1000 ng g^{-1}, there is medium pollution between 1000-2000 ng g^{-1}, and above 2000 ng g^{-1} there is pollution according to total steroid amounts. Accordingly, we can say that there is pollution in these sediment sampling points, MY^{-1} (6479.34 ng g^{-1}), M3 (4548.81 ng g^{-1}), SD1 (4273.99 ng g^{-1}), MKC-D (2368.53 ng g^{-1}), MD73 ($^{-1}$), M20 (2228.73 ng g^{-1}), MY2 (2051.00 ng g^{-1}) and MBC (2004.85 ng g^{-1}). In general, it can be said that there is pollution in the Marmara Sea according to the total amount of steroids. High levels of both total coprostanol + epicoprostanol and total steroid levels indicate that there is fecal and steroid pollution in the Marmara Sea. However, we think that studies should continue in order to reach a definitive conclusion.

Methanol and QuEChERS extraction method were used in the extraction of steroids. Significant results were obtained with methanol extraction in the analysis of steroids in the sediment samples. Because the recovery results were low in the QuEChERS extraction method and the data was better using methanol extraction. As seen in Table 1 and Table 2, the data obtained with methanol extraction was taken into account in the results of this study

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Conflict of Interest: The authors have no conflict of interest to declare.

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