Oceanological and Hydrobiological Studies

International Journal of Oceanography and Hydrobiology

Volume 45, Issue 4, December 2016
pages (605-614)

ISSN 1730-413X
eISSN 1897-3191

Screening of 17α-ethynylestradiol and non-steroidal anti-inflammatory pharmaceuticals accumulation in *Mytilus edulis trossulus* (Gould, 1890) collected from the Gulf of Gdańsk

by

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DOI: **10.1515/ohs-2016-0050**
Category: **Short communication**
Received: **April 11, 2016**
Accepted: **June 09, 2016**

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Abstract

Pharmaceuticals are commonly found in water collected from wastewater treatment plants, fresh water and saline water. Their presence in water may result in constant accumulation in living organisms, and consequently a negative impact on those organisms. Abiotic samples collected from the Gulf of Gdańsk were already proven to be polluted by several classes of pharmaceuticals, but the bioaccumulation was not investigated as far. This study presents the first results on the accumulation of pharmaceuticals in *Mytilus edulis trossulus* (Gould 1890) mussels collected from the Gulf of Gdańsk. The content of target 6 non-steroidal anti-inflammatory pharmaceuticals (ibuprofen, flurbiprofen, diclofenac, paracetamol, naproxen, ketoprofen) and 17α-ethynylestradiol in water and tissue samples was determined. The selected pharmaceuticals (paracetamol, flurbiprofen, 17α-ethynylestradiol) were found only in the largest individuals. The in situ BAFs calculated for paracetamol and flurbiprofen were 2850 and 16154 l kg^{-1} (dry weight), respectively, suggesting a high potential for the bioaccumulation of these compounds. The synthetic hormone 17α-ethynylestradiol was also found in the collected mussels at the concentration of 310 ng g^{-1} of dry weight. Generally, the examined mussels showed poor condition and it is highly likely that the accumulated pharmaceuticals are one of the important factors contributing to this.

Key words: pharmaceutical residues, pharmaceutical bioaccumulation, *Mytilus* sp., 17α-ethynylestradiol (EE2), non-steroidal anti-inflammatory drugs (NSAIDs)

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Introduction

Mytilus edulis is a widespread species in European waters and belongs to the key species of the Baltic Sea (Norling and Kautsky 2008). This results from the fact that mussels are very intense filtrators. *M. edulis* obtain organic matter suspended in the water (about 100 µm in diameter) by siphons. The particles may be inorganic, small living organisms, both phyto- and zooplankton, as well as bacteria, while charged substances could be dissolved in water or have a colloidal nature. Consequently, chemical substances could be accumulated in the mussel body. The rate of accumulation in young mussels is higher than in older animals with a larger body, while the amount of accumulated chemical compounds is usually higher in older individuals. Mussels can filter for up to 24 h per day (Riisgård et al. 2011). The rate of filtration is affected by the access to food, the condition of animals, physiological condition and the development stage of gametes (Pierscieniak et al. 2010). It was proven that filtration carried out by mussels contributes to water cleaning (Edebo et al. 2000; Newell 2004; Odd Lindahl et al. 2005), but it also contributes to the accumulation of pollutants in the mussel tissue (Pimiä et al. 1997). Both filter-feeding behavior and relatively long life cause greater exposure to toxic compounds as compared to other invertebrates, such as crustaceans. Several classes of pollutants have been proven to have high accumulation potential, such as PCB (PolyChlorinated Biphenyls), TBT (Tributyltin), PAH (PolyAromatic Hydrocarbons), heavy metals and pesticides (Dabrowska et al. 2013; Galassi et al. 2008; Widdows et al. 2002). Furthermore, toxic chemical compounds produced by algae are also accumulated by mussels (Strogyloudi et al. 2006; Svensen et al. 2005). This phenomenon means that mussels can be used as indicators of toxic compounds in the aquatic environment, which makes them a useful tool to monitor the water quality (for example Mussel Watch by the Marine Resources Committee). Obviously, the accumulated pollutants often have a negative impact on the condition and the growth rate of a mussel (Halldórsson et al. 2005; Widdows et al. 1997).

Pharmaceuticals are referred to as new emerging pollutants, because their presence has been investigated relatively recently and their impact on the environment is not fully explored. Non-steroidal anti-inflammatory drugs (NSAIDs) are some of the most often investigated and detected pharmaceuticals in water samples all over the world (Nödler et al. 2014). They are present in raw and treated wastewater (Nikolaou et al. 2007), surface water – rivers (Camacho-Muñoz et al. 2010), lakes (Li et al. 2010), estuaries (Lara-Martin et al. 2014), ground water (Sui et al. 2015) and finally in drinking water (Caban et al. 2015). This situation is connected with the high mass of marketed painkillers, especially those without prescriptions, incomplete metabolism in the human body and low efficiency of the removal of pharmaceuticals in conventional wastewater treatment plants (WWTP). It should be added that the consumption of pharmaceuticals, especially NSAIDs, in Poland is among the highest in the world (Willert 2007).

Pharmaceuticals are known to be absorbed by water organisms and to negatively affect their physiology and metabolism (Ericson et al. 2010; Oskarsson et al. 2014; Schmidt et al. 2011). Some negative effects include: a lower growth rate, an increase in the mortality of individuals, an increase in oxygen consumption and excretion. The effects of NSAIDs on a mussel as a non-target organism are different than well-known inflammatory effects on the human body (Gagne et al. 2005). In addition, they can be transferred upward through trophic levels (the process of biomagnification) (Oskarsson et al. 2014). The first assessments of the chronic effect of low doses of pharmaceuticals on mussels have been performed (Cleuvers 2003; Parolini et al. 2011). There is a risk that toxic substances negatively affect the byssus, hindering its adhesion (Lachance et al. 2008). Furthermore, the low salinity of the Baltic Sea makes the organisms living there highly sensitive to hazardous substances (Kautsky et al. 1997).

The biggest WWTP in Pomerania, discharging waters to the Gulf of Gdańsk, is Gdańsk-Wschód, and high concentrations (µg l^{-1}) of NSAIDs were determined during the last four years in raw and treated wastewater in this plant (Caban et al. 2014; Migowska et al. 2012). Furthermore, the same compounds were detected in seawater collected from the Gulf of Gdańsk, at a level of several dozen ng l^{-1} (Borecka et al. 2015; Pazdro et al. 2016). Bearing this information in mind as well as the fact that selected pharmaceuticals have the established potential for accumulation (Nikolaou et al. 2007), the occurrence of high concentrations of NSAIDs in organisms living in the Gulf of Gdańsk is highly possible.

To verify this statement, a screening test was conducted. As mentioned above, the test organisms of the *Mytilus edulis trossulus* species and water samples were collected from the part of the Gulf of Gdańsk, which was already proven to be polluted by pharmaceuticals. Subsequently, the samples were chemically analyzed to determine the presence of six NSAIDs (paracetamol, diclofenac, ibuprofen, flurbiprofen, ketoprofen, naproxen)

and EE2 (17α-ethynylestradiol, synthetic steroidal hormone) (Table 1) and to verify the bioaccumulation potential for target analytes. Bioaccumulation is normally presented using factors. The first one is the Bioaccumulation Factor (BAF), defined by the ratio of a contaminant in a given organism to its concentration in the environment in a state of equilibrium, where the organism can absorb a contaminant through the ingestion of food and the dissolved phase as well as through direct contact. The second one is the Bioconcentration Factor (BCF) – equivalent to BAF, but with no dietary intake of the contaminant, mostly investigated in laboratory conditions. In our study, BAF factors for target pharmaceuticals were calculated.

Materials and methods

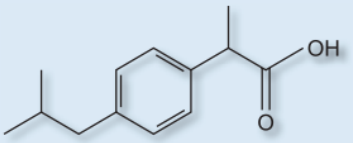
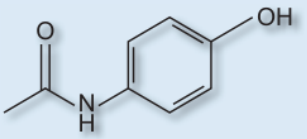
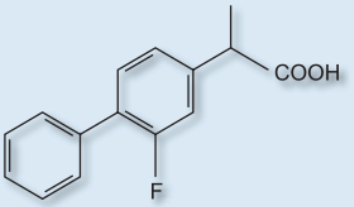
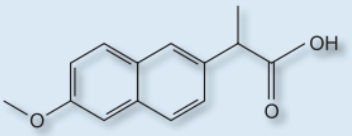
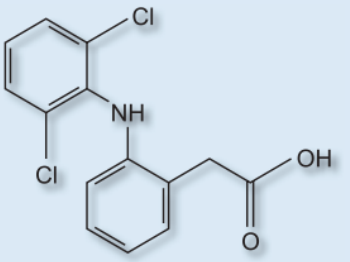
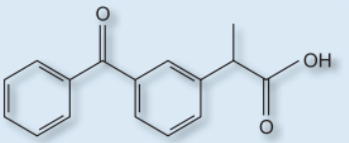
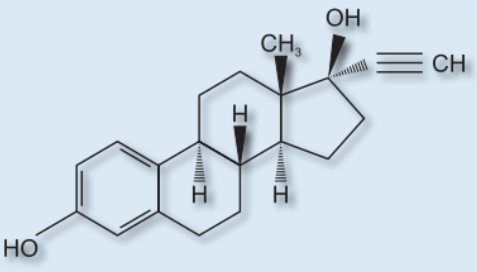
Organic solvents were purchased from Chempur (Piekary Śląskie, Poland), while standards (min. 98% of purity) from Sigma Aldrich (Germany).

Collection and characterization of water samples and organisms

Both water samples and mussels were collected from the Gulf of Gdańsk in October 2015. The geographical coordinates were 54°39′N 18°34′E. The sampling site is located in the outer part of Puck Bay (part of the Gulf of Gdańsk). This area can be impacted by

Table 1

Chemical structures and basic characteristics of target pharmaceuticals

Compound	Chemical structure	Compound	Chemical structure
Ibuprofen (IBU) M = 206.28 g mol^{-1} pKa = 4.91 log Kow = 3.97		Paracetamol (PAR) M = 151.16 g mol^{-1} pKa = 9.38 log Kow = 0.46	
Flurbiprofen (FLUR) M = 244.26 g mol^{-1} pKa = 4.22 log Kow = 4.11		Naproxen (NAP) M = 230.26 g mol^{-1} pKa = 4.15 log Kow = 3.18	
Diclofenac (DIC) M = 296.13 g mol^{-1} pKa = 4.15 log Kow = 4.51		Ketoprofen (KET) M = 254.28 g mol^{-1} pKa = 4.45 log Kow = 3.12	
17α-Ethynylestradiol (EE2) M = 269.4 g mol^{-1} pKa = 10.4 log Kow = 3.67			

the stream of pharmaceuticals from the mouth of a sewer "Dębogórze" (WWTP). Furthermore, brine waters are discharged near the sampling site. Both streams, treated wastewaters and brines, can affect the condition of mussels, while these waters contain a large load of organic matter and salts (Wolowicz & Sokolowski 2006). The organisms were collected at a depth of 12 m. The water temperature was 15.1°C, whereas the salinity was 7 PSU. Water samples were collected in glass bottles. Mussels were collected using a dredge. The lengths of individuals were measured with a Digmatic caliper (accuracy of 0.01 mm), and samples were divided into four groups: 1.1-2 cm, 2.1-3 cm, 3.1-4 cm, and 4.1-5 cm. Mussels were dried on filter paper and the bodies were dissected from the shell and weighed. The wet mass was determined using a Mettler Toledo balance XS 2005 Dual Range with an accuracy of 0.001 g. In order to determine the condition of the animal, the dry weight (W)/length (L) ratio of individuals was determined using Laglers' formula (Wolowicz & Sokolowski 2006), where a – the proportionality constant or intercept and b – the exponent. The prepared material was frozen at -80°C and then lyophilized using the Steris Lyovac GT2 lyophilizer to a constant weight.

Analytical protocol

The water samples were extracted using the solid-phase extraction technique (SPE). The 1500 ml samples were filtered and extracted using Disk H2O-Philic DVB speedisks (J.T. Baker), manufactured for the fast and efficient extraction of high-volume water samples. The washing step was done using hexane (15 ml), while elution using methanol (15 ml) (Caban et al. 2015). The extracts were then concentrated and transferred to chromatographic vials. The water samples were analyzed in duplicate.

The mussel tissue dry material was analyzed separately for each size category, starting with homogenization using a mortar. Then 1 g of material was extracted using pressurized liquid extraction performed by a Dionex ASE 200 extractor (1500 psi, 100°C) and the water:methanol mixture (1:1, v:v). The extracts (20 ml) were diluted using pure water to obtain 500 ml and cleaned up using the already described SPE procedure (using Strata-X, Phenomenex, 200 mg).

The internal standard 2-methylantracene was added to all extracts. After the evaporation of the solvent, the derivatizing reagent (BSTFA +1% TMCS) was added and the derivatization reaction took place in a hitting block (60°C) for 30 min. After the reaction and ambient cooling, the obtained samples were analyzed using a gas chromatograph coupled with a mass spectrometer (GC-MS). The SIM mode (selected ion monitoring) was used for the purpose of quantitative and qualitative analysis. All of the GC-MS parameters were previously optimized (Kumirska et al. 2015). The appropriate instrumental validation was done and the validation parameters were satisfied. The recovery experiment was also conducted using spiked samples and the recovery was min. 90% for the water samples and 60% for the solid samples for all target analytes. The method detection limits (MDLs) and the method quantification limits (MQLs) were in the range of 1 to 10 ng g^{-1} and 3 to 31 ng g^{-1} of dry weight tissue, respectively. MDLs and MQLs of pharmaceuticals in water samples were between 1-4 ng l^{-1} and 4-12 ng l^{-1}, respectively. The precision of the determination of pharmaceuticals in both water and solid samples was below 17%. The blank water samples did not contain analytes.

Determination of the bioaccumulation factor

The bioaccumulation factor (*BAF*) was calculated using the equation:

$$BAF = \frac{C_{org}}{C_w}; \qquad (\text{l kg}^{-1})$$

where: C_{org} is the concentration of the analyte in the mussel tissue sample (ng kg^{-1}), C_w is the concentration of the analyte in a water sample (ng l^{-1}). The BAF was calculated using wet and dry mass, because both types of BAF calculation were reported in the literature.

Results

Table 2 presents the numbers of sampled individuals of *Mytilus edulis trossulus* and the total wet and dry mass of the tissue material in each size category. The highest number of mussels was obtained for medium sized individuals, while the lowest (only 10) for the largest and oldest organisms. The highest content of water was obtained for the class of 3.1-4.0 cm.

To show the condition of the investigated individuals, the regression equation estimating the relation between the shell length (L) and the tissue dry weight (W) of *M. edulis trossulus* was calculated as $0.007L^{2.1304}$, ($r = 0.708$, $p<0.001$, $n= 365$). This results is comparable with the previous reports for this species. For example, in the period from February 1997 to February 1998, the relationship between the length

Table 2

Numbers and masses of sampled individuals of *Mytilus edulis trossulus*

Mussel size categories (cm)	Number of individuals	Total wet mass in size categories (g)	Total dry mass in size categories (g)	% of water
1.1 - 2.0	63	8.765	1.350	84.6
2.1 - 3.0	151	60.467	5.600	90.7
3.1 - 4.0	141	103.551	8.200	92.1
4.1 - 5	10	7.810	1.020	86.9
Total	365			

and the dry mass of individuals from the coastal waters was dry weight = $0.004L^{2.297}$ (r=0.730, p<0.001, n=426) and dry weight = $0.0002L^{1.726}$ (r=0.662, p<0.001, n=428) in Sopot and Gdańsk, respectively (Wolowicz & Sokolowski 2006).

The SPE-GC-MS(SIM) protocol was used for the analysis of water samples collected from the same place as mussels. Of the seven pharmaceuticals tested, the presence of three analytes was confirmed. PAR was found at a concentration of 28 ng l^{-1}, KET at 25 ng l^{-1}, while FLUR at 13 ng l^{-1}. The relative standard deviation between repetitions was about 10%. The residues of pharmaceuticals (PAR, FLUR, EE2) were detected only in the 4.1-5 cm size category and consequently, only the results for this class are presented in Table 3. The determined concentrations of pharmaceuticals are in the range of 80-310 ng g^{-1} of dry weight of mussel tissues.

The BAF can be calculated when the analyte is present both in the water and tissue samples. In the case of our study, BAFs values were calculated for PAR and FLUR. The BAF is preferably calculated using dry weight (U.S. EPA, 2000), thereby these values will be discussed.

Discussion

The determined concentration levels of pharmaceuticals in water samples were similar as in the previous experiments conducted by our department (Caban et al. 2014). PAR is one of the most frequently determined pharmaceuticals in wastewater and surface water in Poland (Caban et al. 2014; 2015), because of its common use in our population as a painkiller and an anti-inflammatory drug. In addition, PAR is the most polar compound and thus better soluble in water than other NSAIDs (Table 1). PAR as well as other NSAIDs can be accumulated in the sediments of estuaries (Stewart et al. 2014). This environment is a contact zone between fresh and saline water, and pollutants which enter with fresh water as soluble fractions can precipitate in a saline environment, because of the salting-out effect. PAR, despite low lipophilicity and low potential for accumulation, was determined at a high concentration in the tested mussel tissue. The high tendency of PAR to absorb was also reported for the fresh-water mussels *Dreissena polymorpha* in treated wastewater, in the study about the use of bivalves in the biofiltration process in wastewater management (Binelli et al. 2014) (other NSAIDs were also studied).

Other commonly used NSAIDs: IBU and DIC were not detected in our study, while these compounds were detected in different organisms (phytoplankton, zooplankton, mussels, snails, bivalves, common carp, lake anchovy, crucian carp) in Lake Taihu in China, as well as in water samples collected from this environment (Xie et al. 2015). IBU was detected at the highest concentration, i.e. about 100 ng l^{-1}. In mussels living in lakes, IBU was not detected, while BAF calculated for DIC was equal to 70 l kg^{-1} (Xie et al. 2015). In the case of FLUR, the presented study is the first one that investigated this pharmaceutical in seawater and mussels, thereby the results cannot be compared.

The presence of PAR in the tested mussel tissue is frightening, because of the fact that the cyto-genotoxicity of paracetamol on mussels has

Table 3

Concentrations of pharmaceuticals found in *Mytilus edulis trossulus* (ng per wet and dry mass of sample) and obtained BAFs

Mussel size categories	Found pharmaceuticals	Concentrations		BAF (using wet weight)	BAF (using dry weight)
		ng g^{-1} of dry weight	ng g^{-1} of wet weight	l kg^{-1}	
4.1-5 cm	PAR	80	10.5	373	2850
	FLUR	210	27.5	1718	16154
	EE2	310	40.4	-	-

been proven (Parolini et al. 2010). PAR and other NSAIDs – salicylic acid, were detected in *Mytilus edulis* which were deployed in a 6-week cage experiment performed at four stations in the Belgian coastal zone. PAR was detected in organisms collected from one sampling point at a concentration of 115 ng per g of dry weight, while salicylic acid in all samples at concentrations of 33-229 ng per g of dry weight. In our study, the concentration of PAR was 80 ng g^{-1} d.w., thus less than in the reference study. One study reported a lack of DIC in mussels, while it was frequently measured in seawater (the case of a year-long study in Ireland) (McEneff et al. 2014).

EE2 is an estrogen responsible for the disruption of the serotonin receptor and cyclooxygenase mRNA expression in *Mytilus edulis* (Cubero-Leon et al. 2010) and can reduce the secondary sexual characteristics of exposed organisms even at a low concentration (ng l^{-1}) by mimicking the natural analogue (McEneff et al. 2013). EE2 was not found in water samples, while it existed in the tested tissues. This can be explained by the fact that this compound has lipophilic properties and it can be accumulated in a water body in a solid suspended fraction, rich in organic matter. The presence of EE2 in mussels collected from the Gulf of Gdańsk was previously reported – the concentration was about 1 ng g^{-1} of wet weight (Hallmann et al. 2016).

The calculated BAFs can be compared to the categorization criteria of different protocols and organizations (Table 4).

Bearing in mind only a log K_{ow} parameter, only FLUR and DIC can be defined as bioaccumulative (according to U.S. EPA 1996, U.S. EPA 2010). Based on BAFs calculated in our study using a wet weight, it can be stated that PAR and FLUR are not bioaccumulative in *Mytilus edulis*. On the other hand, BAFs calculated using a dry weight (preferred by U.S. EPA) indicated FLUR (BAF 16154 l kg^{-1}) and PAR (2850 l kg^{-1}) as bioaccumulative. Considering the REACH regulations (Regulation of the European Parliament and Council Regulation EC No. 1907/2006, "Registration, Evaluation and Authorisation of Chemicals"), FLUR would be a very bioaccumulative substance. However, REACH specified the BCF factor measured in the laboratory, not BAF.

In the case of EE2, the concentration in mussels was high, but because it was not found in water samples, BAF cannot be calculated for this compound. In the study by Pojana et al. (2007), which tested endocrine disrupting the compounds in freshwater mussels, BCF values for EE2 ranged from 1300 to 1500 l kg^{-1} (Pojana et al. 2007). This means that this synthetic estrogen has a very high potential for bioaccumulation.

The measured concentrations of target pharmaceuticals can potentially affect the condition of mussels. In the work of Ericson et al. (2010), *Mytilus edulis trossulus* mussels were exposed to diclofenac, ibuprofen and propranolol at a concentration ranging from 1 to 1000 µg l^{-1}, and the scope for growth (SFG) was lower than in the control. In addition, mussels had lower byssus strength and lower abundance of byssus threads resulting in a reduced ability to attach to the underlying substrate. Mixtures of pharmaceuticals were also tested and a synergistic effect was observed. Another NSAID, i.e. IBU was proved to induce antioxidant stress and endocrine disruption in the *Mytilus galloprovincialis* mussel (Gonzalez-Rey and Bebianno 2012). However, it should be noted that concentrations of pharmaceuticals measured in water in this study were well below the reference values.

It was reported that DIC at a concentration often found in water can induce tissue-specific biomarker responses in *Mytilus galloprovincialis* (Gonzalez-Rey and Bebianno 2014). *Mytilus* mussels are sensitive to low pharmaceutical concentrations in their surrounding environment, therefore they can be used

Table 4

Guidelines for bioaccumulation categorization (review in Arnot et al. 2006)

Regulatory program	Criteria	Categorization	Reference
Canadian Environmental Program Act (1999)	BAF or BCF ≥ 5000 l kg^{-1} or log K_{ow} > 5.0	Bioaccumulative	Government of Canada 1999
United Nations Environmental Programme Stockholm Convention (2001)		Bioaccumulative	UNEP 2001
Washington State Department of Ecology (2004)		Bioaccumulative	Washington State Department of Ecology 2004
Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) is a European Union (2007)	BCF > 2000 l kg^{-1}	Bioaccumulative	REACH 2007
	BCF > 5000 v	Very bioaccumulative	
US EPA (1996)	BAF > 2000 l kg^{-1}, or log K_{ow} > 4	Bioaccumulative	United States Environmental Protection Agency 1996

to assess the impact of treated wastewater discharged into natural water. For example, significant antioxidant, genotoxic and lysosomal responses were closely observed in *Mytilus trossulus* caged in the Gulf of Finland at the point of the WWTP discharge (Dabrowska et al. 2013). In the same study, NSAIDs were determined in water at a concentration 12-39 ng l^{-1}. This concentration is similar to that determined in our study. Similar studies were performed using freshwater mussels (Binelli et al. 2014; Gagné et al. 2012).

It should be added that metabolites of pharmaceuticals in mussel tissues were not determined. It is known that the same path of metabolism for pharmaceuticals occur in both human and mussel bodies, and glucuronide, sulfate conjugated and hydroxy metabolites of NSAIDS can be found in water organisms (Kallio et al. 2010; Netherton 2011).

Conclusion

The preliminary screening of six non-steroidal anti-inflammatory pharmaceuticals (ibuprofen, ketoprofen, diclofenac, paracetamol, flurbiprofen, naproxen) and synthetic hormone 17α-ethynylestradiol in *Mytilus edulis trossulus* (Gould, 1890) collected from the Gulf of Gdańsk provides the first information about the bioaccumulation of target, new emerging pollutants in this region. KET, FLUR and PAR were detected in water samples (several ng l^{-1}). The presence of PAR, FLUR and EE2 was confirmed in mussel tissues, but only in the largest individuals.

The presence of EE2 in the tested mussels is frightening, especially when this information is coupled with fact that much more anthropogenic substances can be found in mussels living in coastal areas of Europe (Álvarez-Muñoz et al. 2015, Olenycz et al. 2015). Mixtures of chemical compounds with the most endocrine-disrupting potential most likely have a negative impact on a water organism. The *Mytilus edulis* mussels already suffer from numerous pathologies, like "gill disease", the cause of which remains unknown (Smolarz et al. 2006). They are also affected by a large load of heavy metals from the Vistula River, which makes the Gulf of Gdańsk one of the most polluted regions of the Baltic Sea (Rainbow et al. 2000). The biodiversity of this region is already low, thereby special attention should be taken to investigate the factors that can worsen the situation. Due to the fact that mussels live near the bottom of the sea and have contact with sediments rich in different hazardous substances, their condition is disturbed by the mixture of pollutants. The need for monitoring of NSAIDs and estrogens in the Baltic Sea is supported by several organizations. HELCOM (Helsinki Commission – Baltic Marine Environment Protection Commission) indicated pharmaceuticals as hazardous substances, the knowledge about which is unfortunately still limited (HELCOM Helsinki Commission, 2010). In Europe, the Water Framework Directive (WFD; Directive 2000/60/EC) and the following Directive 2013/39/EU introduced the monitoring of three pharmaceutical compounds: diclofenac and two hormones (17α-ethinylestradiol and 17β-estradiol), which were placed on a watch list of emerging pollutants.

The presented preliminary study (one study site, single samples and organisms) provides the first information that the bioaccumulation of pharmaceuticals in the Baltic mussels is highly possible. More reasonable conclusions can be presented when further data become available. To conclude, there is a strong need for further research and comprehensive assessment of the impact of pharmaceuticals on *Mytilus edulis* as an important species in the Baltic Sea.

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