Effect of 17 α -Ethinylestradiol on the Time Needed for Males and Females of *Gammarus tigrinus* Sexton, 1939 to Re-couple

Monika Wisniewska and Anna Szaniawska

Department of Experimental Ecology of Marine Organisms, Institute of Oceanography, University of Gdansk, Gdynia 81-378, Poland

Abstract: The aim of presented study was to determine the behavioural response of precopulatory pairs of *Gammarus tigrinus* exposed to the 17 α -ethinylestradiol (EE2). It was hypothesized that 17 α -ethinylestradiol would increase the time needed for a male to re-capture a female. Hypothesis was tested in a laboratory with 20 precopula pairs exposed to different treatment conditions. Paired animals were exposed to two different compound concentrations of 50 ng·L⁻¹ and 500 ng·L⁻¹. The control and solvent control was artificial sea water and artificial sea water with ethanol (the diluent for EE2). The couples were tested several times under different experimental treatments. The obtained results indicate that EE2 affects the precopulatory mate guarding behaviour of *Gammarus tigrinus*. EE2 in concentration of 50 ng·L⁻¹ and 500 ng·L⁻¹ prolonged the time needed for re-pairing. However, relative to the controls only EE2 in concentration of 500 ng·L⁻¹ significantly prolonged the time needed for male-female re-coupling. In summary, EE2 affects the reproductive behaviour of *Gammarus tigrinus*.

Key words: *Gammarus tigrinus*, 17 α -ethinylestradiol, reproductive behavior, re-pairing.

1. Introduction

The last decades have brought a growing interest in environmental Endocrine Disruptor Compounds (EDC) known as substances that may interfere with the body's endocrine system and produce adverse developmental, reproductive, behavioural, neurological and immune effects in humans and populations of other organisms worldwide [1]. Endocrine disruption may be caused by a wide range of natural and anthropogenic substances, including xenoestrogens that imitate estrogen, 17 α -ethinylestradiol (EE2).

Estrogens are sex hormones occurring in the environment. They are exuded by humans and animals with urine [2] in varying amounts that depend on the age, gender and reproduction level of organisms [3]. Estrogens are not completely eliminated in the water treatment process [4]. They are present in aquatic environment in sediments, groundwater, surface water,

waste water and even drinking water [5].

17 α -ethinylestradiol is a synthetic compound, which is commonly used as the main component of oral contraceptive formulations and hormone replacement therapies [6]. It may be exposed to bioaccumulation and biomagnification processes [7]. EE2 can affect different groups of organisms, amphipods, chironomids, cladocerans and copepods [8]. It was found that EE2 can induce changes in the reproductive behaviour, the population size and the sex ratio in amphipod *Gammarus pulex* [9, 10]. It also disturbs maturation of the germ cell and spermatogenesis, impairs the development, induces the hermaphroditism and changes in the length of antennae 1 and 2, and gnathopod 2 in *Hyaella azteca*, EE2 may cause mouthpart deformities and accelerated development of chironomid *Chironomus riparius* [11, 12]. In the case of Copepoda, EE2 inhibits naupliar development of *Tisbe battagliai* [13]. The concentration of EE2 in the Baltic waters varies depending on the region. Samples collected from sites located in the inner coastal waters

---

Corresponding author: Monika Wisniewska, master, main research field: experimental ecology of marine invertebrates. E-mail: ocemwi@ug.edu.pl.

showed higher estrogenic activities compared to those collected at sites located in the outer coastal waters [14]. Data from the German coastal waters show that EE2 was 6 ng·L⁻¹ (in inner Wismar bay), while the 17 β -estradiol (E2) was recorded with the concentration below 0.2 ng·L⁻¹ [15]. Unfortunately, there is no information published on EE2 concentration from the other parts of the Baltic Sea. Furthermore, there has been little interest in determining the effect of this hormone on Baltic crustaceans.

To examine the impact of 17 α -ethinylestradiol, tests were performed on the *Gammarus tigrinus*—an alien species in European waters. The *Gammarus tigrinus* occurs both in marine and freshwater ecosystems throughout the world, including the Baltic Sea [16, 17]. *Gammarus tigrinus* is a bottom-dwelling omnivore, therefore, it is relatively tolerant to pollution. *Gammarus tigrinus* has a specific reproduction behaviour and great reproductive capacity [18, 19]. In the Gulf of Gdansk, the population of the *Gammarus tigrinus* is stable and sometimes replaces native species: *Gammarus salinus*, *Gammarus zaddachi* and *Gammarus duebeni* in areas of their distribution [20].

Reproductive behaviour of amphipoda directly precedes the act of copulation, hence, they affect the reproductive success and the probability of offspring, which in turn affects the probability of population survival. Gammaridae have a specific reproductive behaviour—the precopulatory guarding time when a male is carrying a female. When a male establishes a physical contact with a sexually attractive female, he places her lengthwise beneath his body in the position of the precopulatory amplexus [21]. The male can swim whilst carrying the female [22]. The precopula time precedes the external fertilization which can occur only immediately after female moulting [23]. Prevention or disruption of precopulatory guarding can be a sensitive indicator of environmental stress [24]. Previous studies demonstrate that changes in the precopulatory mate guarding behaviour of gammaridae

are correlated with the concentration of a pollutant to which they are exposed [10]. These studies were carried out on freshwater gammaridae—*Gammarus pulex*. There is no information about the response of euryhaline species, thus, the main objective of this work was to determine the effect of the synthetic estrogen on the reproductive behaviour of *Gammarus tigrinus* Sexton, 1939. For this purpose, it was examined whether forcibly separated precopula specimens of the *G. tigrinus* will re-couple under the temporary influence of 17 α -ethinylestradiol. This in turn will help to determine whether the re-capture of precopula animals can be related to the concentration of a pollutant.

2. Material and Methods

2.1 Test Organisms

Specimens of *Gammarus tigrinus* used in the study were obtained from the Gulf of Gdansk (the southern Baltic Sea) from a depth of 1 meter, between May and July of 2013. Acclimatization to experimental conditions was established as follows: an initial two-week interval during which animals were put into a container provided with small mussel shells for shelter and water at a temperature of 19 °C, salinity of 7 PSU and constant oxygenation about 100%. The light regime of 16 hours of light followed by 8 hours of darkness was applied. During the acclimatization process, the animals were fed three times a week on soft tissue (*Mytilus edulis trossulus*) and green algae (*Enteromorpha* sp. or *Cladophora* sp.).

2.2 Stock Solution

The stock solution of 17 α -ethinylestradiol (Sigma Aldrich, product E4876) used for the experiment was prepared by dissolving 0.5 mg of EE2 per litre of 96% ethanol (Chempur Poland, product 423964203). Experimental solutions were prepared by dilution of stock solutions using artificial sea water.

2.3 Experimental Design

In the experiment, 20 paired males and

non-egg-bearing females of *Gammarus tigrinus* were used. Specimens were of a similar length depending on sex: 8 ± 0.5 mm for males and 6 ± 0.5 mm for females. The organisms were fed for the last time one day before the exposure. They were exposed to the control containing artificial sea water, the solvent control containing artificial sea water with ethanol (the diluent for EE2), EE2 in the concentration of $50 \text{ ng}\cdot\text{L}^{-1}$ (the highest recorded dose in the environment [25]) and $500 \text{ ng}\cdot\text{L}^{-1}$ (the laboratory dose, about 1/10,000 value of EE2 $\text{LC}_{48-50 \text{ h}}$). The final content of ethanol in the solvent control and in the EE2 treatments was 0.001% of the water volume. Before the exposure, the paired animals were separated by leaving them on a paper towel until the male releases the female, and then put into an appropriate container. The time needed for re-attachment was measured. Each pair was examined four times under different experimental treatments. When the couple did not re-attach during 15 min of observation, the results were not included in the calculation. Time of 15 min was the limit time for animals response. There were 20 repetitions for each concentration.

2.4 Statistical Analysis

The concentration-response relationship was analysed using ANOVA test at a significance level of $p < 0.05$. The differences in the time required for re-coupling in different concentrations were analysed using the non-parametric Wilcoxon test for dependent samples at a significance level of $p < 0.05$. All analyses were performed using STATISTICA 10 (Statsoft Poland).

3. Results

Based on the results, the data can be divided into

two groups depending on the range of the minimum and maximum reaction time (Table 1). The first group represents the control and solvent control, the second group represents the treatment group (treatment with EE2 in the concentration of $50 \text{ ng}\cdot\text{L}^{-1}$ and $500 \text{ ng}\cdot\text{L}^{-1}$). In total, 75% of the results for the controls were in the range of 0 sec to 100 sec. In contrast, 75% of the results for samples treated with the $50 \text{ ng}\cdot\text{L}^{-1}$ EE concentration are between 2 sec and 150 sec and for the $500 \text{ ng}\cdot\text{L}^{-1}$ EE2 concentration between 50 sec and 300 sec.

The average times needed for the animals to re-couple (Fig. 1) were 50 sec and 65 sec for the control and solvent control, compared to 114 sec and 168 sec with hormone treatment of $50 \text{ ng}\cdot\text{L}^{-1}$ and $500 \text{ ng}\cdot\text{L}^{-1}$ concentration respectively. The concentration-response comparison showed a statistically significant difference (ANOVA test with $F_{3,71} = 5.7$, $p < 0.005$). This result indicates that the reaction time increases with an increasing concentration of EE2.

The Wilcoxon signed-rank test indicates significant differences ($p < 0.05$) in the reaction time between animals treated with control conditions (control and solvent control) and with a concentration of $500 \text{ ng}\cdot\text{L}^{-1}$ EE2 ($p = 0.00084$ and $p = 0.00649$, respectively) and also a significant difference between animals treated with control conditions and a concentration of $50 \text{ ng}\cdot\text{L}^{-1}$ EE2 ($p = 0.03858$).

Moreover, the number of animals' pairs with the response time longer than in the control was checked (Fig. 2). Relative to the control, the reaction time for animals treated with solvent control conditions and a concentration of $50 \text{ ng}\cdot\text{L}^{-1}$ EE2 was longer in about 70% of the exposed pairs. This data shows that ethanol is masking out the difference. It also can be a reason why there were not statistical difference in

Table 1 The minimum and maximum time of animals re-attachment in different experimental concentrations.

Treatment conditions	Minimum (s)	Maximum (s)
Control	2	133
Solvent control	1	172
$50 \text{ ng EE2}\cdot\text{L}^{-1}$	2	443
$500 \text{ ng EE2}\cdot\text{L}^{-1}$	6	445

Effect of 17 α -Ethinylestradiol on the Time Needed for Males and Females of *Gammarus tigrinus* Sexton, 1939 to Re-couple

Fig. 1 Effects of the different EE2 concentrations on the time needed by males and females of *Gammarus tigrinus* to re-pair (the figure shows the mean reaction time (bar) $\pm$ SD, the numbers of pairs in each concentration are indicated at the base of the bars, *—statistically significant differences with $p < 0.05$ (Wilcoxon signed-rank test)).

Fig. 2 Differences in the reaction time of the *Gammarus tigrinus* in the various concentrations of EE2 (the figure shows the percent of pairs (bar) that indicated longer or shorter time needed to re-attachment in comparison with the control, the number of pairs used in each concentration was 20, *—statistically significant differences between concentration of 500 ng·L⁻¹ and other treatment conditions with $p < 0.05$ (other test of significance)).

re-coupling time between animals exposed to solvent control and concentration of 50 ng·L⁻¹. However, the effect of EE2 is apparent at the higher concentration because difference is amplified to a point where ethanol can't mask it.

4. Discussion

The behavioural response is frequently used as a tool in the ecotoxicological tests. Changes in the behaviour may indicate a change in the environmental conditions [26]. The effect of contamination on the animal behaviour can be reflected in the impact on the locomotion [27], feeding, ventilation [28] and reproductive activity like precopula mating and separation [9]. The precopulatory behaviours have a considerable ecological significance because they are crucial for the mating success [29].

In the international literature, there are many references concerning the impact of various natural and anthropogenic substances on the behaviour of the common freshwater shrimp *Gammarus pulex* [30-34]. *Gammarus tigrinus* is a common species in the Baltic Sea, therefore, it was used in this study to determine the effect of the chemical stressor, i.e. 17 α -ethinylestradiol, on the reproductive behaviour.

The right choice of research methods has been verified during the preliminary studies [35]. In terms of the reproductive behaviour, so far, the time required for the separation of organisms under the influence of a stress factor has been researched [9]. This study was an attempt to verify the response of the precopula *Gammarus tigrinus* during the exposure to EE2. The time required for re-coupling of precopula individuals can be considered as a measure of the behavioural response of an organism to a stressor.

In this study, the significant effect of EE2 on the precopulatory guarding behaviour of *Gammarus tigrinus* was observed. Compared to the both controls, the presence of estrogen in the water at the concentration of 500 ng·L⁻¹ increases the time needed for a male to re-capture a female. Based on the lack of

statistically significant differences between the solvent control and the concentration of 50 ng·L⁻¹, it can be assumed that ethanol also affect the behaviour to a small extent. Watts et al. [9] conducted a similar research on *Gammarus pulex*. They checked the Indirect Separation Time (IST50) and the Re-pairing Time (RT50) under EE2. They exposed animals to this compound prior to measurements of the effect exerted by the stressor on the precopulatory behaviour. In this study, the specimens were not acclimatized to EE2 before exposure. The *Gammarus tigrinus* re-pairing success was greater than 90% at all treatment concentration, while the re-attachment of *Gammarus pulex* after 4 hours of separation was > 70% at all treatment concentrations [9]. Despite no prior exposure, the significant effect of EE2 was observed for the concentration in ng·L⁻¹, while Watts et al. [9] indicate a significant effect on RT50 for EE2 in the concentration higher than 1 mg·L⁻¹.

Based on the similar number of pairs exhibited prolonged re-capture time in 50 ng·L⁻¹ and in solvent control, it can be concluded that changes in the response of animals are mostly caused by ethanol not by the hormone. Therefore, ethanol from the solvent control was also a stressor for precopula pairs of *Gammarus tigrinus*. The prolonged re-capture time in 19 out of 20 pairs exposed to EE2 concentration of 500 ng·L⁻¹ may indicate that this concentration has a significant effect on the organisms, much stronger than the concentration occurring in the natural environment.

To sum up, the time needed for re-coupling increased with the increasing concentration of 17 α -ethinylestradiol in water, so, this hormone affects the reproductive behaviour of *Gammarus tigrinus*. Disorders in reproductive behaviours may lead to changes in the likelihood of fertilization and, consequently, the production of a new generation. Not only the time needed for the separation of precopula animals but also the time needed for re-pairing can be a good indicator of the behavioural reaction to a

pollutant to which they are exposed. However, further research with a longer exposure time is necessary. The future studies will address this problem as well as the effects of the stress factor—EE2 on the reproduction of *Gammarus tigrinus*.

Acknowledgements

This work was supported by the National Science Centre Poland under Grant number UMO2011/01/N/ST10/06885 and University of Gdansk under Grant number 538-G220-B282-13.

The publication is financed from European Social Fund in as a part of the project “Educators for the elite—integrated training program for Ph.D. students, post-docs and professors as academic teachers at University of Gdansk” within the framework of Human Capital Operational Programme, Action IV. This publication reflects the views only of the author, and the funder cannot be held responsible for any use which may be made of the information contained therein.

References

- [1] Jaser, W., Severin, G. F., Jutting, U., Juttner, I., Schramm, K. W., and Kettrup, A. 2003. “Effects of 17 α -Ethinylestradiol on the Reproduction of the Cladoceran Species *Ceriodaphnia Reticulate* and *Sida Crystalline*.” *Environment International* 28: 633-638.
- [2] Lange, I. G., Daxenberger, A., Schiffer, B., Witters, H., Ibaretta, D., and Meyer, H. H. D. 2002. “Sex Hormones Originating from Different Livestock Production System: Fate and Potential Disrupting Activity in the Environment.” *Analytica Chimica Acta* 473: 27-37.
- [3] Lucas, S. D., and Jones, D. L. 2006. “Biodegradation of Estrone and 17 β -Estradiol in Grassland Soil Amended with Animal Wastes.” *Soil Biology and Biochemistry* 38: 2803-2815.
- [4] Zwiener, C., Glauner, T., and Frimmel, F. H. 2000. “Biodegradation of Pharmaceutical Residues Investigated by SPE-GC/ITD-MS and On-Line Derivatization.” *Journal of High Resolution Chromatography* 23 (7/8): 474-278.
- [5] Campbell, C. G., Borglin, S. E., Green, F. B., Grayson, A., Wozei, E., and Stringfellow, W. 2006. “Biologically Directed Environmental Monitoring, Fate, and Transport of Estrogenic Endocrine Disrupting Compounds in Water : A Review.” *Chemosphere* 65: 1265-1280.
- [6] Hogan, N. S., Duarte, P., Wade, M. G., Lean, D. R. S., and Trudeau, V. L. 2008. “Estrogenic Exposure Affects Metamorphosis and Alters Sex Ratios in the Northern Leopard Frog (*Rana Pipiens*): Identifying Critically Vulnerable Periods of Development.” *General and Comparative Endocrinology* 156: 515-523.
- [7] Lai, K. M., Scrimshaw, M. D., and Lester, J. N. 2002. “Prediction of the Bioaccumulation Factors and Body Burden of Natural and Synthetic Estrogens in Aquatic Organisms in the River Systems.” *Science of The Total Environment* 289: 159-168.
- [8] Hutchinson, T. H. 2002. “Reproductive and Developmental Effects of Endocrine Disrupters in Invertebrates: In Vitro and in Vivo Approaches.” *Toxicology Letters* 131: 75-81.
- [9] Watts, M. W., Pascoe, D., and Carroll, K. 2001. “Survival and Precopulatory Behaviour of *Gammarus Pulex* (L.) Exposed to Two Xenoestrogens.” *Water Research* 35 (10): 2347-2352.
- [10] Watts, M. W., Pascoe, D., and Carroll, K. 2002. “Population Responses of the Freshwater Amphipod *Gammarus Pulex* (L.) to an Environmental Oestrogen, 17 α -ethinylestradiol.” *Environmental Toxicology and Chemistry* 21: 445-450.
- [11] Vandenberg, G. F., Adriaens, D., Verslycke, T., and Janssen, C. R. 2003. “Effects of 17 α -ethinylestradiol on Sexual Development of the Amphipod *Hyalella Azteca*.” *Ecotoxicology and Environmental Safety* 54: 216-222.
- [12] Watts, M. W., Pascoe, D., and Carroll, K. 2003. “Exposure to 17 α -ethinylestradiol and Bisphenol A—Effects on Larval Moulting and Mouthpart Structure of *Chironomus Riparius*.” *Ecotoxicology and Environmental Safety* 54 (2): 207-215.
- [13] Andersen, H. R., Wollenberger, L., Halling-Sørensen, B., and Kusk, K. O. 2001. “Development of Copepod Nauplii to Copepodites—A Parameter for Chronic Toxicity Including Endocrine Disruption.” *Environmental Toxicology and Chemistry* 20 (12): 2821-2829.
- [14] Beck, I. C., Bruhn, R., and Gandrass, J. 2006. “Analysis of Estrogenic Activity in Coastal Surface Waters of the Baltic Sea Using the Yeast Estrogen Screen.” *Chemosphere* 63: 1870-1878.
- [15] Beck, I. C., Bruhn, R., and Gandrass, J. 2006. “Bioassay-Directed Fractionation for Analyzing Estrogens in Surface Waters of the German Baltic Sea.” *Acta Hydrochimica Hydrobiologica* 34: 560-567.
- [16] Leppakoski, E., and Olenin, S. 2000. “Non-native Species and Rates of Spread: Lessons from the Brackish Baltic Sea.” *Biological Invasions* 2: 151-163.
- [17] Ba, J., Hou, Z., Platvoet, D., Zhu, L., and Li, S. 2010. “Is *Gammarus Tigrinus* (Crustacea, Amphipoda) Becoming

- Cosmopolitan through Shipping? Predicting Its Potential Invasive Range Using Ecological Niche Modeling.” *Hydrobiologia* 649: 183-194.
- [18] Sutcliffe, D. W. 1993. “Reproduction in *Gammarus* (Crustacea, Amphipoda): Female Strategies.” *Freshwater Forum* 3: 26-64.
- [19] Pinkster, S., Smit, H., and Brandse-De Jong, N. 1977. “The Introduction of the Alien Amphipod *Gammarus tigrinus* Sexton, 1939, in the Netherlands and Its Competition with Indigenous Species.” *Crustaceana Supplement* 4: 91-105.
- [20] Szaniawska, A., Łapucki, T., and Normant, M. 2003. “The Invasive Amphipod *Gammarus tigrinus* Sexton, 1939, in Puck Bay.” *Oceanologia* 45 (3): 507-510.
- [21] Sutcliffe, D. W. 1992. “Reproduction in *Gammarus* (Crustacea: Amphipoda): Basic Processes.” *Freshwater Forum* 2 (2): 102-128.
- [22] Kunz, P. Y., Kienle, C., and Gerhardt, A. 2010. “*Gammarus* Spp. in Aquatic Ecotoxicology and Water Quality Assessment: Toward Integrated Multilevel Tests.” *Reviews of Environmental Contamination and Toxicology* 205: 1-76.
- [23] Bollache, L., and Cezilly, F. 2004. “State-Dependent Pairing Behaviour in Male *Gammarus pulex* (L.) (Crustacea, Amphipoda): Effects of Time Left to Moults and Prior Pairing Status.” *Behavioural Processes* 66: 131-137.
- [24] Dixon, M. J., and Shaw, P. J. 2011. “Watercress and Water Quality: the Effect of Phentyl Isothiocyanate on the Mating Behaviour of *Gammarus pulex*.” *International Journal of Zoology* 2011: 1-9.
- [25] Ying, G. G., Kookana, R. S., and Ru, Y. J. 2002. “Review Article. Occurrence and Fate of Hormone Steroids in the Environment.” *Environment International* 28: 545-551.
- [26] Sornom, P., Felten, V., Medoc, V., Sroda, S., Rousselle, P., and Beisel, J. N. 2010. “Effect of Gender on Physiological and Behavioural Responses of *Gammarus Roeseli* (Crustacea Amphipoda) to Salinity and Temperature.” *Environmental Pollution* 158: 1288-1295.
- [27] De Lange, H. J., Noordoven, W., Murk, A. J., Lüring, M., and Peeters, E. T. H. M. 2006. “Behavioural Response of *Gammarus pulex* (Crustacea, Amphipoda) to Low Concentration of Pharmaceuticals.” *Aquatic Toxicology* 78: 209-216.
- [28] Felten, V., Charmantier, G., Mons, R., Geffard, A., Rousselle, P., and Coquery, M. et al. 2008. “Physiological and Behavioural Response of *Gammarus pulex* (Crustacea, Amphipoda) Exposed to Cadmium.” *Aquatic Toxicology* 86: 413-425.
- [29] Blockwell, S. J., Maund, S. J., and Pascoe, D. 1998. “The Acute Toxicity of Lindane to *Hyalella Azteca* and the Development of Sublethal Bioassay Based on Precopulatory Guarding Behavior.” *Archives of Environmental Contamination and Toxicology* 35: 432-440.
- [30] Poulton, M. J., and Pascoe, D. 1990. “Disruption of precopula in *Gammarus pulex* (L.)—Development of a Behavioural Bioassay for Evaluating Pollutant and Parasite Induced Stress.” *Chemosphere* 20 (3-4): 403-415.
- [31] McCahon, C. P., and Poulton, M. J. 1991. “Lethal and Sub-lethal Effects of Acid, Aluminium and Lime on *Gammarus Pulex* during Repeated Simulated Episodes in a Welsh Stream.” *Freshwater Biology* 25 (1): 169-178.
- [32] McCahon, C. P., Poulton, M. J., Thomas, P. C., Xu, Q., Pascoe, D., and Turner, C. 1991. “Lethal and Sub-lethal Toxicity of Field Simulated Farm Waste Episodes to Several Freshwater Invertebrate Species.” *Water Research* 25 (6): 661-671.
- [33] Malbouisson, J. F. C., Young, T. W. K., and Bark, A. W. 1994. “Disruption of Precopula in *Gammarus Pulex* as a Result of Brief Exposure to Gamma-hexachlorocyclohexane (Lindane).” *Chemosphere* 28 (11): 2011-2020.
- [34] Malbouisson, J. F. C., Young, T. W. K., and Bark, A. W. 1995. “Use of Feeding Rate and Re-pairing of Precopulatory *Gmmarus Pulex* to Assess Toxicity of Gamma-hexachlorocyclohexane (lindane).” *Chemosphere* 30 (8): 1573-1583.
- [35] Wisniewska, M., and Szaniawska, A. 2013. “The Influence of 17 α -Ethinylestradiol on Reproductive Behaviour of *Gammarus Tigrinus* (Sexton, 1939)—Preliminary Study.” In *The Functioning and Protection of Water Ecosystems—Threats, Protection and Management of Water Resources*, edited by Rosinska, J., and Neuman, M. Poznan, Poland: Bonami.