1. Introduction

Heavy metals, widely used in industry, have been accumulated in increasing quantities in recent years as contaminants in all components of the biosphere especially in marine ecosystems[1,2]. Heavy metals have the potential to be lethally dangerous even in small quantities[3]; thus, it is very important to understand the mechanisms of entry of contaminants into marine waters and their availability[4] and potential effects on biota and ecosystems[5].

With respect to the Marine Strategy Framework Directive 2008/56/EC (MSFD), the relevant qualitative descriptor for contaminants for determining Good Environmental Status (GES) is Descriptor 8 which states that ‘concentrations of contaminants are at levels not giving rise to pollution effects’, while Descriptor 8.2.1 describes a specific indicator for pollution effects that ‘Levels of pollution effects on the ecosystem components concerned, having regard to the selected biological processes and taxonomic groups where a cause/effect relationship has been established and needs to be monitored’. Here, research support is expected for a better understanding of the relationship between pressures and their effects and impacts on the marine environment, and for deriving operational indicators for GES assessment. The MSFD is aiming at the protection of the environment against contaminants and is to identify eventual environmental problems which hinder the achievement of GES and the identification of their cause and thus the sources of chemical contamination[6]. This status stresses the necessity to research not only short term toxic effects of these metals but also the long term implications of low levels of exposure on the structure and balance of ecosystems.

The main emphasis of this study is on heavy metal pollution, a subject which is of particular interest because of the essential requirement of organisms for trace quantities of many metals and the fine balance between requirement and excess, disturbance of which results in markedly deleterious effects. Essential metals are components of many biologically important macromolecules and are frequently the active site in these molecules[7]. Cu, for example, is a component in the blood pigment haemocyanin, and in
cytochrome-c. In addition, free metal ions function as coenzymes in many cellular processes[8,9].

There is still little information on the ways probably by which heavy metals affect the structure of ecosystems as a result of interaction between affected and unaffected species. The present study aimed to investigate the effect on Crangon crangon (Linnaeus, 1758) (C. crangon) and Syngnathus acus (Linnaeus, 1758) (S. acus) of copper, which is essential in trace quantities, yet markedly toxic even at quite low concentrations.

2. Materials and methods

2.1. General information concerning C. crangon

C. crangon survives for 4 years and reaches a maximum length of 80 mm, maximum weight of 6.464 g. Reproduction period of C. crangon is between February and October and all month. Ovigerous females of C. crangon are the most abundant in May and September. The size at 50% sexual maturity for females is 53.5 mm in total length off Sinop Peninsula in the Black Sea[10,11]. C. crangon has a geographic distribution ranging through the North Sea and Baltic Sea, the Mediterranean Sea, Black Sea and the White Sea. They live largely at depths from 0 to 50 m. The distribution of C. crangon is affected by environmental factors such as temperature, habitat characters and varied seasonal migration models[12]. It favours areas of brackish water with mud or sand substrata and strong water movement. Experiments have shown that C. crangon is found in its richest abundance in sediment with particles intermediate in size between silt and coarse sand, and this is presumably because this permits the most efficient and rapid burial. C. crangon has been identified as a major predator in areas of shallow sea, one of the most abundant members of the macro-fauna and an important component of the food web. There is a change in its diet as the size of the shrimp increases. Small individuals predate meiofauna, particularly ostracods and harpacticoids, also nematodes and benthic foraminifera. Large ones predate macro-fauna, having a significant effect on the population of amphipods, worms, schizopods (i.e. Mysidacea and Euphausiacea), snails, young bivalves and young fish. Most of C. crangon do not survive in the first year; some individuals, however, survive for 2 or more years, and very occasionally, may attain an age of 5 years and reach a maximum length of about 90 mm.

2.2. General information concerning S. acus

S. acus is a fish found in the coastline of Sinop Peninsula on sand and amongst algae and eel-grass. S. acus feeds mainly on small crustaceans[13]. The body of greater pipefish is narrow and elongated, of verrucous appearance. The head has a long muzzle over more than half the length of it, and a small bump on the nape of the neck. The greater pipefish has pectoral fins, a dorsal fin and a small caudal fin. Its colour is brown with green or black vertical stripes, but it may vary depending on the environment[14].

2.3. Collection of animals

C. crangon appears during the winter and spring and the number of individuals increases to reach their highest abundance in mid-winter and mid-spring[15]. Specimens of C. crangon were collected from Sinop Peninsula in 2013 (Figure 1).

All individuals of C. crangon were captured at the depth of 10 m with mud or sand substrata. Specimens were sampled by beam trawl with length of 3 m and 10 mm cod-end mesh size. The specimens clearly recognized as C. crangon were separated and total length (TL) (from tip of rostrum to the tip of telson along the mid dorsal line) of each specimen was measured to the nearest 0.1 mm using vernier callipers. They were separately placed in biologically filtered clean seawater with 3 cm depth of the clean sediment into plexiglas experimental stock tanks (30 cm × 30 cm × 20 cm) at temperature of 21 °C. Specimens of C. crangon were fed with Artemia salina.

Specimens of S. acus were collected during spring and autumn in 2013. Specimens were captured by beam trawl with 3 m length and 20 mm cod-end mesh size, at a depth of 10–20 m. The sizes of the brood-stock were 9 to 13 cm. It has been observed that gravid greater pipefish in stock aquarium gave birth a day later (approximately 20–24 h). New-borns seemed to have absorbed the yolk sac. Aquarium experiments have shown that new-borns were benthic, remaining close to the bottom. New-borns were measured with a 0.1 mm precision by callipers and the mean total length was determined as (20.00 ± 0.71) mm (min: 18 mm and max: 22 mm). After they were fed with Artemia salina nauplii, which were opened at 30‰ salinity, in the morning and evening for 6 day, the experiment was started with greater pipefish.

2.4. Experimental protocol

Clean sediment was collected from the same area and washed through a 500-μm mesh sieve into a tank to remove any associated...
macrofauna, and then washed again at least 3 times with clean seawater before used in subsequent experiments[16,17]. Clean sediment was added to the test tanks to create a 3-cm deep layer.

In order to evaluate the effect of copper on these organisms, it is necessary to have understanding of the role of the metal in general and the relationship between species. An excess of the particular metal is likely to have an effect on the balance of biological function of the organisms. Therefore, first of all short term (10 days) experiments were carried out in order to define lethal and sub-lethal concentrations of copper. The results were used as a basis for choosing test concentrations for the further experiments (21-day experiments)[16,17].

Stock solutions of MERC grade chemicals, copper (II) sulphate (CuSO₄·5H₂O), were prepared in seawater and diluted as required. Three replicated series of concentrations and controls were used and all control and test solutions were aerated[16-23]. Temperature, dissolved oxygen, salinity and pH were measured in all experiments and all replicates, and treatments were applied with the same ecologic factors to reduce any potential physiological stress on the organisms encountered during bioavailability of chemicals[16,17]. Test solutions were not changed. Damaged animals were not used[16,17,20].

In all statistical tests, the significance level was set at $P = 0.05$. If data were normally distributed and variances homogeneous, the appropriate parametric test (e.g. Students $t$-test) was used, otherwise, non-parametric tests (e.g. Mann-Whitney test) were employed. When more than two samples were tested, One-way ANOVA was used. If ANOVA results were significant, the control and treatment means were compared by Tukey test to determine which treatment(s) differed from which[24].

3. Results

The water quality measurements showed that the average temperature of the water was $(20 \pm 1) ^\circ C$, salinity $(17.5 \pm 1.0)\% c$, pH $8.1 \pm 0.2$ and dissolved oxygen $(7.1 \pm 0.2)\text{ mg/L}$. These values were not statistically different between the controls and the treatments and replicates. Average size of $S.\ acus$ specimens used in the experiment was $(26.80 \pm 0.95)\text{ mm}$ (minimum and maximum lengths were 24 and 30 mm). The size of the $C.\ crangon$ specimens is showed in Figure 2. There was no mortality in all the controls for all species, indicating that the holding facilities, water, uncontaminated sediment and handling techniques were acceptable for conducting toxicity tests, as required in the standard EPA/COE protocol where mean survival should be $\geq 90\%$[25].

Animals were checked daily for mortality. Survival of all species decreased with increasing copper concentrations in seawater. The mean survival values for animals are shown in Figures 3 and 4.

In the present study on the second day, 80% of the shrimp treated at 20 mg/L died. However, at the end of the 13th day, all of the shrimps exposed to 2 mg/L or less of copper were alive. All of the pipefish died at the end of the 3rd day treated at 5 mg/L. The results showed that $S.\ acus$ was more sensitive to copper than $C.\ crangon$. 

![Figure 2. Size of C. crangon.](image)

**Figure 2.** Size of $C.\ crangon$. RTL: Total length with rostrum; CL: Carapace length; AL: Length of abdomen; W: weight.

![Figure 3. Mean number of C. crangon surviving in seawater containing different copper concentrations.](image)

**Figure 3.** Mean number of $C.\ crangon$ surviving in seawater containing different copper concentrations.

![Figure 4. Mean number of S. acus surviving in seawater containing different copper concentrations.](image)

**Figure 4.** Mean number of $S.\ acus$ surviving in seawater containing different copper concentrations.

4. Discussion

Cu is essential to many organisms but is potentially harmful at some levels of exposure[16,17,26-28]. The results of the present study agree with those of toxicity studies on other invertebrates[16,17,20].
concentrations in shrimps [18, 19, 26]. Bat et al. [19] found that non-essential metal Pb is significantly less toxic to Palaemon adspersus than copper, which is an essential metal. It may be owing to geography that the sites have high background Cu levels [7, 29] and invertebrates may have developed either a physiological or genetic adaptation or a combination of both to some metals [8, 9, 30, 31]. Similarly, Bat et al. [32] indicated that another essential metal Zn was more toxic to the polychaete worm Hediste diversicolor than the non-essential metal Pb.

In some cases the effects of excess metal may not be immediately apparent, if, for example, it is bound in an inert status. This kind of concealed effects will exert stress on cell function, the effects of which may only be obvious over an extended period of time, or when an organism is exposed to contaminant which may give an additional stress. The fact that metals act at the cellular level by disrupting enzymatic processes explains both why they are toxic at even very low concentrations, and the great variety of detrimental effects on different organisms. The Laizhou Bay is potentially contaminated by metals from industrial discharges and metal concentrations in shrimps Crangon affinis indicated that the metal pollution induced disturbances in osmotic regulation and energy metabolism and reduced anaerobiosis, lipid metabolism, and muscle movement [33].

Cu occurs naturally as metallic copper, copper sulphide and copper oxide [29, 34]. In industrialized areas heavy metals including copper are an important component in waste water and the input of copper into the environment by this route may exceed the natural input by several orders of magnitude [35, 36]. For example China is a major country in shrimp production and copper sulphate is usually used to eliminate filamentous algae and phytoplankton in shrimp farms [18]. Guo et al. [18] reported that copper treatment induced dose and time dependent toxicity to shrimp Litopenaeus vannamei.

In many amphipods and polychaetes, particulate-bound metal ions are the primary source of metal uptake, especially for suspension and deposit feeding animals which ingest large quantities of particulate organic material as food [37]. For benthic organisms, metal-contaminated sediment is probably a major source of metal uptake [38, 39]. In these organisms metal uptake is either from contact with the contaminated sediment or from the interstitial water, which may contain high concentrations of soluble metal as a result of leaching [16].

The aim of the present study was to evaluate the effects of Cu at known concentrations on two organisms C. crangon and S. acus which have been shown to have good potential for sediment toxicity test and are likely to be used in the future studies. Survival rate of both organisms decreased with increase in the Cu concentrations in the water. Here, both C. crangon and S. acus have been shown to be a suitable test species to assess heavy metal toxicity using static 10-day and 21-day bioassays. Several toxicity testing methods have been developed since the EPA/COE testing protocol was devised [25], involving a great variety of test species [37]. However, little is known about the effects of specific contaminants on the bioassay response. The present study has confirmed the potential of both species for toxicity bioassays, both species meeting most of the criteria required [16] for suitable toxicity test organisms.

**Conflict of interest statement**

We declare that we have no conflict of interest.

**Acknowledgments**

This study was supported by the Department of Hydrobiology, Fisheries Faculty, Sinop University (Grant No. S.049). This study was presented in the 3rd International Symposium on EuroAsian Biodiversity (SEAB-2017), Faculty of Biology, Belarusian State University, Minsk, Belarus, 5–8 July 2017.

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