Ecotoxicological Impact Assessment of Micro-Sized Coal Particles on Zooplanktonic Crustacean *Artemia Salina*

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Abstract. Micro-sized coal particles caused pollution of the aquatic environment in the area of marine coal terminals. In this work, we investigated the impact of micro-sized coal particles (<20 µm, 20-100 µm, 100-250 µm, and 250-500 µm fractions) on the viability of zooplanktonic crustacean *Artemia salina*. The nauplii of *A. salina* were exposed to particles of four different types of coal at concentrations from 100 to 5000 mg/L at static and shaking conditions. During 96 h of exposure, there were no pronounced toxicological effects of coal in the used concentrations. However, the observed absorption of the coal particles in the guts of *A. salina* may cause various abnormalities in the longer-term experiment.

1. Introduction
Coal dust is relatively well understood as a source of atmospheric pollution [1-4]. However, its particles are also accumulated in the marine environment. Coal particles get into water due to blowing from the territory of marine coal terminals, during loading operations and transferring using a conveyor or other uncovered equipment. While coal is kept in store in open piles, dust and particles can be released into the seawater by windy conditions, cyclones, and monsoon rainfall [5].

Coal particles have a different effect on flora and fauna [6], such as 1) shading effect; 2) physical damage, caused by the presence of solid matter; 3) releasing of toxic components, such as trace metals or coal-derived hydrocarbons. All these effects could be both sub-lethal and lethal for marine organisms [7-10], but the toxic level will substantially depend on the duration of exposure, type of coal, and its concentration in water [11].

Despite a long history of research in seawater contamination by coal, less attention is paid to its toxicological effects on organisms. Also, the effects of various types of coal are underexplored [11].

This research aimed to assess the influence of four types of coal on crustacean *A. salina*. We chose *A. salina* as a common test-object used in ecotoxicology.
2. Materials and methods

2.1. Coal samples
In his research, we took four types of coal loading in marine coal terminals in the ports of Nakhdoka and Vostochny:
- lignite coal, rank 1B (Primorsky Krai);
- bituminous coal, lean, rank T (Kuzbass);
- bituminous coking coal, lean-caking, rank OS (Kuzbass);
- anthracitic coal (Kuzbass).

The classification of coal is given following the assignment of coal types in Russia and USA [12].

Preparation of coal samples was executed in the Education Scientific Center of Nanotechnology, Polytechnic Institute (School), FEFU, Russia. Coal samples were ground with agate mortar and pestle (150 mm in outer diameter) to obtain the particles with the required grain size distribution. Grinding was performed manually instead of a ball grinder to avoid metal interference, which is an important factor for further toxicological analysis.

Particle size distribution was analyzed with laser diffraction using Analysette 22 NanoTec plus laser particle sizer (Fritsch GmbH, Germany). The measurements were run at the settings of “coal/water at 20°C” with particle size range 0.01 – 2000 µm in 5 iterations. The results were analyzed with MaS control software (Fritsch GmbH, Germany) using the Mic equation of state. Coal samples were diluted with sterilized seawater to obtain the stock concentration of 5000 mg/L and then were homogenized using Sonopulse 3100 HD ultrasonic homogenizer (Bandelin electronic GmbH & Co. KG, Germany) at 22 kHz, 100 watts for 30 minutes.

2.2. Test organism
In this work, we used the nauplii of zooplanktonic crustacean A. salina as a test organism. Naturally, A. salina lives only in lakes and ponds with high salinity, the adult male reaches 8-10 mm long, female 10-12 mm. Cysts turn into larvae water swimmer, nauplia, in 24-36 hours [13]. A. salina is widely used in toxicity tests of the nanomaterials [14] like TiO2, AgTiO2 [15], polymeric, lipid-based and drug nanoparticles [16], silver nanoparticles (AgNPs) [17,18], silver nanowires (AgNWs) [18] and others.

The cysts of A. salina were purchased locally and hatched in a glass bowl with filtered sterilized seawater with salinity 33 ± 1‰, and pH 8.0 ± 0.2. The nauplii were hatched after 24 h of incubation at a temperature of 20 ± 2 °C with a light cycle of 16/8 h. The hatched nauplii were transferred in a bowl with fresh filtered sterilized seawater for bioassays.

2.3. Toxicity test
Preparation of the nauplii and toxicity test were conducted in accordance with ISO TS 20787 (2017) with a few changes (test duration prolonged up to 96 h). Concentrations of 0 (control), 100, 500, 1000, 2000, and 5000 mg/L for each sample of coal were selected after conducting a series of pre-tests. Each concentration was carried out in quadruplicate. The nauplii of A. salina (10 animals per well) were transferred into a 96-well plate with related coal concentration. The volume of liquid in each replication was 200 µL. During the exposure period, the temperature was 25 ± 1 °C with a light cycle of 16/8 h, and there was no feeding of the nauplii. The dead and alive Artemia were counted after 24, 48, and 96 h of exposure. For investigation the ingestion of coal particles by A. salina after 96 h of exposure we used optical microscope Axio Observer A1 (Carl Zeiss, Germany). All the test was performed under two conditions: static and shaking plate on the orbital shaker SkyLine (ELMI, Latvia) with 250 rpm.

2.4. Statistical analysis
Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, USA).
3. Results and discussion
Coal samples, used in the previous investigations of toxicological effects on marine organisms, had a different distribution of particle size, such as $<63 \, \mu m$ [19,20], $<40 \, \mu m$ [21], $<425 \, \mu m$ [22].

The particle size distribution of four coal types used in this research is represented in Table 1. We selected four main groups of the particle size distribution ($<20 \, \mu m$, $20$-$100 \, \mu m$, $100$-$250 \, \mu m$, $250$-$500 \, \mu m$) with a prevailing number of particles (from 97.5 to 99.8 %). The number of particles in the size range $>500 \, \mu m$ was insignificant (from 0.2 to 2.5 %). The percentage distribution of particles was from 19 to 33.7 % in each of the four main groups, the median was 23.5. It means that test-organisms were exposed to particles of each group approximately equally in the ecotoxicological experiment.

| Type of coal | Particles size distribution, % |
|--------------|--------------------------------|
|              | <20 \, \mu m | 20-100 \, \mu m | 100-250 \, \mu m | 250-500 \, \mu m | Median diameter, \mu m |
| Lignite coal, rank 1B | 28.3 | 23.2 | 23.8 | 24.3 | 28.3 |
| Bituminous coal, lean, rank T | 22.1 | 21.1 | 19.9 | 34.1 | 22.1 |
| Bituminous coking coal, lean-caking, rank OS | 29.7 | 27.4 | 21.1 | 21.6 | 29.7 |
| Anthracitic coal | 33.7 | 19 | 20.1 | 26.6 | 33.7 |

Table 1. The particle size distribution of four coal types.

Figure 1. Effects of coal on the viability of A. salina in the test with shaking plate: lignite coal, rank 1B (a), bituminous coal, lean, rank T (b), bituminous coking coal, lean-caking, rank OS (c), anthracitic coal (d). ND – the counting of nauplii was obstructed due to the high turbidity of the liquid.
We registered no pronounced toxicological impact of coal in different concentrations (Figures 1, 2). In the test with a shaking plate (Figure 1), we observed low mortality of A. salina for 24 and 48 h of exposure (0% for lignite coal and 10% for other types of coal). However, crustacean mortality was significant in the control group (up to 60%) for 96 h. The observed result suggests that the test with the shaking plate was not relevant due to high mortality rates in the control group.

Figure 2. Effects of coal on the viability of A. salina in the static test: lignite coal, rank 1B (e), bituminous coal, lean, rank T (f), bituminous coking coal, lean-caking, rank OS (g), anthracitic coal (h). ND – the counting of nauplii was obstructed due to the high turbidity of the liquid.

Figure 3. Microscope images of live Artemia nauplii showing absorption of the coal particles in the guts: control (a), lignite coal, rank 1B (b), bituminous coal, lean, rank T (c), bituminous coking coal, lean-caking, rank OS (d), anthracitic coal (e).
In the static test (Figure 2), we did not point out significant mortality for 24 and 48 h. This result suggests that in common these species are resistant to coal presence in the water. The response was registered only for 96 h of exposure, and the most pronounced effect on the viability of A. salina nauplii was observed for lignite coal (viability decreased up to 70% with concentrations 100 mg/L and 1000 mg/L in 96 h test) and bituminous coking coal (viability decreased up to 70% with concentrations 1000 mg/L and 5000 mg/L in 96 h test). We suggest that this could be caused by bituminous volatile compounds and phenols, which content in the lignite coals is rather more than in other types of coal [11]. It was reported that volatile organic compounds can inhibit the growth of algae while reducing zooplankton biomass [11, 23]. Bituminous coal contains a significant quantity of PAHs [11,24], which is mentioned for coking coals [25]. PAHs bioavailability could cause toxic effects on marine organisms [26, 27].

Investigations of coal toxicological effects on marine organisms have shown different results, for example, in the experiments with crabs Cancer magister [28] and mussels Villosa iris (7-week test) [22] there was no response for exposure of coal, and mortality was not observed, but effects on the viability of the embryos and larvae of corals Acropora tenuis was revealed in 72-h test (the highest mortality rate was 26 %) [20]. Mostly sub-lethal effects of coal are investigated in experiments, such as growth inhibition of fish [19], structural changes and damage to fish gills [29], oxygen consumption for fish [29] and mussels [21], necrosis of mussel gill tissues [22], fertilization of corals [20]. We believed that the responses to long-term, low level, chronic exposures to coal, which are occurred close to marine coal terminals, need to be evaluated in future studies in addition to acute exposure assessment.[20].

Based on the results of this study we suggest that crustacean A. salina is sufficiently resistant to the toxicological effects of coal on its viability. At the same time, as shown in Figure 3, we observed pronounced accumulation of the coal particles in nauplii guts after 96 h of exposure, which can lead to evolving of different abnormalities in long-term experiments and can be investigated in the future.

4. Conclusions
Our research showed no significant effects of four types of coal on the nauplii of A. salina during 96 h of exposure. The viability of the nauplii decreased to 70% in the tests with lignite coal, bituminous coal, bituminous coking coal, and to 80% in the test with anthracitic coal in static conditions. At the same time, we observed the uptake of coal by A. salina nauplii during the experiment. It can result in evolving abnormalities and revealing of sub-lethal effects, which should be investigated in further studies to address the issue of understanding of the mechanisms of micro-sized coal particles accumulation and chronic toxicity in aquatic organisms.

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