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# Bioassay of the estuary waters of the Chernaya River (Bay of Sevastopol, Black Sea) using the brine shrimp *Artemia* (Crustacea: Brachiopoda)

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The area of Sevastopol Bay is a heterogeneous system, with the estuary of the Chernaya River in the apical part. To assess the ecological status of various parts of the bay and estuary in the summer season of 2018, we determined the oxygen content, salinity, pH, Eh, as well as the proportion of nauplii hatched from the cysts of *Artemia* sp. in water samples from seven coastal areas, differing in location, hydrological conditions and level of anthropogenic influence. For different areas, hydrochemical indices of the water samples tested and hatching values of larvae from crustacean eggs are shown to be different; these were significantly lower in the most polluted water samples from the estuary area. The possibility of using a set of methods of hydrochemical analysis and biotesting to improve the information content of assessments of the ecological status of coastal sea waters is discussed, using the example of the Bay of Sevastopol and the estuary of the Chernaya River.

*Keywords:* estuary, pollution, *Artemia*, cysts, salinity, oxygen, redox potential, biotesting.

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## Introduction

The Bay of Sevastopol is a complex system, which includes about 20 bays, differing in their topography, hydrological regime, coastal infrastructure and level of anthropogenic impact (Fig. 1). Its length is more than 7.5 km, its area 7.96 km$^2$; the maximum depth is 21 m. The Bay of Sevastopol is separated from the open sea by an artificial dock, which greatly hinders its water exchange and contributes to the accumulation of various pollutants in the water and in the sediments. The main pollutants of the Bay of Sevastopol are petroleum products, heavy metals, nutrients, organochlorine compounds, phenols and surfactants (Belyaeva, 2012; Kopytov et al., 2010; Subbotin et al., 2007). This situation is typical for most coastal waters of the Crimea. At the same time, the main sources of pollution are municipal wastewaters, which currently account for 83% of the total volume of discharge of effluents from the Crimean Peninsula and enter the marine environment both directly from the coast and through rivers. In 2016, 3.067 tons of oil products, 8.167 tons of synthetic surfactants, 10 370 tons of sulphates, 25 650 tons of chlorides, and 1 721 tons of nitrates were dumped into the rivers of the Black Sea basin. The volume of wastewater containing pollutants was 84.7% in 2014 and 75.8% in 2016 (Ivanyutin and Podovalova, 2018).

The level of pollution in the coastal areas may vary, including in the Bay of Sevastopol, as there are currents in it that carry pollutants to different areas where they settle to the bottom. Therefore, it is sometimes quite difficult to determine the ecological status of marine areas based only on the determination of pollutant concentrations in them. In addition, various compounds can interact with each other and with components of seawater, exhibiting synergistic or antagonistic effects.

Ultimately, the area of Sevastopol Bay gradually narrows and terminates with an estuary, where the Chernaya River enters into it (Fig. 1). Until the mid-twentieth century, the uppermost part of the Bay of Sevastopol was almost rectangular in shape and ended with the extensive Inkerman Marsh, with the mouth of the Chernaya River along its southwestern border. In the 1950s, after draining the swamp and dredging, the Inkerman estuary was formed in its place, characterized by complex coastal infrastructure, with an area of about 0.18 $km^2$, a predominant depth of 5 to 8 m, and a maximum depth of 8.6 m.

The estuary was connected to the bay by the deep-water (up to 8.4 m) Chernorechensky channel. Approximately 100 m upstream from the place where the river enters into the Inkerman estuary, the channel of the Chernaya River was straightened and deepened to 5–6 m; at present, the depths have decreased to 1.5–3 m. As a result of these significant anthropogenic changes, the only typical estuary in Crimea was formed. It is characterized by an increase in salinity of the water from the surface to the bottom and towards the sea, with a pronounced wedge of salt water penetrating relatively far upstream and two layers due to the large density gradient between the desalinated surface and saline bottom waters. The position of its boundaries is quasistationary, conditionally drawn in the lower reaches of the Chernaya River (on average about 1800 m from the mouth) and at the exit from the Chernorechensky channel to the bay, and completely covers the Inkerman estuary (Boltachev and Karpova, 2012). This area, called Inkerman, is of particular environmental interest, as the combination of natural and anthropogenic factors created a unique ecosystem that allows the integral influence of these components on the living conditions of aquatic organisms to be analyzed. Sea and fresh waters are mixed here, the salinity and temperature of which can vary both during the year and throughout the day.

The coastal territory of Inkerman has a complex infrastructure: it includes the release of water from a state district power station, an enterprise for the disassembly of old and recycled ships (“cutting wall”), ship parking, the “Avlita” loading and transshipment complex through which various cargoes are transported, etc. Thus, along with the natural features of this water area, due to the mixing of sea and fresh waters, the waters are also polluted as a result of intensive economic activity on the coast, including urban facilities. At the same time, data on pollution of this water area are practically absent. The active dynamics of the movement of water masses and the compounds of anthropogenic origin contained in them creates certain difficulties for determining the ecological state of individual sections of the bay. In this respect, bioassay methods can be useful for solving these problems.

Currently, to assess the toxicity of natural waters, simply determining the presence of certain pollutants in them and establishing their concentrations, is not sufficient. The data obtained may not reflect the true picture of the ecological state of water bodies with a constantly changing hydrological and hydrochemical regime, which include estuaries. Therefore, bioassay methods are increasingly being applied using various aquatic organisms. One of these is *Artemia* sp. (Crustacea: Branchiopoda), a typical saltwater inhabitant. *Artemia* is a non-selective filtrator. With an effective system of osmoregulation, it is able to tolerate quite sharp fluctuations in salinity. This crustacean can exist in an environment with a salinity of less than 70 g/l, if there are no predators in the biotope, and also survives at a salinity of more than 250 g/l, producing cysts. Due to the synthesis of special enzymes capable of retaining oxygen, *Artemia* can survive oxygen decrease in water down to critical values. Most geographic races of *Artemia* are adapted to exist at temperatures of 6–35 °C,


**Fig. 1.** Sevastopol Bay and sampling stations. 1 – Inkerman Bridge; 2 – estuary; 3 – “cutting wall”; 4 – end of the channel; 5 – Bay of Sukharnaya; 6 – Konstantin’s Ravelin; 7 – Cape Tolstiy.

corresponding to the salinity and ionic composition of the habitat (van Stappen, 1996). The life stages of the crustacean are very sensitive to various types of pollution, and therefore, *Artemia* is recommended as a test object for assessing the state of natural waters and for monitoring natural sea and salt basins (van Steertegem and Persoone, 1993; United States environmental protection agency, 1983). As "endpoints" in toxicological studies, the proportion of *Artemia* cysts from which nauplii hatched, their survival over time, changes in their morphological characteristics, as well as the behavior, swimming rate of adults, and their biochemical parameters are taken into account (Bagshaw et al., 1986 ; Brix et al., 2006; Neumeyer et al., 2014).

Based on the above data, it is possible to predict the formation of various levels of pollution of the tested water areas of the Sevastopol Bay and changes in the hydrochemical parameters of water, which have a direct impact on the state of the habitat of aquatic organisms. The purpose of this work was to study some physico-chemical parameters of water in various areas of the Bay of Sevastopol and their bioassay using the hatching parameters of larvae from *Artemia* cysts.

## Materials and methods

Water samples were taken in August 2018 in different areas of the Bay of Sevastopol at 7 stations: station 1 – Inkerman Bridge, directly adjacent to the mouth of the Chernaya River; station 2 – estuary; station 3 – "cutting wall"; station 4 – end of the canal; station 5 – Bay of Sukharnaya; station 6 – water area near Konstantin's Ravelin, located behind an artificial dock; station 7 – the water area near Tolstiy Cape in the open sea (Fig. 1). The temperature was measured directly in water using a HANNA Instruments Check Temp-1 electronic thermometer (Russia). The hydrogen pH, redox potential (Eh), and the concentration of oxygen dissolved in water were determined in laboratory conditions using an Expert-001 analyzer (Econix-Expert Moexa CoLtd, Moscow, Russia) using the corresponding selective electrodes from Volta (St. Petersburg, Russia).

Water salinity was measured using a PAL-06S LTrHA GO refractometer (Japan) and expressed in ppm (‰). Water samples taken in different parts of the Bay of Sevastopol were stored in a refrigerator at +4 °C before determining the above parameters, measurements were carried out no later than 4–6 hours after sampling.

### *Bioassay of water using the parameters of hatching nauplii from Artemia cysts*

For bioassay water samples from different parts of the Bay of Sevastopol, we used *Artemia* cysts collected on the coast of the salt Saki Lake (Saki, Crimea). Collected crustacean cysts were washed according to generally accepted recommendations (El-Magsodi et al., 2005; van Stappen, 1996), dried, and then placed in test samples taken from different parts of the bay. As a control, cysts were incubated in sea water with a salinity of 18‰, taken at a distance of 2 nautical miles from the coast (control 1), as well as under standard conditions (control 2, incubation medium with a salinity of 35‰) and kept at a temperature of +25 °C for 48 hours with occasional stirring. The proportion of nauplii hatched from cysts was determined as the ratio of the number of nauplii hatching after 24 and 48 hours to the number of cysts placed in the incubation medium. In addition, the percentage of hatching of *Artemia* nauplii in the tested water samples was determined in relation to the control under standard conditions (35‰) and in water from a 2-mile zone (18‰).

### *Statistical analysis of the results*

All hydrochemical determinations were performed in triplicate. The number of nauplii hatching from *Artemia* cysts was determined in five replicates; calculation for each sample was carried out three times. Statistical differences between the studied parameters were established using the Student's t-test at the level of values Bridge and $p < 0.05$ (Khalafyan, 2008). Correlation analysis was performed using the CURFVIT computer program (version 2.10-L).

## Results

The obtained results showed that the oxygen content in the studied water samples varied within 7.37–9.26 mg/l (Fig. 2). The highest values were noted in the coastal waters of Konstantin's Ravelin (station 6, 9.26 mg/l), and the lowest at the Chernaya River mouth in the Inkerman area (station 1) and at the "cutting wall" (station 3). The pH values changed to a lesser extent, but even in this case, showed noticeable decrease to 7.49–7.51 in water samples taken at stations 1 and 3 compared with other samples, in which they were above 7.61. Salinity varied in the range of 15–19‰, and was significantly lower in two sampling locations, at stations 1 and 3, as in previous cases. The same trend was observed for the Eh indices, but only for water samples from station 1. There is no correlation between oxygen content and salinity. At the same time, a moderate relationship was found between the oxygen concentration in water and pH and Eh ($r$ = 0.44–0.56), as well as between salinity, pH, and Eh ($r$ = 0.44–0.52). However, a high correlation was noted between pH and Eh ($r$ = 0.97).

Thus, the research results showed certain differences between the studied hydrochemical characteristics of the tested water areas of the Bay of Sevastopol.

As the bioassay of the quality of water samples using *Artemia* cysts showed, the proportion of nauplii hatched from them in the studied areas was also different (Fig. 3). After 24 hours and 48 hours of incubation, the percentage of hatched larvae remained almost unchanged in the tested water samples taken at station 7 at Cape Tolstiy (14.3 ± 1.3% and 16.9 ± 2.9%), in water samples from stations 5 (11.0 ± 1.6 and 11.0 ± 2.6%)

and 1 (16.0 ± 1.7 and 15.0 ± 2.5%, respectively), but this indicator increased significantly after 48 hours in samples taken from Konstantin's Ravelin at station 6 (11.7 ± 1.4% after 24 hours versus 28.7 ± 4.5% after 48 hours). The same tendency, but less pronounced, was revealed when determining the hatching of nauplii from cysts in the water of stations 3 (13.1 ± 1.2% and 17.3 ± 2.3%), 2 (9.8 ± 1.2% and 14.8 ± 2.5%) and 4 (11.7 ± 1.8% and 15.2 ± 2.1%, respectively).

A comparative analysis of the proportion of hatched nauplii from *Artemia* cysts in the tested water samples from different sites of the Bay of Sevastopol and control values also revealed some features (Table 1). The percentage of hatching nauplii from *Artemia* cysts, incubated for 24 hours in all experimental samples, did not differ much, or tended to decrease compared with the data obtained for control 1. After 48 hours, this trend manifested itself to a greater extent, and the percentage of hatching nauplii from crustacean eggs in all experimental variants was reduced compared to control 1 by 7–64%. At the same time, the most clearly established regularity was expressed when testing water samples from stations 1–5 and 7.

A comparative analysis of the proportion of hatched nauplii from *Artemia* cysts in the water of the tested water areas in relation to control values 2 (incubation medium with a salinity of 35‰) also did not show significant differences after 24 hours of incubation. After 48 hours, the picture was slightly different, but the differences were also not significant. At the same time, one can note a tendency towards an increase in the proportion of hatching nauplii from *Artemia* cysts incubated in water from the water area of station 6 near Konstantin's Ravelin, but a slight decrease in this indicator in water samples from station regions 1–5.

Thus, the research results showed a different effect of the incubation medium, which was used water from different parts of the Bay of Sevastopol, on the hatching of larvae from *Artemia* cysts, used as a test organism. Moreover, in most cases, the tested water samples from different regions of the Bay of Sevastopol and especially from Inkerman waters had an inhibitory effect on hatching of larvae from cysts. No significant correlation was found between the hydrochemical parameters of water and hatching of nauplii from *Artemia* cysts, with the exception of a moderate correlation between pH, Eh and the percentage of hatching of nauplii from cysts after 24 hours ($r$ = 0.45 and 0.39, respectively).

## Discussion

The above study showed significant differences in the tested hydrochemical parameters of the coastal waters of the Bay of Sevastopol. A comparative analysis of the studied parameters showed that the oxygen content in the water was higher in the open part of the sea, that is, at station 7 adjacent to Cape Tolstiy, in the Bay of Sevastopol at station 6, located near Konstantin's Ravelin, and at station 5 in the Bay of Sukharnaya, while in the direction of the estuary (stations 4 to 1), its value decreased. The same trend was noted for the values of the redox potential Eh. Along with the active reaction of the pH environment, this indicator has a significant impact on the ecological state of the reservoir. The intensity of photosynthesis, respiration, and redox processes depends on the values of Eh (Hargrave et al., 2008). It is known that in natural water bodies Eh ranges from −400 to +700 mV, which is determined by the combination of all the oxidation and reduction processes that occur in

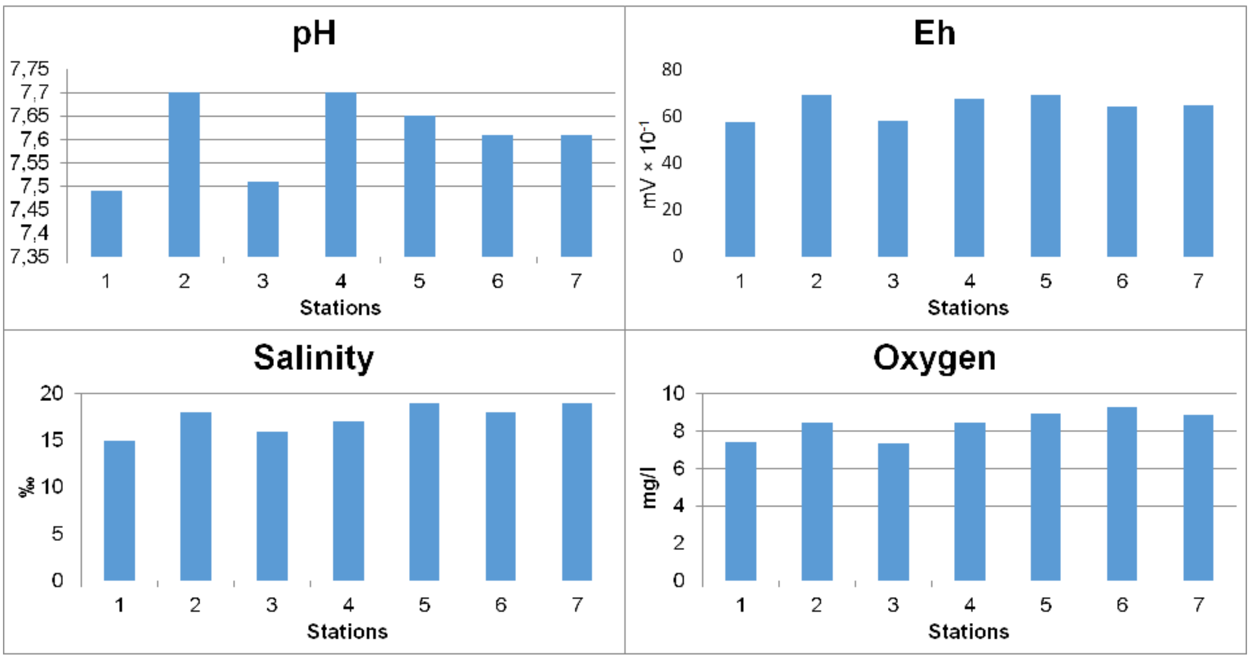


**Fig. 2.** Hydrochemical parameters of water samples from different areas of the Bay of Sevastopol.

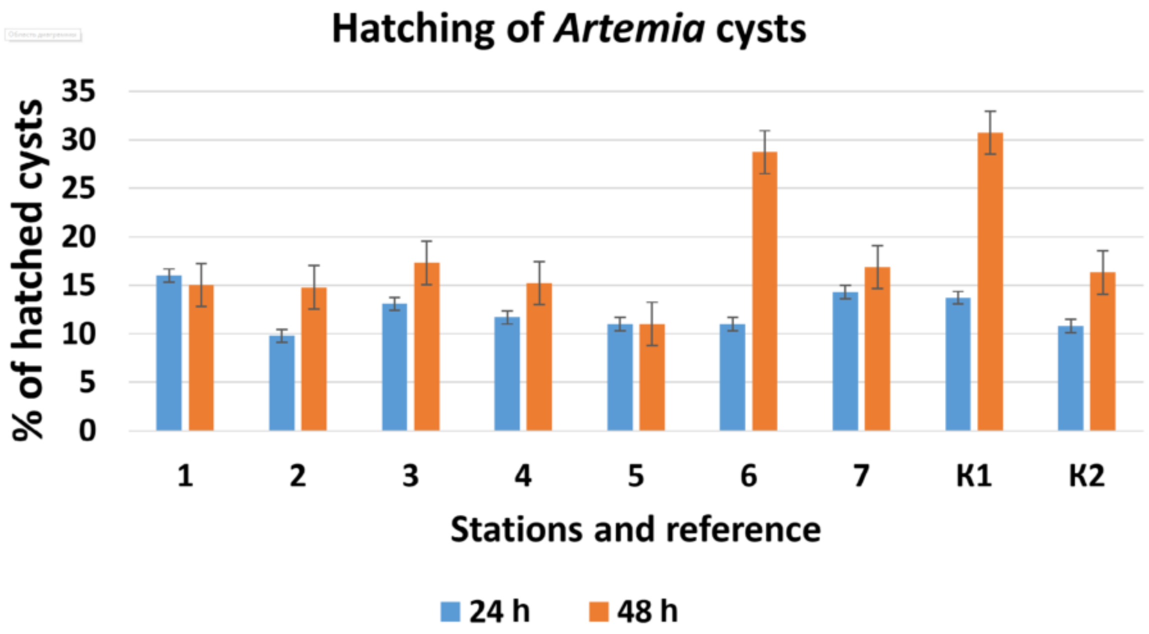


**Fig. 3.** Hatching nauplii from cysts (mean ± standard deviation, *n* = 15) in water from different areas of the Bay of Sevastopol. 1–7 – sampling stations, K1 – control 1 (cysts were incubated in Black Sea water with a salinity of 18‰ taken in a 2-mile zone); K2 – control 2 (cyst incubation occurred under standard conditions in water with a salinity of 35‰).

them. In the studied waters of the Bay of Sevastopol, this indicator varied insignificantly in the range from −57.7 to −69.6 mV.

Oxygen in natural water has the most powerful oxidizing ability, and hydrogen has a reducing ability. Both oxidizing and reducing reactions constantly occur in water. When a reservoir is contaminated and a large amount of mineral and organic substances gets into it, or when dead plants and animal matter decomposes, the redox potential can increase sharply. This indicator also increases the eutrophication process, which is due to the intensive development of phytoplankton and the active life of microalgae in the marine environment (Ciglenecki and Cosovic, 1996). Oxidative processes reduce the pH of the water, while reducing processes contribute to its increase. In turn, the pH affects the value of Eh. The redox potential has lower values in the bottom layers of water. At the surface of the soil it is greater than in the soil itself. Since pH values fluctuate throughout the day, the value of Eh also changes, which also depends on temperature (Fedorov et al., 2011; Plavsic et al., 2011).

In the Bay of Sevastopol, adjacent to the delta of the Chernaya River, the water temperature on the surface varies during the year from 0 to 26.6° C, and at the bottom does not drop below 4 °C in winter; salinity ranges from 3.25 to 16.13‰ on the surface and from 14.74 to 17.36‰ in the bottom layer. This estuary is characterized by high dynamics of thermohaline characteristics, which, depending on the hydrometeorological conditions, can vary both horizontally and vertically in different time intervals from several tens of minutes or hours, to seasons. It was shown that at a distance of about 380 m from the modern mouth of the river, the salinity of water on the surface varied from 4.90 to 15.04‰ during the year, and from 14.34 to 17.42‰ at the bottom. The extreme temperatures of the surface water layer varied from 0 °C with ice formation in winter to 27.6 °C in summer. An analysis of the interannual dynamics of thermohaline characteristics revealed trends in increasing water salinity both in the bottom and, to a greater extent, in the surface layer and a significant decrease in the amplitude of fluctuations of this indicator in the estuary as a whole (Boltachev and Karpova, 2012).

The studies also showed a difference in the hydrochemical characteristics of water samples taken in different areas of the Bay of Sevastopol. If the hydrochemical parameters were similar in the coasts of Konstantinov's Ravelin (station 6) and at Cape Tolstiy (station 7), then they changed significantly with distance from the open sea. This resulted in a decrease in salinity and oxygen content, which is probably due to the direct influence of the runoff from the Chernaya River. However, in addition to this, the impact of anthropogenic activity on the ecological state of coastal waters should be taken into account, which is especially evident in the Bay of Sevastopol water area in the Inkerman area at stations 1–5.

The mouth of the Chernaya River receives agricultural drainage of 600000 $m^3$ per year (Ovsyanyi et al., 2001). It is clear that the pollutants contained in this enter the Chernaya River and its course – to the Bay of Sevastopol. They contain from 48% to 52% mineral nitrogen and phosphorus. The concentration of nitrates in the storage pond is 5–10 times higher than in the water of the Bay of Sevastopol. An additional contribution to the pollution of this part of

**Table 1.** Hatching of nauplii from *Artemia* cysts in the tested water samples from Bay of Sevastopol with respect to control 1 (Black Sea water from a 2-mile zone with a salinity of 18‰) and control 2 (standard incubation medium with a salinity of 35‰), taken as 100%; n/s – the difference is not significant in relation to control.

| Sampling area | % to control 1 | | % to control 2 | |
|---|---|---|---|---|
| | 24 hours | 48 hours | 24 hours | 48 hours |
| Station 1 | +16.8 (n/s) | −44.0 ($p < 0.01$) | +48.1 (n/s) | −8.0 (n/s) |
| Station 2 | −28.5 (n/s) | −50.0 ($p < 0.01$) | −10.0 (n/s) | −9.3 (n/s) |
| Station 3 | 0 (n/s) | −52.0 ($p < 0.01$) | +21.3 (n/s) | +6.0 (n/s) |
| Station 4 | −15.0 (n/s) | −50.0 ($p < 0.01$) | +8.3 (n/s) | −7.0 (n/s) |
| Station 5 | −20.0 (n/s) | −64.0 ($p < 0.01$) | +1.8 (n/s) | −33.5 (n/s) |
| Station 6 | −20.0 (n/s) | −7.0 ($p < 0.01$) | +1.8 (n/s) | +76.0 (n/s) |
| Station 7 | +4.0 (n/s) | −45.0 ($p < 0.05$) | +32.4 (n/s) | +3.6 (n/s) |

the bay consists of municipal effluents, released from ships and metal-cutting enterprises, as well as the discharge of warm water from the state district power station, which are distributed over the water area by the underwater currents characteristic of this bay.

We have previously shown that pollution of the Bay of Sevastopol in the area of the estuary of the Chernaya River reaches significant values, which adversely affects the condition of the inhabitants of this part of the water area (Rudneva et al., 2016). However, it is rather difficult to assess the ecological state of individual coastal areas, since the complex pollution that they experience as a result of large-scale anthropogenic activity on the coast of the bay and in coastal waters, taking into account the peculiarities of the hydrodynamic situation, does not allow us to identify the dominant negative factors.

In this regard, we undertook a bioassay of the studied areas based on the method of calculating the number of hatched nauplii from *Artemia* cysts, which is widely used in ecotoxicological studies (Neumeyer et al., 2014; Yu and Lu, 2018). In this case, the bioassay of water samples allows the quality of the environment for the life of aquatic organisms to be determined.

The results showed that hatching of larvae from brine shrimp eggs turned out to be lower when they are incubated in water samples from the tested sections of stations 1, 2, 4, and 5 compared with the corresponding results obtained when cysts were kept in water from a 2-mile open sea zone (control 1). Obviously, in this case, the studied water samples may contain components that adversely affect the development of crustacean embryos and their exit from the cyst shell. Results obtained by other authors showed that the presence of heavy metals in the incubation medium can influence the hatching of nauplii from *Artemia* cysts (Bagshaw et al., 1986; Brix et al., 2006). In particular, it was noted that cadmium (6.5 and 65 mg/l) and zinc (11.2 and 112 mg/l) blocked hatching of nauplii from cysts even after 48 hours of incubation. Moreover, there was a significant delay in the larval exit process and a change in their further development (Bagshaw et al., 1986). At the same time, the concentration of cadmium in water in the range of 0.01–5 mg/l did not significantly affect the hatching of the larvae of the crustacean, but subsequently the fertility of females obtained in a medium with heavy metal was lower than in the control sample (Sarabia et al., 2003). It was further found that the hatching process was even more affected by the presence of copper ions in water, to which the various ontogenetic stages of the crustacean were very sensitive (Brix et al., 2006). The number of hatched nauplii significantly decreased in the presence of diuron and irgarol (herbicides used against fouling of the hulls of ships, docks and aquaculture structures). Studies of the toxicity of these components for brine shrimps showed a slight decrease in the percentage of hatching of larvae from cysts at an irgarol concentration of 1 and 3 mg/l in the medium, while at a concentration of diuron of 1 and 25 mg/l, a significant decrease in this indicator was observed (Alyuruk and Cavas, 2013). The authors suggested that these components blocked the active center of special serine protease enzymes, which are directly involved in ensuring the successful exit of larvae from the egg. However, no further significant deviations in the development of larvae were recorded.

Dose-dependent effects of inhibition of hatching of nauplii from eggs were established in the presence of diethylene glycol and sodium dodecyl sulfate in the incubation medium (Rotini et al., 2015). Some drugs used in aquaculture, including antibiotics, are also able to inhibit hatching of nauplii from *Artemia* cysts, or alter the pigmentation and survival of larvae (Migliore et al., 1997).

At the same time, it should be noted that salinity also affects hatching of nauplii and their further development, which confirms a comparative analysis of the results obtained by comparison with control indices 2 (incubation of cysts in water with a salinity of 35‰) and is consistent with data from other authors (Neumeyer et al., 2014). In particular, even a slight increase in salinity within acceptable limits (20–35‰) can lead to incomplete hatching or changes in the morphology of larvae. In addition, a change in salinity significantly modifies the toxic effect of compounds in the medium. Thus, a decrease in salinity led to a significant decrease in the toxic effect of copper pyrithione (an analogue of tributilin, used as an antifouling paint for ship hulls) on *Artemia* nauplii (Koutsaftis and Aoyama, 2008).

Thus, the results of the studies allowed us to conclude that the decrease in hatching of nauplii from *Artemia* cysts in water taken from different parts of the Bay of Sevastopol compared to control indicators, where water from the 2-mile zone was used as the medium, indicates a negative effect of the components contained in experimental samples on the process of hatching of larvae from crustacean eggs. In particular, heavy metals, oil products and antifouling agents used in the paint for ships, which enter seawater as a result of their disposal at enterprises located in this area, can be classified as such components.

## Conclusions

The data obtained indicate significant differences in the hydrochemical parameters of coastal waters in different areas of the Bay of Sevastopol, which are determined by both natural factors and the level of anthropogenic impact. Given the specificity of the studied water area, due to the presence of the Chernaya River estuary, a comprehensive study of the physicochemical parameters of the water and its quality assessment using bioassay methods can make a significant contribution to assessing the ecological state of the water area.

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