Measuring systems designed for working with living organisms as biosensors. Features of their metrological maintenance

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Abstract. The paper deals with the use of living organisms as biosensors for integrated assessing of the quality of their habitat. In particular, the paper considers the application of fish, shellfish, and crayfish for the water quality evaluation. By the example of a measuring system with crayfish, the features of metrological maintenance of systems with biosensors are described.

1. Introduction
Living organisms as biosensors in their natural habitat are widely used in solving environmental problems. First of all, this concerns a comprehensive evaluation of the rapid changes in the quality of this habitat which causes its biological danger. Integral behavioral and physiological characteristics, such as the mobility of organisms, frequency of respiratory movements and/or heart rate, are taken as parameters being measured directly.

In particular, the quality of the aquatic environment mainly takes into account the content of alkali and alkaline earth metal salts, humic and fulvic acids, dissolved oxygen, as well as pH value, the content of heavy metals that are foreign for human organisms (mercury, lead, etc.) and a number of highly toxic organic compounds. Among organic compounds, the most dangerous ones are pesticides, toxins of biological origin and chemical warfare agents. For implementing an instrumental quality control of the environment, the concentration of these compounds is determined individually or for a group of derivatives of the same compound with similar chemical properties. In doing so, the degree of biological hazard is determined by evaluating whether the concentration values exceed the values of the maximum permissible concentrations (MPC) set in regulations.

Regulations setting the procedure for determining the MPC values, on one hand, require lengthy (up to several months) experiments, and on the other hand, they do not allow the synergy and antagonism of influencing various toxicants to be taken into account. In Russia, the MPCs have been approved for at least 1000 toxicants. In water, to provide continuous or automated (with a period of no more than 10 - 30 minutes) instrumental control of them simultaneously, is unrealistic either for technical or economic reasons.
2. Features of the use of hydrobionts as biosensors

Using hydrobionts (aquatic organisms), i.e. organisms living in water, (fish, bivalve mollusks, crayfish, crabs, etc.) as biosensors allows real-time monitoring of the entire spectrum of toxicants of different chemical kind and structure, taking into account synergism or antagonism of their effects. This is due to the fact that in such hydrobionts, all significant biochemical violations directly affect the work of their cardiovascular system, and movement characteristics. To monitor the toxicity of water, continuous recording and analysis of the mobility and frequency of respiratory movements in fish [1], the opening angle and frequency of periodic closing / opening valves in mollusk clam shells [2] and heart rate in crustaceans [3], are used. The use of adult hydrobiont specimens with a relatively long life cycle (several years) as biosensors allows minimizing the effect of age-related changes on the above characteristics.

The first stage of the reaction of aquatic organisms to abrupt variations in the quality of the environment is the process initiated by their sensory system. The further reaction depends on the behavior “program” inherent in a particular type of the biosensor when danger is detected. In many ways, it is identical to the responses to an abrupt change in illumination (“shadow”) or to a short-time high-intensity noise.

Some examples: in bivalve mollusks, the response to exceeding the MPC of a toxicant consists in rapid closing the valves; in crayfish, this reaction lasts less than 1 minute, while the heart rate \( (HR) \) during this time interval compared to the \( HR \) value before the impact, can either decrease by 30 % or increase by 50 %. At the same time, both the qualitative and quantitative characteristics of such a reaction vary from one hydrobiont individual to another, but are well reproduced across time for each of them. In the case of a high concentration of the toxicant entering the water after a time interval inversely proportional to this concentration, a monotonic increase (or decrease) of the value of the integral characteristic under control, e.g., \( HR \), starts.

The change direction depends on the biochemical mechanism of the toxicant influence. Thus, using the example of measuring the \( HR \) value in a wide range of crabs, shrimps and crayfish, it was shown that toxicants creating interference for transporting oxygen, cause the decrease of the \( HR \) value. On the contrary, while exposing to high concentrations of mercury ions which are the strongest nerve poison, the \( HR \) value during the first few hours increases significantly. However, in both cases, while exposing to high concentrations of toxicants, there is a trustworthy difference between the values of the \( HR \) measured during such an impact and before it.

If one accepts that at a crayfish rest state, under stable temperature (18 - 20 °C), the \( HR \) value fluctuates within the range of \( \pm SD \) \( (SD \) is a standard deviation) around its average value \( HR_{av} \) and, e.g., for \( SD/HR_{av} \approx 0.1 \), then a 30 % increase or decrease of \( HR \) compared to \( HR_{av} \) can be considered as the sign of reliable changes of this parameter. Observations have shown that such an event under conditions of stable water quality can be recorded once per 3 - 4 hours. A similar situation takes place for both the value of the opening angle and frequency of the periodic closing / opening the valves of mollusks. Therefore, 30 % deviation of these characteristics from their average values is accepted as the criterion of a reliable deviation of a hydrobiont specimen behavior from the norm in a measuring system [2]. The coincidence of such deviations for both parameters in more than 50 % of the total number of mollusks in the system within 10 – 20 minutes is accepted as a criterion for changing the quality of water under control.

It should be noted that the above measuring systems for monitoring the quality of the aquatic environment are the “threshold” systems, since if the presence of significant concentrations of a toxicant in water causes an increase in the hydrobiont activity or circulation of its hemolymph, this fact further accelerates the toxicant inflow.

3. Measuring system designed to work with crayfish

The measuring system assigned for water quality control [3], installed at all water intakes of water supply stations of St. Petersburg, consists of 6 - 8 measuring channels (MC) of the same type, in which the crayfish are used as biosensors, \( HR \) being used as the measurand.
The principle of operation of a MC of a bio-electronic fiber-optic system is based on laser irradiating the outer surface of a crayfish in the zone of its heart localization followed by recording a radiation flux scattered in the opposite direction. To irradiate the outer surface and receive an optical signal carrying information about cyclic heart contractions of the crayfish, a thin fiber-optic sensor is used, which is non-invasively fixed on the crayfish shell. The flexibility and lightness of the fiber optic sensor provides the freedom of unhindered movements of the crayfish.

The change of no less than 30 % of the HR value compared with $HR_{av}$, at least, in 3 crayfish for 10 minutes, was taken as a criterion for changing the quality of water. Without a change in water quality, the probability of such an event is estimated by statistics no more than once per 1.5 months, and at the coincidence in 4 crayfish, the above takes place once per 2.5 years.

It was established experimentally that the HR change in response to toxicants with dangerous concentrations, varies between different biosensor specimens within the limits of ±10 %, and the values of $SD/HR_{av}$ vary from 10 to 30 %. From this, it follows that to select specific specimens of biosensors, to use the criterion of the minimum values of both $HR_{av}$ and $SD/HR_{av}$ is necessary.

It was also found that the closer the HR value of a crayfish at a rest state is to the minimum value $HR_{rest}$, which is characteristic for a healthy crayfish at a comfortable water temperature $t$, the lower is the threshold of demonstrating a short-term sensory response, as well as the threshold of a response to "shadow" and noise. Thus, for the crayfish such as Astacys leptodactylus, in the temperature range from 16 to 26 °C, the value $HR_{rest} = (2.3t - 4)$ beats/min. This is due to the fact that in the same crayfish, the priorities of the biochemical resources distribution change depending on its functional state.

Thus, it was found that the closer the crayfish state in the daytime is to the rest state, the more pronounced is its sensory response and the smaller is its $SD$ value. To achieve the state of the most complete rest of the crayfish, two conditions should be fulfilled: a non-invasive method of recording control characteristics (in this case, the optical method of receiving the cardioactivity signal is used) and the absence of interference for free movements (in this case, a flexible fiber-optic sensor is applied).

4. Metrological maintenance of measuring systems intended for work with crayfish
For successful functioning of such systems, not only properly working measuring equipment is necessary, but also a “workable” set of biosensors selected for this purpose according to certain criteria.

“Metrological health” of cancers in their application as biosensors, is provided by the following procedures carried out at the stage of preparing the MC for use and in the course of their operation:

1. Selection of crayfish based on their external (visual) parameters.

The need to control all 10 cancer limbs to be present is associated with experimentally established dependencies of the cancer’s HR reaction changes in response to the addition of a number of model toxicants to water if such injuries are present. At the same time, there is a deterioration in the functional state of cancer, which is also manifested in an increase in $HR_{av}$ in cancer in conditions of “operational rest” compared to the $HR_{rest}$ values of (determined by the formula $HR_{rest} = 2.3t - 4$) and an increase in the concentration threshold of the sensory induced HR reaction in response to the model toxicants used (sodium, potassium and calcium chlorides, mineral and organic acids). The cancer antennal integrity monitoring is necessary concerned to normal operation of the primary chemo receptors. The selection of the approximately same weight (length) crayfish species Astacys leptodactylus: 30 - 40 g (100 - 120 mm), caught in the same water body, allows one to select the cancers to approximately the same age and biological properties and with the prospect of further growth within 1 - 2 years.

2. Selection of crayfish based on the level of signals resulting from cyclic contractions of the heart.

Comparison of the value of amplitudes A of pulsations caused by changes in the intensity of scattered light by the single-chamber heart tissues of cancer its compression/relaxation cycle for different instances of cancers show significant (up to 10 times) differences when using the same instance of a optical fiber and pulsation converter. In the instance of the same cancer at the same values of HR and water temperature, the A values vary in the range of ±20 %, only.
Figure 1a is a real example of a HR 20-second interval for the recorder’s output ripple variation under the “test pulse” overlay and shows how the individual components of such ripple are determined in table 1.

**Table 1.** The example for a group of 12 Astacus leptodactylus cancers (n = 1 - 12) output ripple variation at (20 °C, stress, \(HR = 100 - 120\)) when using the same instance of an optical fiber and pulsation converter.

| n   | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | 11  | 12  |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| A, V| 0.8 | 0.83| 0.54| 0.40| 0.45| 0.65| 0.45| 0.38| 0.40| 0.36| 1.3 | 1.1 |
| T, V| 1.4 | 1.0 | 1.38| 1.1 | 1.2 | 1.17| 1.21| 1.17| 1.25| 1.30| 1.3 | 1.4 |
| A/T | 0.54| 0.8 | 0.41| 0.36| 0.33| 0.54| 0.33| 0.27| 0.33| 0.23| 1.0 | 0.8 |
| A/N | 5.3 | 5.5 | 3.6 | 2.7 | 3.0 | 4.3 | 3.0 | 2.5 | 2.7 | 2.4 | 8.6 | 7.3 |

The following symbols are used in table 1: A - the amplitude of the cardio activity periodic signal pulsation; T - the amplitude of the "test pulse" caused by a change in (1.6±0.2) % of the laser radiation intensity (and the flow of light scattered by the outer wall of the cancer core and collected by the optical probe, respectively); A/T - the ratio of these amplitudes; A/N - the ratio of the amplitude of the signal of cardiac activity to the amplitude N of irregular "noise" caused by fluctuations in the light intensity, scattered by the cancer’s core.

**Figure 1.** (a) - The example of the optical pulsation curve. The values of peak height T, amplitude A and double noise amplitude 2N shows the result of the real crayfish testing. The typical T, A and N values relations are presented in table 1. (b) - The example of the HR dynamics for the crayfish. The arrows 1 note the time of the 3 min mechanical stress action beginning. The HR\(_{rest}\) levels, HR\(_{test}\) levels and 2SD value, used in the table 2, are pointed out.

The table 1 data show that the variability ratio of the maximum value to the minimum value for the amplitude of the ripple A (1.3/0.36 = 3.6) practically determines the variability of A/T (1.0/0.23 = 4.3) and variability of A/N (8.6/2.4 = 3.6) - for the used samples of cancers. We have found previously [4], that the value of the measurement error HR is determined mainly by the value of the ratio A/N. Therefore, cancer selection to improve the accuracy of the measurement of the value of HR is to be made on the basis of the highest value A.

3. Use of individual settings of each MC in accordance with the physiological characteristics of the crayfish (HR and SD values at the rest state).
Almost all cancers are suitable as biological sensor to detect cases of accidental pollution of natural water by high concentrations of toxicants causing stress and 2 - 3 times increase in HR (see figure 1b), except those being in a state of severe stress, in which HR is already maximal HR_{rest}. But when exposed to lower concentrations of toxicants there is a short-term (1 - 5 minutes) "sensory response", in which even the maximum value of HR is at the midpoint of the interval between HR_{rest} and HR_{test}. In this case, the statistical criterion: (HR - HR_{rest}) > 3SD_{rest} may be used as the criterion of reliability of detection of such reaction against the background of fluctuation of HR value. A substantial individual variability of HR requires individual updates for each of the cancer the average value of HR_{rest} and value SD_{rest} (20 s) - for of HR values distribution, based on the number of heart beats over a interval of 20 second. Measurements for a large sample of cancers in the used temperature range 14 - 20 °C showed that the values of SD_{rest} (20 s) vary from day to day in the range (3 - 7) beats/min, almost the same for all cancers. In this case, a short-term (20 s) increase in the HR value relative to the average HR_{rest} value of more than 20 - 25 beats per minute characteristic of the sensory response can be reliably detected.

Average HR_{rest} values at the same temperature vary in the range of ±25 % for different cancers. A typical example of variation of the ratio of individual values to the average for the group value HR_{rest}/HR_{rest}(av) for different cancers № 1 - № 4 of the same size: № 1 - (1.20-1.25), № 2 - (1.02-1.07), № 3 - (0.75-0.80) and № 4 - (0.93-0.96).

"Metrological health" of cancers in the process of operation is ensured by periodic control testing of their reaction to the effects of characteristic toxicants, which enables making sure that a quantitative characteristic of the response to the selected test impact is maintained.

The rapid (30 - 40 seconds) increase in the concentration of sodium chloride in water to a final value of 30 g/l, but without changing the water circulation regime (see figure 1b) is used as crayfish periodic control test. At the test all cancers showed a rise in HR to values HR_{tox} equal to HR_{test} during the first 5 minutes - see table 2.

**Table 2.** An example of the maximum HR values achieved at a temperature of 20 °C for the same instances of Astacys leptodactylus cancers for one day under mechanical stress (HR_{test}) - after a short term "suspension" in water and under chemical stress (HR_{tox}) - with exposure to 30 g/l sodium chloride.

|    | 1     | 2     | 3     | 4     | HR_{rest}, beat/min | HR_{tox}/HR_{rest} |
|----|-------|-------|-------|-------|--------------------|---------------------|
|    | 97±1  | 84±1  | 96±1  | 87±1  | 91±6.5             | 1.07±0.02           |
|    | 104±1 | 89±1  | 95±1  | 84±1  | 93±7.0             | 1.06±0.02           |
|    |       |       |       |       | 1.02±0.02          | 0.99±0.02           |
|    |       |       |       |       |                    | 0.96±0.02           |
|    |       |       |       |       |                    | 1.020±0.045         |
|    |       |       |       |       |                    | 0.045               |

Exposure to high (30 g/l) concentrations of sodium chloride creates a strong "osmotic shock" first of all - for the cancer’s gills tissues. This causes cancer "avoidance", reaction with the maximal hormonal activation of motor activity possible for this temperature so leading to HR maximum values. Therefore - the average values of HR_{tox}, obtained under such influence [5], are to be the same, as the values of HR_{test} achieved during "handling" or short-term "suspension" above the bottom level in the aquarium water. This allowed to use more sophisticated periodic control of compliance the HR, maximum possible for one biosensor, with the temperature determined “normal” values HR_{test}. The fourfold repetition of the test procedure "suspension" within 2 - 3 weeks under the same conditions (17.5 °C), showed that 3 "healthy" cancers for the specified time period HR_{test} values (122±10), (113±6) and (102±12) beat/min vary from day to day (±SD) as significantly as individual HR_{test} values vary in this group with each of 4 tests: (117±17), (105±16), (116±6) and (111±9) beat/min. But weakened by cancer (deceased through 7 days after the test) have shown significantly lower HR_{test} values (87±8) beat/min, than the average group HR_{test} values of the "healthy" cancers - (112±10) beat/min.

Studies conducted in order to develop a biosensor simulator necessary for specifying and checking the metrological characteristics of the MCs of the systems intended for measuring the HR of crayfish, show that diffuse reflection from surface crayfish tissues is well reproduced in case of using a fluoroplastic plate of a certain thickness as a simulator of these tissues. When the optical sensor holder is mounted on such a plate instead of the crayfish shell, the signal entering the receiving fiber
corresponds to the signal characteristic for a statistical average crayfish under the absence of contractions of its heart. In this case, the variable component of the signal is determined by the optical and electronic noise of all the elements included in the MC, which allows evaluating the level of this noise and controlling this level later in the process of operation.

To create a modulated optical signal that simulates the crayfish heartbeat, a fluoroplastic plate is used in the form of a sector, which is located at a certain distance from the plate simulating the superficial crayfish tissues. When this plate is rotated, a signal is generated that is similar to the signal arising from contractions of the crayfish heart. Frequency of rotation is set by a measurement standard. Comparison of this frequency and the frequency measured by the system equipment makes it possible to determine the error of the HR measurements. To check whether the optical properties of the simulator plate lie within the permissible limits, a coefficient of directed transmittance is measured with the help of a spectrophotometer.

In the course of operation of the system, in each MC, the automatic metrological self-check is carried out. It is based on monitoring the characteristic associated with the SD values of the crayfish at the rest state and at the state of deviation from the norm. If this characteristic exceeds the specified limits, a signal of the metrological malfunction of the corresponding MC is generated.

5. Conclusion
The above issues related to the metrological maintenance of the system intended for measuring the crayfish HR are also important of other measuring systems designed for working with biosensors. In the course of their operation, the necessity exists to monitor the biosensor performance as well as simulate the biosensor signals while determining the metrological characteristics of the systems.

References
[1] Sydor W J, Miller W R, Cairms J. and Gruber J D 1982 Use a box and line plots to assess fish ventilatory behavior in biological monitoring systems Can. J. Fish. Aquat. Sci. 39(12) 1719
[2] Zwart D D, Kramer K J M and Jenner H A 1995 Practical experiences with the biological early warning system mossel monitor Environ. Toxic. Water 10 237
[3] Kholodkevich S V, Ivanov A V, Kurakin A S, Kornienko E L and Fedotov V P 2008 Real time biomonitoring of surface water toxicity level at water supply stations J. Environmental Bioindicators 3(1) 23
[4] Lyubimtsev V, Kholodkevich S, Druzhinin I and Kurakin A 2017 Bioelectronic system with metrological self-check intended for diagnostics of acute water toxicity Pribory 10(208) pp 34-39 (In Russian)
[5] Lyubimtsev V, Kholodkevich S, Taymanov R and Sapozhnikova K 2017 Bio-electronic System for Megapolis Water Supply Monitoring 13th Int. Symp. on Measurement Technology and Intelligent Instruments (ISMTII 2017) (Xi’an China) pp 102-103