ISSN 0096-3925, Moscow University Biological Sciences Bulletin, 2009, Vol. 64, No. 3, pp. 115–120. © Allerton Press, Inc., 2009.
Original Russian Text © D.N. Matorin, L.B. Bratkovskaya, O.V. Yakovleva, P.S. Venediktov, 2009, published in Vestnik Moskovskogo Universiteta. Biologiya, 2009, No. 3, pp. 28–33.

Biotesting of Water Toxicity According to the Ratio of Microalgae Consumption by Daphnia Detected with Chlorophyll Fluorescence

D. N. Matorin, L. B. Bratkovskaya, O. V. Yakovleva, and P. S. Venediktov

Department of Biophysics, Moscow State University, Moscow, 119992 Russia
e-mail: matorin@biophys.msu.ru

Received February 5, 2008

Keywords: Daphnia magna, fluorescence of chlorophyll, biotechnology, ecology

DOI: 10.3103/S0096392509030067

The daphnia test, based on an assessment of survival, fecundity, and quality of progeny in the investigated aqueous samples is one of the main tests used for setting the quality of natural and waste waters in many countries around the world (US. EPA, 1994). This is due to fact that daphnia, an important part of freshwater zooplankton, have a short life cycle, are easily cultivated under laboratory conditions, and have a high sensitivity to toxicants of a different nature (Filenko, 1988; Filenko et al., 2004). In our country the daphnia test is a major standard method for the determination of the occupational exposure limit (OEL) in fish industry water bodies (*Rukovodstvo*, 2002). In several works (Matorin et al., 1990; Tsvylev, Sokolova, 1986) it was shown that one of the primary daphnia reactions towards the toxicants is a change in their nutrition activity taking place long before the possible death of the test-object.

Like the majority of planktonic crustaceans, according to its feeding mechanism, daphnia belong to filtrators. The process of feeding is related to continuous oscillatory movements of the thoracic legs; the inner side of these legs is densely covered with thin bristles that serve for the holding of the particles suspended in water. A significant portion of the food consumed by daphnia—in addition to the simplest, bacteria, and detritus—constitutes planktonic microalgae (Sushchenya, 1975).

One of the highly sensitive methods for tracking the concentration of algae is the method of rapid chlorophyll fluorescence (Matorin, Venediktov, 1990). The intensity of a constant fluorescence F_0 with a high correlation coefficient corresponds to the total content of pigments at a low level of excitation of the photosynthetic apparatus of phytoplankton and correlates with the abundance of algal cells (Matorin et al., 2004). Thus, upon the calibration of the yield of the constant fluorescence (F_0) it is possible to do a quick and low labor-intensive (1–2 min) determination of the algae concentration in the sample within the range of 1×10^3 cells/ml to 10×10^6 cells/ml. At present, these measurements can be carried out on the different fluorometers developed at the Department of Biophysics, Moscow State University, and on the instruments of some other firms.

This paper reports the results of the studies of the main features of daphnia feeding under normal conditions and in the presence of toxic substances obtained with the abovementioned fluorescence techniques. Some practical recommendations for the use of the rates of filtration of daphnia as a sensitive indicator of the toxic effect of pollutants were proved.

MATERIAL AND METHODS

The test objects were *Daphnia magna* grown according to the conventional hydrobiologic procedure (Filenko et al., 2004). Daphnia were fed with the culture of green algae *Chlorella vilgaris* grown in cultivators at 24°C on 18% Uspenskii's medium with the illumination of 30 $\mu E\ m^{-2}\ c^{-1}$ of the FAR day light luminiscent lamps. Before the experiments, the algae were growing throughout the day. The density of the culture corresponded to 3×10^5 cells/ml. Prior to the experiments chlorella in the exponential phase of growth was centrifuged to separate it from the cultural medium.

The consumption of chlorella by daphnia was estimated according to the decrease of the yield of the constant fluorescence (F_0) directly proportional to the concentration of the algae cells in solution. The measurements of chlorophyll fluorescence in algae suspension was carried out on the impulse fluorophotometer developed at the Department of Biophysics, Biological Faculty of Moscow State University, and intended for the measurement of highly diluted suspensions of microalgae. Some experiments were car-

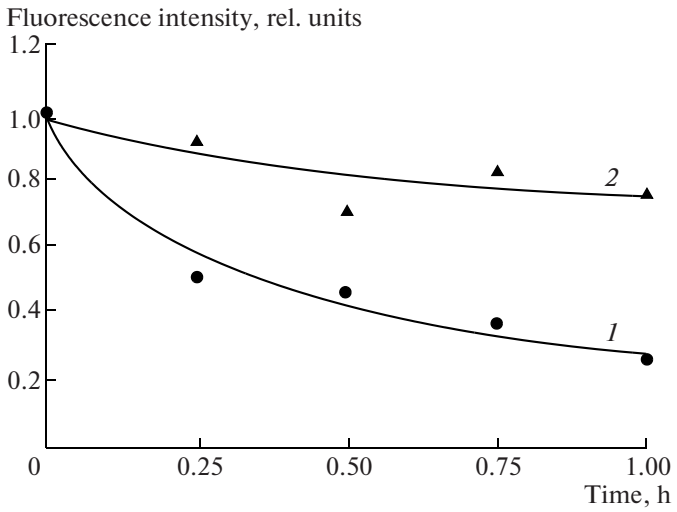

Fig. 1. The influence of methyl mercury salts (10^{-7} M) on the rate of daphnia feeding (prior to the measurements daphnia were incubated with methyl mercury salts for 1 h); *1* is blank and *2* is in the presence of the toxicant.

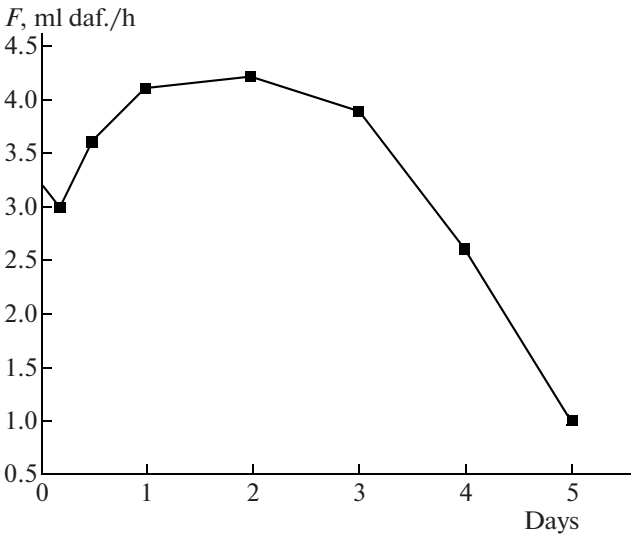

Fig. 2. Dependence of the filtering rate F (ml/h) of daphnia on the fasting time prior to the experiment.

ried out on a Toksi-Ram laboratory fluorometer (Heinz Walz GmbH, Germany).

The following toxicants were used in the toxicological experiments: methylmercury (MeHg), copper sulfate ($CuSO_4 \cdot 5H_2O$), tripropyl–tin chloride ($(C_3H_7)_3SnCl$, TPTC), zinc sulfate ($ZnSO_4$), lead chloride ($PbCl_2$), potassium dichromate ($K_2Cr_2O_7$), and Diuron® (N'–(3,4-Dichlorophenyl)-1,1-dimethylurea). In each case the experiments were repeated three times.

RESULTS AND DISCUSSION

The addition of chlorella in solution containing daphnia resulted in a decrease of the fluorescence signal accompanying algae consumption (Fig. 1). At chlorella concentrations below 3×10^5 cells/ml fluorescence signal decreased exponentially.

The concentration of chlorella cells (C_t) at any given moment t could be calculated as: $C_t = C_0\exp(-kt)$, (1), where C_0 is the initial concentration of chlorella and k is the constant of the chlorella concentration decrease in the sample. An instantaneous rate of the algae concentration decrease (at t approaching 0) is $dC/dt = -C_0k$. Given that $k = \ln(C_t/C_0)t^{-1}$ (2) and $dC/dtC_0 = dV/dtV$ (3), where V is the total volume of the sample containing daphnia and dV/dt is an instantaneous rate of filtration of daphnia (denoted as F). At constant F, the average rate of daphnia filtration is $F = \ln(C_t/C_0)V/nt$, where n is the number of daphnia in the sample. The value of F, or the rate of filtration, is expressed as milliliter per daphnia per hour (ml/daf. h) and refers to the volume of water filtered by daphnia in the sample within an hour. In the further experiments, we used the Eq. (3) to assess the activity of daphnia feeding.

Increase of linear dimensions of the daphnia's body (L, mm) results in an increase of the daphnia filtration rate. The dependence of F on L is described as: $F = 0.32L^{1.83}$, $r = 0.94$, and $P < 0.05$. The rate of filtration in adult animals (5–6 mm) reaches 8–10 ml/h for daphnia with a mean F of 6–7 ml/h or 150–200 ml/day. A high level of variability in F (CV = 57%), typical for adult daphnia, reduces their importance for toxicological experiments, since it requires a large number of repetitions in order to obtain reliable results. Already one-day old ($L = 1$ mm) juvenile daphnia is able to eat the algae. The rate of filtration in one-day juveniles did not exceed 0.3 ml/h and was also characterized by a great variation (CV = 42%). The most stable F (CV = 21%) was found for young daphnia of a medium size ($L = 3.5$–4 mm, 6–8 day specimens). Their average F was 3.5–4 ml/h. The decline of F of such daphnia down to 2–2.5 ml/h and below indicated the adverse conditions of cultivation; with this in mind, all the following experiments were carried with the medium size daphnia (3.5–4 mm).

The optimum planting density of daphnia (V/n)—4–5 ml per hour per daphnia for a feeding period of one hour (20–25 ml per 5 daphnia)—could be calculated from Eq. (3), providing that the daphnia, with $F = 4$ ml/daf. h, will consume 60–70% of algae present in the sample within an hour.

Dependence of F on the duration of daphnia fasting is shown in Fig. 2. In the first day of fasting F reached a maximum level and stabilized (CV = 21%). Further fasting (up to 3 days) did not affect F. By the end of the fifth day F dropped to 0 and daphnia died. Thus, to maximize F and its stabilization before the

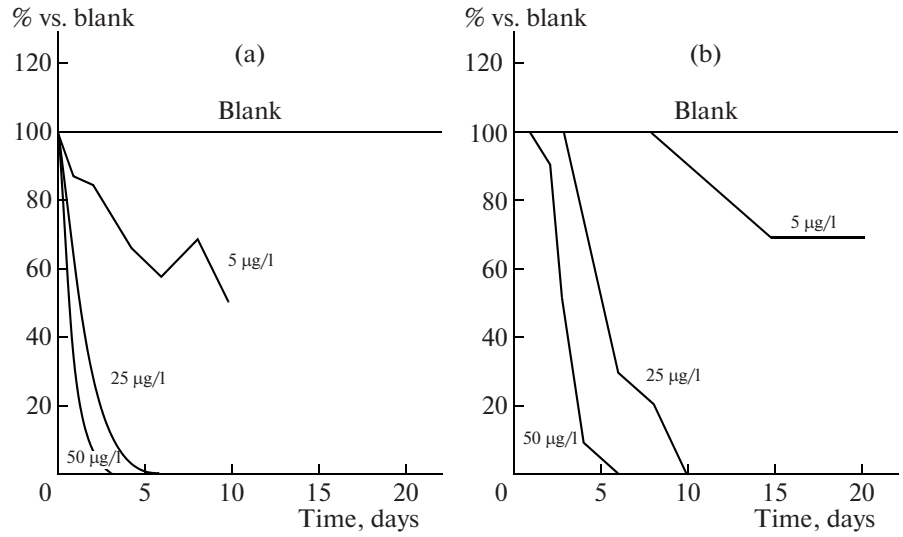

Fig. 3. Influence of TPTC on the (a) filtering rates (ml/h) and (b) survival of daphnia (all parameters as % of blank).

experiments daphnia should be kept in the examined aqueous solution for one day (24 h) without the addition of feeding.

The rate of filtration in daphnia depended on the concentration of food. At concentrations of algae in the sample below $3–5 \times 10^3$ cells/ml daphnia hardly ate. The frequency of the filter apparatus movements was not different from normal and was approximately 200 cycles per minute. If the average speed of filtering on daphnia remains 4 ml/h (0.3 μl per 1 cycle), then, at an algae concentration of $3–5 \times 10^3$ cells/ml, in one cycle, daphnia filters out 1–2 cells of chlorella, that it may lose, not to form the food lumps. The inability of daphnia to meet their food needs at algae concentrations below 5×10^3 cells/ml was noted in the works of other authors (Filenko et al., 2004). At an algae concentration range of $30–300 \times 10^3$ cells/ml filtration rate remains constant. In this density range the number of cells consumed by daphnia by the time t (denoted as K) increases linearly with the increase of the concentration of the algae cell. The maximum K for each algae concentration could be calculated as: $K = \Phi v\ C$ (4), where Φ is the number of the filtering apparatus cycles, v is the volume of water filtered by daphnia per cycle, C is the concentration of algae cells in the sample. Since daphnia extracts algae from the solution of a constant volume with a constant rate, i.e. $\Phi v\ C = \text{const}$, from formula (4) it is evident that the concentration of the filtered food in the range of $30–300 \times 10^3$ cells/ml increases linearly.

Increase of the algae density above 300×10^3 cells/ml is accompanied by a decrease of F; at least for the first 1–2 h no changes of the filtering apparatus work were observed. The decrease of F was proportional to the increase of the algae concentration. The quantity of the consumed algae reached its maxima of approximately 800×10^3 cells/h and then remained constant regardless of the increase in the density of the algae cells. From these data it is possible to conclude that at algae cell concentrations higher than a certain C_{max} in a given time t daphnia are unable to filter more food than K_{max} cells and, therefore, loose the "excessive" filtered cells back to the solution. For the algae concentrations above C_{max}, $K = \Phi v\ C - P$ (5), where P is the number of the algae cells lost by daphnia during the time t and $P = K v\ (C\ C_{max}/C_{max})$.

Since at high concentrations of food daphnia consume a constant number of cells at regular time intervals, the fluorescence signal over time decreases almost linearly. At food concentrations above 2×10^6 cells/ml the filtering apparatus of algae gets clogged leading to daphnia death. Based on the presented data, for feeding daphnia in the further experiments we selected an optimal concentration of chlorella of 100×10^3 cells/ml, corresponding to a constant value of F.

In the course of daphnia incubation, the environmental factors (temperature, pH, oxygen content, etc.) were the same as for the cultivation of other crustaceans. When feeding daphnia one should avoid the illumination of samples with intense light. Daphnia are very sensitive towards mechanical damage and the planting of them in the examined aqueous samples should be done carefully. If these two conditions are met, the rate of filtration of daphnia calculated according to the Eq. (3) could be used as one of the most sensitive indicators of daphnia activity in toxicological experiments.

One of the primary reactions of daphnia towards toxins was the decrease of their filtering rate (F) and, thus, the decrease of the quantity of the consumed algae cells (K) (Figs. 1 and 3). The visual observation

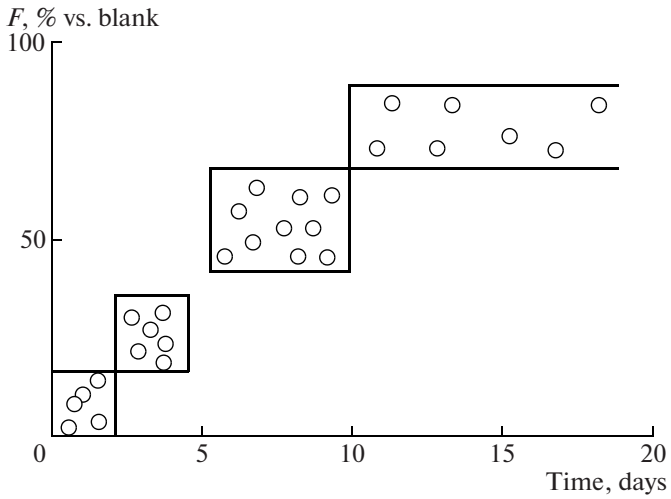

Fig. 4. Dependence of LT_{50} of daphnia in the presence of the toxicants on filtering ration F (ml/h) after 24 h after the toxicant addition.

of poisoned daphnia showed that the decrease in F is generally consistent with the decrease of the frequency of motion of their filtering apparatus; however, no strict correlation between these parameters was found. At high concentrations of the toxicant, corresponding to the complete suppression of F (daphnia stopped eating), the rate of their thoracic legs movements decreased approximately 3–4 times. Some toxicants, such as TPTC, on the other hand, stimulated the movements of the thoracic legs regardless the decrease of F. On the other hand, $ZnCl_2$ and $PbCl_2$ slightly decreased the amplitude of the thoracic legs movements (the span of the grip) without any changes in the movement frequency; nevertheless, these toxicants caused a significant decrease of F.

The discrepancy between the work of the filtering apparatus and the value of F increased with the increase of the algae cell concentration in the sample for all the examined toxicants. Thus, it was concluded that the effects of the toxicants on F and K were related not only to inhibition of the algae cells filtering (decrease of Φ and/or v) but also to the efficiency of its digestion, i.e., with the decrease of the maximum number of algae eaten by intoxicated daphnia at a given amount of time and a corresponding number of the "lost" algae cells (filtered but not eaten). Thus, intoxication reduces the food requirements of daphnia in a given amount of time and F could be used to characterize the food activity of the crustaceans.

Maximum concentrations of the examined substances that did not affect the filtering rate of daphnia in the one-day experiments

Toxicant	Concentration, μg/l	OEL for fish industry waters
Mercury	0.3	0.1
Copper	5	1
Led	10	100
Potassium bichromate	40	–
Zinc	10	10
TPTC	2–3	1

The dynamics of toxicant action (for example TPTC) on the survival and F of daphnia in the experiments lasted for up to 20 days and is presented in Fig. 3. It is evident that a reliable inhibition of F at toxicants concentrations corresponding to the death of daphnia was observed already in the first 24 h of the experiments. Further dynamics of F had a complex fluctuating character. However, a general tendency of decrease of the daphnia filtering rates with time was clearly observed. An 80–100% suppression of F typically was observed 1–4 days before the death of daphnia (depending on the toxicant and its concentration).

Based on the experiments with various toxicants, we tried to establish a relationship between the degree of inhibition of F in daphnia in the daily experiments and the time of death of half of the daphnia (LT_{50}) in the long-lasting experiments (Fig. 4). No correlation was found between the inhibition of F in the initial stages of intoxication and LT_{50}, defined as a combined result of experiments with the toxicants of a different nature. Dependence of F on LT_{50} provided a basis for a preliminary estimation of the sample toxicity. At suppression of F above 70% vs. blank in the first day of experiment, LT_{50} did not exceed 5 days and corresponded to the sample of an acute toxic effect on crustaceans (Filenko et al., 2004). If in the first day of the experiment F of daphnia was suppressed by no more than 50-60%, the sample had no acute toxic effect but had a chronic effect on daphnia, while its LT_{50} was in the range of 5–6 to 20 or more days. It should be noted that the lack of effect in the daphnia experiments, or an increase (stimulation) of F in the first day of the experiment did not unambiguously indicate the complete safety of the sample. More specific results require experiments of extended duration.

The experiments with the various toxicants demonstrated that the maximum concentrations the toxicants that did not cause a reliable inhibition of F in the daily experiments were close to the strictest OEL established for these substances (table). The table and Fig. 5, presenting the results of the probit–analysis (Zhmur, 1997) of the toxic effects of the certain substances on the considered parameter F, show that daphnia are highly sensitivity towards most of the examined toxicants. The data indicate a high efficiency of the proposed test and a possibility of its application for operational control of toxicity of natural and anthropogenic waters.

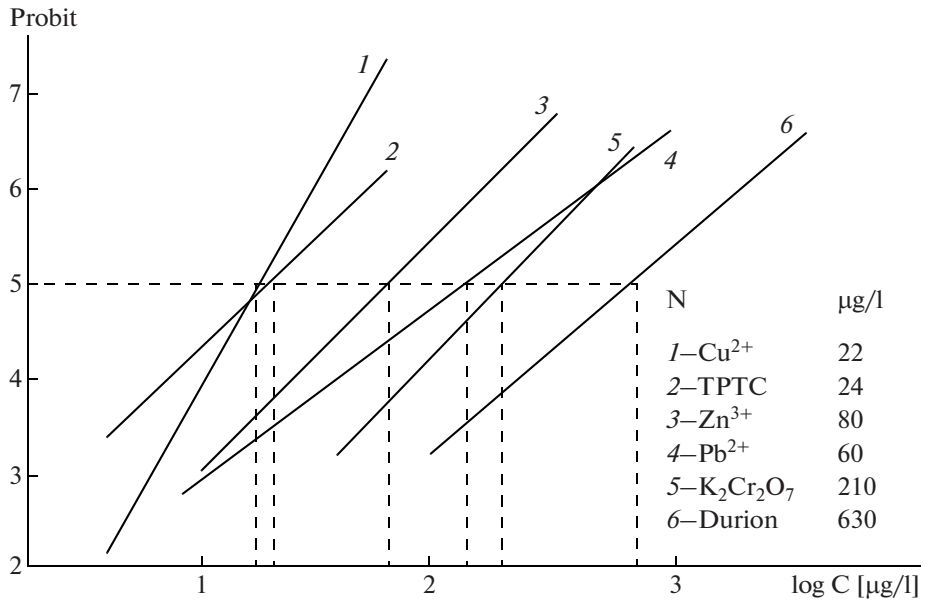

Fig. 5. Results of probyt-analysis of the toxical effects of the substances on the activity of daphnia feeding in the one-day experiments.

CONCLUSIONS

The above described protocol for the estimation of water toxicity from the rate of algae consumption by daphnia registered fluorometrically provides a high reliability of biotesting (Matorin, 2007). Maximum concentrations of toxicants detected by this method in daily experiments were close to the corresponding OEL values for fish industry bodies. It is important to note that the same fluorometric instrumentation enables the estimation of toxicity influence on the photosynthetic activity of microalgae. Using both methods, we conducted a study of detoxifying properties of humic substances of different genesis towards heavy metals, herbicides, and polycyclic aromatic hydrocarbons (Perminova et al., 2001a, 2001b; Yudov et al., 2005). Thus, it is now possible to use the same instrument for the detection of water toxicity towards the two major components of the ecosystems: microalgae, which are the primary producers of organic material in this media, and planktonic crustaceans, their major consumers, which are, in turn, a food for fish and other organisms of higher trophic levels.

An integrated biotest using algae and daphnia could be implemented in a network of environmental monitoring to control the toxicity of industrial water sewers and the incoming quality of the wastewaters going into biological treatment, to monitor the quality of the water treatment, and for periodic surveys of pollution of selected environmentally important waters. The ultimate goal of the introduction of biotesting is the creation of automatic water quality biomonitoring. The Department of Biophysics, Moscow State University, is developing a portable test-fluorometer for biotesting and the assessment of microalgae under pollution conditions.

The equipment used in the present work was also tested in the monitoring of the Moscow river and Lake Issyk-Kul, and to indicate the contamination with residues of dioxins in the Bay of Nachang (South Vietnam).

ACKNOWLEDGEMENTS

The work was supported by Ministry of Education grants RNP.2.1.1.1699 and 2008-02-1, 2-03-12. We acknowledge V.A. Osipova for assistance in preparing the manuscript.

REFERENCES

10-Day Chronic—*Daphnia magna* or *Daphnia pulex*, US EPA, 1994, SOP no. 2028. Available from: http://www.ert.org/products/2028.PDF

Filenko, O.F., *Vodnaya toksikologiya* (Water Toxicology), Chernoglovka, 1988.

Filenko, O.F., Isakova, E.F., and Chernomordina, A.V., Characteristics of the Effect of Potassium Bichromate on Generations and Model Populations of Lower Crustaceans, *Aktual'nye problemy vodnoi toksikologii* (Topical Problems of Water Toxicology), Flerov, B.A., Ed., Borok, 2004, pp. 176–194.

Matorin, D.N., Assessment of Toxicity of Waters by the Rate of Microalga Consumption by Daphnia Recorded by Chlorophyll Fluorescence, in *Bioindikatsiya i biotestirovanie* (Bioindication and Biotesting), Melekhov, O.P. and Egorov, E.I., Eds., Moscow, 2007, pp. 253–255.

Matorin, D.N. and Benediktov, P.S., Chlorophyll Luminescence in Microalgal Cultures and in Natural Phytoplankton Populations, *Itogi Nauki Tekhn., Ser. Biofizika* (Advances in Science and Technology, Ser. Biophysics), Moscow: VINITI, 1990, vol. 30, pp. 49–100.

Matorin, D.N., Antal, T.K., Ostrowska, M., Rubin, A.B., Ficek, D., and Majchhrowski, R., Chlorophyll Fluorometry as a Method for Studying Light Absorption by Photosynthetic Pigments in Marine Algae, *Oceanologia*, 2004, vol. 46, no. 4, pp. 519–531.

Matorin, D.N., Vavilin, D.V., and Benediktov, P.S., On the Possibility of using Fluorescent Methods for Studying Feeding of Crustaceans, *Biol. Nauki*, 1990, issue 1, pp. 147–152.

Perminova, I.V., Grechishcheva, N.Yu., Kovalevskii, D.V., Kudryavtsev, A.V., Petrosyan, V.S., and Matorin, D.N., Quantification and Prediction of the Detoxifying Effects of Humic Substances Related to Their Chemical Binding to Polycyclic Aromatic Hydrocarbons, *Environ. Toxicol. Chem.*, 2001a, vol. 35, pp. 3841–3848.

Perminova, I.V., Gretschishcheva, N.Yu., Petrosyan, V.S., Anisimova, M.A., Kulikova, N.A., Lebedeva, G.F., and Matorin, D.N., Impact of Humic Substances on Toxicity of Polycyclic Aromatic Hydrocarbons and Herbicides, in *Humic Substances and Chemical Contaminants,* Clapp, C.E. and Senesi, N., Eds., St Paul, USA, 2001b, pp. 276–287.

Rukovodstvo po opredeleniyu metodom biotestirovaniya toksichnosti vod, donnykh otlozhenii, zagryaznyayushchikh veshchestv i burovykh rastvorov (A Guide to Determination of Toxicity of Water, Bottom Sediments, Pollutants, and Mud Fluid by Biological Testing Method), Moscow, 2002.

Sushchenya, L.M., *Kolichestvennye zakonomernosti pitaniya rakoobraznykh* (Quantitative Consistent Patterns of Feeding of Crustaceans), Minsk, 1975.

Tsvylev, O.P. and Sokolova, S.A., Trophic Activity of Planktonic Phytophages—A Rapid and Sensitive Indicator of Aquatic Medium Quality, in *Voprosy sravnitel'noi fiziologii i razrabotki teoreticheskikh osnov biotestirovaniya* (Problems of Comparative Physiology and Development of Theoretical Bases of Biological Testing), Yaroslavl, 1986, pp. 11–13.

Yudov, M.V., Zhilin, D.M., Pankova, A.P., Rusanov, A.G., Perminova, I.V., Petrosyan, V.S., and Matorin, D.N., Synthesis, Metal-Binding Properties and Detoxifying Ability of Sulphonated Humic Acids, Chapter 24, in *Use of Humic Substances to Remediate Polluted Environments: From Theory to Practice*, Perminova, I.V., et al., Eds., the Netherlands: Springer, 2005, pp. 485–498.

Zhmur, N.S., *Gosudarstvennyi i proizvodstvennyi kontrol' toksichnosti vod metodami biotestirovaniya v Rossii* (State and Production Control of Water by Biological Testing Methods in Russia), Moscow, 1997.