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Diclofenac sodium aqueous systems at low concentrations: Interconnection between physicochemical properties and action on hydrobionts

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ABSTRACT

Diclofenac sodium (DS) is a widely used nonsteroidal anti-inflammatory drug (NSAIDs). NSAIDs are poorly removed during standard wastewater treatment. The consequences of the presence of NSAIDs in rivers and lakes at 10^{-11}–10^{-8} mol/L are not yet established; therefore, ecotoxicologists have focused their efforts on studying the effect of low-concentration NSAIDs on fish and hydrobionts, and also on predicting the potential risks to humans. Literature provides some information about the bioeffects of some NSAID solutions in low concentrations but there is no physicochemical explanation for these phenomena. Studying the physicochemical patterns of DS solutions in the low range of concentrations and establishing an interconnection between the solutions' physicochemical properties and bioeffects can provide a conceptually new and important source of information regarding the unknown effects of DS. The physicochemical properties and action of DS solutions on *Ceriodaphnia affinis* cladocerans, *Paramecium caudatum* infusoria, *Chlorella vulgaris* unicellular green algae, as well as on the growth of the roots of *Triticum vulgare* wheat seeds, were studied in the calculated concentration range of 1×10^{-3}–1×10^{-18} mol/L. The relationship between these phenomena was established using the certified procedures for monitoring the toxicity of natural water and wastewater. It was shown for the first time that water solutions of DS are dispersed systems in which the dispersed phase undergoes a rearrangement with dilution, accompanied by changes in its size and properties, which affects the nonmonotonic dependences of the system's physicochemical properties and could cause nonmonotonic changes in action on hydrobionts in the low concentration range.

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Introduction

Diclofenac sodium (DS) belongs to the group of non-steroidal anti-inflammatory drugs (NSAIDs), and is one of the most frequently studied drugs, with clinically proven mechanisms of action and effectiveness (Barkin, 2015; Shimanovsky et al., 2010; Ku et al., 1985). NSAIDs have a single point of application as their basis – a cascade of reactions associated with the transformation of arachidonic acid, the central biochemical mechanism of inflammation. The effect of NSAIDs is aimed at inhibiting cyclooxygenases – the key enzymes of arachidonic acid metabolism, which transform it into prostaglandins. The change in blood concentration of cyclooxygenases and inflammatory mediators, i.e. prostaglandins, accounts for the main therapeutic effects inherent in this group of drugs: analgesic, anti-inflammatory, and antipyretic (Barkin, 2015; Shimanovsky et al., 2010; Ku et al., 1985). Recently, evidence has been found that NSAIDs, including DS, also provide preventive and curative effects against malignant tumors (Pantziarka et al., 2016).

Typical daily DS doses for treatment of rheumatoid diseases, musculoskeletal system and postoperative pain, as well as those used in oncology, are within the range of 50–150 mg (Barkin, 2015; Pantziarka et al., 2016; Gan, 2010; Vyshkovsky, 2006). In the case of administering a 100 mg DS dose, its plasma concentration reaches 0.5–1 μg/mL or 4.2×10^{-6}–8.5×10^{-6} mol/L (Vyshkovsky, 2006). When used at such doses, NSAIDs suppress the biosynthesis of not only anti-inflammatory prostaglandins, but, due to lack of selectivity, also reduce the concentrations of other fractions of these active signaling molecules; in particular, of those prostaglandins that have a protective effect on gastrointestinal mucosa. Therefore, treatment with NSAIDs is often accompanied by considerable side effects related to gastrointestinal lesions (Baigent et al., 2013).

Administering of NSAIDs at commonly used doses results in their placement within the group of pharmaceutically active compounds (PACs) that pollute the aquatic environment, a growing concern of ecotoxicologists worldwide (Xia et al., 2017; Gonzalez-Rey and Bebianno, 2014; Memmert et al., 2013; Gröner et al., 2015; Guler and Ford, 2010; Hoeger et al., 2005; Hong et al., 2007; Quinn et al., 2011; Guiloski et al., 2015). NSAIDs, including DS, are poorly removed during standard wastewater treatment and, as a result, are detected in aquatic environments at concentrations ranging from nanograms (ng/L) to micrograms (μg/L) per liter, i.e., at about 10^{-11}–10^{-8} mol/L.

The outcomes of a study of the DS effects in concentrations of 10^{-8}, 10^{-7}, 10^{-6} mol/L on the breeding and motor activity of *Danio rerio* zebrafish show that it is potentially neurotoxic and significantly affects the motor activity of zebrafish embryos (Xia et al., 2017). The effects of DS in concentrations between 3×10^{-11} and 3×10^{-8} mol/L on the behavior of the marine amphipod *Echinogammarus marinus* were investigated (Guler and Ford, 2010). There was a trend towards a decrease in geotaxis score after DS exposure, which may indicate toxic effects of the drug resulting in reduced activity. An investigation of DS effects on brown trout (*Salmo trutta fario*), a salmonid species native to German rivers, exposed to DS in concentrations of 1.6×10^{-9}–1.6×10^{-7} mol/L for 7, 14 and 21 days (Hoeger et al., 2005), showed that DS affects kidney and gill integrity and selected immune parameters in brown trout (*Salmo trutta fario*).

Diclofenac exposure at 3×10^{-9} mol/L, an environmentally relevant concentration, is thought to have the potential to cause cellular toxicity and elicit estrogenic effects in Japanese Medaka, *Oryzias latipes* (Hong et al., 2007). The tested biomarkers represented gene toxicity, reproductive and endocrine function, and biotransformation, and indicated that diclofenac can cause cell toxicity, including possible carcinogenic and apoptotic effects within non-target species.

The acute and chronic toxicity of DS were assessed for three freshwater species (*Daphnia magna*, *Pseudokirchneriella subcapitata*, *Lemna minor*) using standardized toxicity tests, with toxicity found in the non-environmentally relevant mid 3×10^{-6} mol/L concentration range (Quinn et al., 2011). Results observed from biomarkers indicated that DS can cause significant stress at environmentally relevant concentrations, acting primarily through oxidation pathways with significant destabilization of the lysosomal membrane.

The effects of trophic exposure to diclofenac at concentrations of 6×10^{-10}–6×10^{-8} mol/L were studied in the male freshwater fish *Hoplias malabaricus* (Guiloski et al., 2015). Antioxidant enzyme activity and bio-transformation were evaluated in liver and gonads. The results suggest that DS causes oxidative stress in liver and reduced testosterone levels, which can have a negative impact on aquatic organisms.

The consequences of the presence of PACs in rivers and lakes at such concentrations are not yet established; therefore, ecotoxicologists have focused their efforts on studying the effect of low-concentration NSAIDs on fish and hydrobionts, and also on predicting the potential risks to humans (Munro et al., 2019; Xia et al., 2017; Gonzalez-Rey and Bebianno, 2014; Memmert et al., 2013; Gröner et al., 2015; Guler and Ford, 2010; Hoeger et al., 2005; Hong et al., 2007; Quinn et al., 2011; Guiloski et al., 2015).

One way to reduce the negative effects a drug is to decrease the therapeutic dose (Shimanovsky et al., 2010; Hinz et al., 2005). It is believed that the use of lower doses can ensure the selectivity of the drug, while avoiding adverse side effects and achieving comparable therapeutic efficacy. However, it has been established that the effects of many drugs at low concentrations may differ significantly from those at therapeutic doses, both in terms of direction and mechanism of action (Ku et al., 1985; Burlakova et al., 2003; Voronina and Molodavkin, 1999; Mattson and Calabrese, 2009; Khunderyakova et al., 2012; Sergeeva et al., 1997; Denisov, 2016). Pharmacological profiles of drug solutions administered at low concentrations usually have a complex form and exhibit a number of features, such as nonmonotonic dose–effect dependencies, the presence of a "silent zone," where the biosystem is practically insensitive to the effects of the drug, bioeffect sign change, synergism with drugs at standard doses, etc. (Shimanovsky et al., 2010; Mattson and Calabrese, 2009).

This behavior is typical not only for many drugs, but also for most biologically active compounds (BACs). In general, a serious problem in objective assessment of the bioeffects of highly diluted aqueous solutions of biologically active compounds is the absence of their physicochemical justification (Shimanovsky et al., 2010; Burlakova et al., 2003; Mattson and

Calabrese, 2009). In this regard, the development of physicochemical models of the effect that BAC solutions at low calculated concentrations have on living systems is a critical scientific task.

Recently, it has been established that highly diluted aqueous solutions of many BACs are self-organized dispersed systems that undergo "domain-nanoassociate" and "nanoassociate-nanoassociate" rearrangements of the dispersed phase with dilution at 25–60°C, accounting for nonmonotonic concentration dependences of physicochemical properties that correlate with the biological activity of such systems (Konovalov and Ryzhkina, 2014; Konovalov et al., 2015; Ryzhkina et al., 2014, 2015, 2017, 2018, 2019). It was demonstrated that molecular-water structures called supramolecular domains (Sedlák, 2006; Sedlák and Rak, 2013) normally form at sufficiently high concentrations of BACs in the range of $1.0–1 \times 10^{-5}$ mol/L both in the presence and in the absence of low-frequency background electromagnetic fields (EMF). In contrast, nanoassociates are formed at much lower calculated concentrations, and exhibit higher water content. It has been established that the extreme values of nanoassociate parameters (particle size (*D*) within 100–400 nm, ζ-potential (electrokinetic potential) from –1 to –20 mV), physicochemical properties and bioeffects of the systems are observed at practically the same concentration intervals. This allows us to experimentally prove the relation between the nonmonotonic nature of the bioeffects and the transformation of the dispersed phase (Konovalov and Ryzhkina, 2014; Konovalov et al., 2015; Ryzhkina et al., 2014, 2015, 2017, 2018, 2019).

Literature provides some information about the bioeffects of DS, accompanied in the low concentration range by nonmonotonic dependences, a silent zone, and a change in pharmacological profile, but there is no explanation for these phenomena (Shimanovsky et al., 2010; Sergeeva et al., 1997; Denisov, 2016; Xia et al., 2017; Gröner et al., 2015; Guler and Ford, 2010; Hoeger et al., 2005; Guiloski et al., 2015).

This paper studies the self-organization, physicochemical properties and action of DS solutions on *Ceriodaphnia affinis* cladocerans, *Paramecium caudatum* infusoria, *Chlorella vulgaris* unicellular green algae, as well as on the growth of the roots of *Triticum vulgare* wheat seeds in the calculated concentration range of $1 \times 10^{-3}–1 \times 10^{-18}$ mol/L, establishing the relationship between these phenomena using methods of dynamic light scattering (DLS), conductometry, as well as the certified procedures for monitoring the toxicity of natural waters and wastewater.

The chosen test objects belong to classes of organisms of different levels of organization: (1) unicellular (*C. vulgaris* unicellular green algae, *P. caudatum* infusoria) and (2) multicellular vegetable (roots of *Triticum vulgare* wheat seeds) and animal (*Ceriodaphnia affinis* cladocerans) organisms. Such a choice of test objects makes it possible to evaluate the effect of a substance's solutions under various schemes of its metabolism in living organisms. Algae, infusoria and cladocerans are organisms that are in the early stages of the trophic chain of aquatic biocenoses. A change (decrease or a sharp increase) in the number of each of them can lead to a change in the number or death of more highly organized hydrobionts, and degradation of the biocenosis as a whole. Therefore, these test objects are widely used in toxicological studies.

1. Materials and methods

1.1. Chemicals

DS (purity > 98.5%) was purchased from Sigma–Aldrich, China. Freshly prepared ultra-pure de-ionized water with resistance 18.2 MΩ/cm (Simplicity, Millipore) was used for preparing solutions. The initial substrate's solution of concentration of 1×10^{-3} mol/L was prepared from DS diluted with this water. Sample solutions (10 mL) were prepared via sequential decimal dilution, with each solution kept 20 hr after dilution (Konovalov and Ryzhkina, 2014; Konovalov et al., 2015).

1.2. Experimental design

The method of analysis of highly diluted solutions involves the study of self-organization and properties of such solutions in two parallel series (Konovalov and Ryzhkina, 2014; Konovalov et al., 2015). The difference between the first and the second series is that in the first series sample solutions were kept on the laboratory bench (natural conditions) before being studied by physicochemical methods, and in the second in a cylindrical three-layer permalloy container that protected the contents from external electromagnetic fields (EMFs), with screening coefficients of ~1000 (hypo-electromagnetic conditions). Using this method, it is possible to establish a threshold concentration (c_{thr}), below which nanoassociates are formed in solutions, and above – molecular-water domains (Konovalov and Ryzhkina, 2014). The solutions were freed of dust by filtering through Iso-Disc N-25-4 Nylon (Supelco, USA) filters. The solutions were stirred using a minishaker (Shaker lab dancer, IKA, Germany) for 10 sec. Prior to the measurements, the solutions were kept at constant temperature (25 ± 0.1)°C for 1 hr.

1.3. Physicochemical methods

1.3.1. Conductometry

Changes in the electrical conductivity (χ) of the solutions at (25 ± 0.1)°C were determined using a conductometer (inoLab Cond Level 1, WTW, Germany).

1.3.2. Dielcometry

Dielectric permittivity was measured by a Dielectric Constant Meter (BI-870, Brookhaven Instruments, USA).

1.3.3. pH

pH was measured by a pH-meter (inoLab pH 720, WTW, Germany) at (25 ± 0.1)°C.

1.3.4. Dynamic light scattering (DLS)

The particle size (the effective hydrodynamic diameter (*D*) of the kinetically labile particles at the maximum of the distribution curve) was determined on a "Zetasizer Nano ZSP" analyzer (Malvern Instruments, UK) equipped with a 633-nm He–Ne laser and operating at an angle of 173°. The software used to collect and analyze the data was the Dispersion Technology Software version 7.10 from Malvern. Each sample was measured in single-use polystyrene cuvettes (Sarstedt, Germany) with a pathlength of 10 mm. The

measurements were made at a position of 4.65 mm from the cuvette wall with an automatic attenuator and at a controlled temperature of 25 ± 0.1°C. The error bars displayed on the dependences of the size of particles (*D*) were obtained by the standard deviation (SD) of six measurements of the same sample in three parallel experiments.

1.3.5. Electron paramagnetic resonance (EPR)

EPR spectra were recorded on an X-band EPR spectrometer "Elexsys E500" (Bruker, Germany) similarly to Konovalov and Ryzhkina (2014) and Ryzhkina et al. (2015). The test solutions were placed in a cylindrical container with an internal diameter of 1 mm and repeatedly purged with nitrogen at (25 ± 0.1)°C. The work utilized the classical spin probe of 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) (purity 98%, Sigma, China). During the whole irradiation time the EPR spectra of the samples were recorded in the "Rapid scan" mode. All EPR experiments using a spin probe were performed three times. The relative error of the experiment was 20%. The change in the state of the dispersed phase as the calculated DS concentration decreased was judged by the change in rotational correlation time (τ_c), which was determined in accordance with (Berliner and Reuben, 1989).

1.4. Toxicological methods

The toxic action of the DS systems was tested using the certified procedures for the monitoring of toxicity of natural waters and wastewater (Ryzhkina et al., 2017, 2018, 2019). The solutions for the tests on infusoria (*P. caudatum*) and cladocerans (*C. affinis*) were prepared using dechlorinated tap water, and the solutions for the tests on wheat (*Triticum vulgare*) and algae (*C. vulgaris*) were prepared using distilled water.

1.4.1. Biotesting on cladocerans Ceriodaphnia affinis

The method is based on the determination of the mortality and changes in fertility of *C. affinis* when exposed to toxic substances present in the test solution, compared with the control culture in samples that do not contain toxic substances (control). The determination of the toxicity of each sample without dilution and at each dilution was carried out in 10 glass vessels in 2 parallel experiments and accompanied by a series of controls in 10 vessels. The temperature of the ambient air in the laboratory room was determined by the system of general conditioning of the laboratory premises and was equal to (22 ± 2)°C. An additional heating element was installed in the luminostat, which maintains (regulates) the temperature in the range (23 ± 1)°C. Biotesting was carried out in glass vessels with a capacity of 30 mL, which were filled with 15 mL of the test solution, and where a single ceriodaphnia of no more than 24 hr of age was placed. Cladocerans placing started from the control series. Assessing the mortality of ceriodaphnia in the test and control samples was carried out within 48 hr.

The percentage of dead ceriodaphnia (*A*, %) was calculated by Eq. (1):

$$A = \frac{X_c - X_t}{X_c} \times 100\% \quad (1)$$

where, X_c and X_t are the number of surviving individuals in the control and in the test solution, respectively.

The toxicity of DS solutions was determined from the death rate of cladocerans (*Ceriodaphnia affinis*). Acute toxicity was defined as a death rate of at least 50% of cladocerans during 48 hr, providing that the death rate for the reference sample did not exceed 10%. The concentration of DS was considered harmless (not leading to acute toxic effect) if the death rate did not exceed 10%.

1.4.2. Biotesting on infusoria Paramecium caudatum

The method was based on determination of the number of infusoria (*P. caudatum*) when exposed to toxic substances present in the water sample under study, compared with the control. The determination of the toxicity of each sample was carried out in five parallel series. As a control, five parallel batches with cultivation water (dechlorinated water, pH = 7.0–8.0, *t* = (20 ± 2)°C) were used. For biotesting, a micro-aquarium with wells was used, which was placed on the stage of a stereomicroscope. 10–12 individuals were placed in each well; 5 wells were used for control and 5 wells for a solution of a certain concentration, the toxicity of which was being determined. Then, 0.6 mL of cultivation water was poured into the control wells, and 0.6 mL of the test solution was added to the experimental wells. The micro-aquarium was placed in a thermostat and kept for 24 hr at a temperature of 22–24 °C. Next, the numbers of individuals was counted under the microscope.

To assess the acute toxicity of a sample, the percentage change in the number of paramecium (*A*, %) was calculated by Eq. (2):

$$A = \frac{X_d}{X_i} \times 100\% \quad (2)$$

where, X_i and X_d are the number of individuals initially and after 24 hr, respectively.

The acute toxicity of DS solutions on infusoria (*P. caudatum*) was defined as a change of at least 50% in the number of infusoria during 24 hr. The concentration of DS was considered harmless (not leading to acute toxic effect) if the change of number of infusoria did not exceed 10% during 24 hr.

1.4.3. Biotesting on Chlorella vulgaris green algae

The method was based on registering differences in the optical density of the test culture of the *C. vulgaris* green algae grown on a medium that did not contain toxic substances (control) and in the studied solutions.

2 mL of the test culture in 50% Tamiya medium containing 12.5×10^6 cells/mL was introduced into 48 mL glass vessels containing control and test solutions. The contents of each glass were mixed and 6 mL of the mixture was then poured in 4 reactors, which were then placed in a multi-cell cultivator KVM-05 (*T* = (36 ± 0.5)°C, light intensity 60 W/m^2, hold-up time 22 hr). After 22 hr, the optical density (λ = 560 nm) was measured in each reactor.

The relative difference in the average optical density (*l*, %) for each dilution compared with the control was calculated by Eq. (3):

$$l = \frac{D_c - D_t}{D_c} \times 100\% \quad (3)$$

where, D_c and D_t are average values of optical density in the control and test solution, respectively.

The toxicity criterion on algae tests was at least 20% change during 22 hr in the optical density level due to inhibition or stimulation of the algae sample with respect to the reference.

1.4.4. Biotesting on the wheat seeds of Triticum vulgare

The technique was based on measuring the length of the roots of wheat seedlings in the early stages of development. 25 dry disease-free seeds with germination of at least 95% were placed into a Petri dish. Then 5 mL of the test solution was added to each, and distilled water was added to the control Petri dish. All samples were placed in a thermostat for 7 days. Three parallel experiments were then conducted. After the experiment, the length of the longest roots was determined.

The phytotoxic effect (%) was determined by comparing the length (L_{av}, mm) of the roots of the control and experimental seeds:

$$L_{av} = \frac{\sum L_i}{n} \quad (4)$$

where, L_i (mm) is the length of the maximal root of each seed, and n is the total number of seeds used for the experiment.

Phytotoxic action was considered confirmed if the change in the root's growth was at least 20%.

2. Results and discussion

When studying the self-organization of DS aqueous solutions using the DLS method, it was found that within the calculated concentration range of 1×10^{-3}–1×10^{-18} mol/L, they were dispersed systems, which is typical for aqueous solutions of many BACs (Konovalov and Ryzhkina, 2014; Konovalov et al., 2015; Ryzhkina et al., 2014, 2015, 2017, 2018, 2019). At concentrations from 1×10^{-3} to 1×10^{-8} mol/L, the particle size distribution based on light scattering intensity was monomodal, and the systems contained a dispersed phase of about a hundred nanometers in size (Fig. 1a and b). The correlation functions and volume particle size distributions in this concentration range are given in Appendix A Figs. S1 and S2.

In the concentration range of 1×10^{-9}–1×10^{-12} mol/L, particles were not detected by the DLS method (the silent zone), which, as shown earlier by the NTA method, may be associated with a decrease in their number (Ryzhkina et al., 2015). Starting at the calculated concentration of 1×10^{-13} mol/L and up to 1×10^{-18} mol/L, the particles were again identified, with the particle distribution in terms of light scattering intensity being bimodal. In the systems, particles of about tens and several hundred nanometers in size were formed (Appendix A Fig. S3). Below 1×10^{-18} mol/L, the DLS data were incorrect due to the polymodality of the particle size distribution.

A study of self-organization of the DS systems held in a permalloy container showed that in the absence of external low-frequency EMF in the sufficiently high concentration range of DS 1×10^{-3}–1×10^{-7} mol/L, particles were formed (Fig. 1c and d), but the particle size distribution was somewhat different from that of the systems held on the laboratory bench (Fig. 1a and b). At lower concentrations of 1×10^{-8}–1×10^{-18} mol/L, no particles were detected in the systems by DLS.

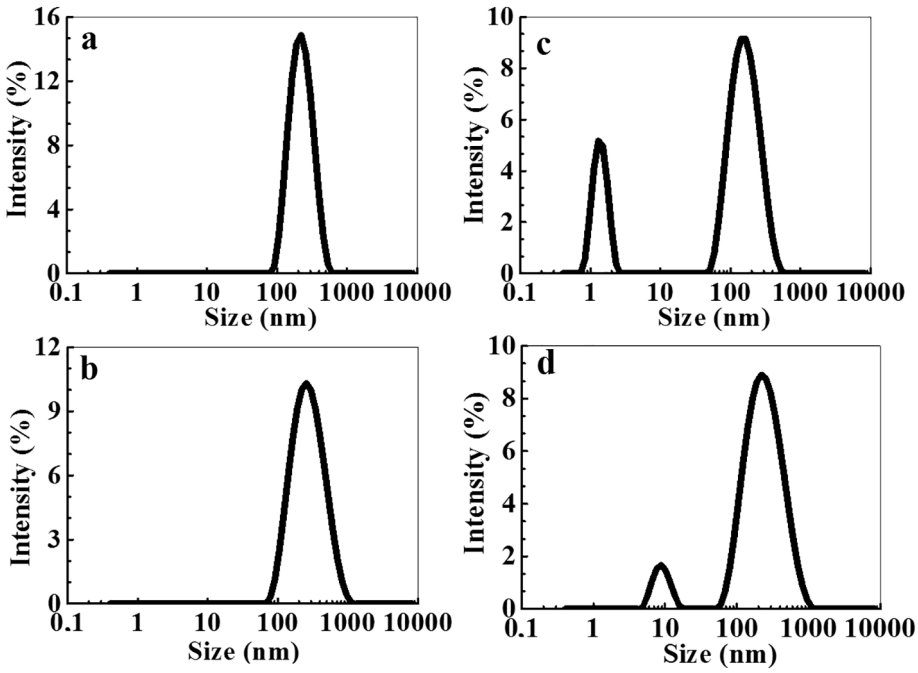

Fig. 1 – Particle size distribution based on light scattering intensity in the diclofenac sodium (DS) systems kept on a laboratory bench (a, b) and in a permalloy container (c, d). Concentration (c, mol/L) of DS as follows: 1×10^{-4} (a, c), 1×10^{-7} (b, d) mol/L, 25°C.

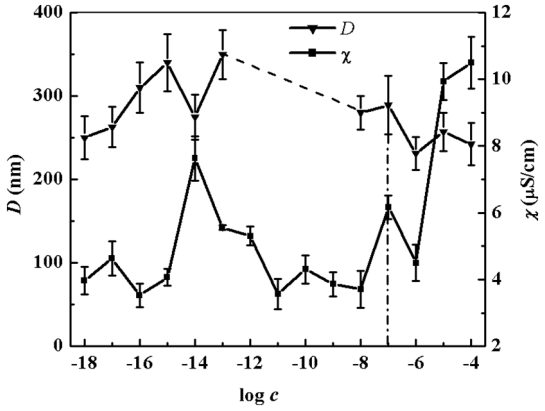

Fig. 2 – Dependence of the size of particles (*D*) and specific conductivity (χ) on the concentration (c, mol/L) of the DS systems, 25°C. Dashed line marks the threshold concentration (c_{thr}).

Therefore, according to Konovalov and Ryzhkina (2014), the concentration of 1×10^{-7} mol/L is the threshold concentration (c_{thr}), i.e., above this concentration in the range of 1×10^{-3}–1×10^{-7} mol/L in DS systems, a domain-type phase is formed (Sedlák, 2006; Sedlák and Rak, 2013); below this concentration, at 1×10^{-8} mol/L and in the range of 1×10^{-13}–1×10^{-18} mol/L, a nanoassociate-type phase is formed. In the range of 1×10^{-9}–1×10^{-12} mol/L there was a silent zone in which DS dispersed phases were not detected by DLS.

Fig. 2 shows the nonmonotonic concentration dependence of the size (*D*, nm) of particles, dominated by the intensity of light scattering, for samples held on a laboratory table, indicating the c_{thr}.

This figure also shows the concentration dependence of the electrical conductivity (χ) of the systems. It can be seen that there is a relation between the *D* and χ dependencies. In the concentration range of 1×10^{-6}–1×10^{-8} mol/L the changes occur symbatically, and in the range of 1×10^{-13}–1×10^{-18} mol/L – in antiphase. The relationship between the parameters of the dispersed phase and the properties of the systems in the range of low calculated concentrations inherent in many BAC systems indicates that the change in the physicochemical properties of solutions is due to the rearrangement of domains and nanoassociates (Konovalov and Ryzhkina, 2014; Konovalov et al., 2015; Ryzhkina et al., 2014, 2015, 2017, 2018, 2019). One of the characteristic features of such systems is that in the silent zone, the values of the systems' physicochemical properties, i.e., χ, pH, dielectric permittivity (Δε), surface tension, etc., approach the values of the solvent, i.e., distilled water (χ = 3 μS/cm, Δε = 0, pH = 6.5).

Fig. 3 shows the nonmonotonic concentration dependences of pH and Δε, where $\Delta\varepsilon = \varepsilon_{solution} - \varepsilon_{solvent}$, with the extrema at 1×10^{-8}, 1×10^{-13} mol/L. In the range of 1×10^{-12}–1×10^{-9} mol/L, the changes in pH and Δε are insignificant, which is due to a very low DS concentration and likely an insufficient number of nanoassociates in this concentration range. This results in the fact that the ε values of the solution approach ε of distilled water, and the Δε values approach zero. In accordance with dielcometry theory (Oehme, 1958), the sharp growth of Δε at 1×10^{-8} and 1×10^{-13} mol/L indicates a rearrangement of the system, which is due to an increase in intermolecular interactions and polarization of the medium. As can be seen from the data in Fig. 2, it is at these concentrations that the nanoassociates are formed. As shown in Lo et al. (2012) and Ho (2014), a special directionality of dipole orientation is realized in the nanoassociates, which in turn leads to an increase in both the nanoassociates' dipole moment and the Δε of the medium.

For a more detailed study of DS system properties, we relied on spin-probe EPR, a sensitive and informative method for studying the structural and dynamic properties of micelles, vesicles, macromolecules, biomembranes, etc., that bind to the probe (Gonsalves et al., 2008; Berliner and Reuben, 1989; Gennis, 1989). The change in the spin probe EPR spectrum, retained in the dispersed phase due to physical interactions, makes it possible to estimate the rotational correlation time (τ_c) characterizing the probe mobility and the nature of the microenvironment in its localization zone.

The EPR method, which is very sensitive to changes in the molecular mobility of the spin probe and the physicochemical properties of its microenvironment, can also become a valuable tool for studying the dispersed phase state's changes while decreasing the calculated concentration of BAC. Since nanoassociates are mainly formed by ordered water dipoles, the orientation and mobility restriction of which vary with dilution, this is likely to affect the values of τ_c associated with the probe nanoassociates. A number of papers on this subject have been based on the example of surfactant systems, in particular sodium dodecyl sulfate (Vijayan et al., 1980) in the range of 1×10^{-1}–1×10^{-5} mol/L and cetyltrimethylammonium bromide (CTAB) in the concentration range of 1×10^{-3}–1×10^{-10} mol/L and temperatures of 25–45°C (Konovalov and Ryzhkina, 2014; Ryzhkina et al., 2015).

In this paper, DS systems were studied in a wide range of concentrations, using a 2,2,6,6-tetramethylpiperidin-1-oxyl (TEMPO) probe analogous to (Konovalov and Ryzhkina, 2014; Ryzhkina et al., 2015; Vijayan et al., 1980). To prove the binding of the TEMPO molecules by the dispersed phase, the DS/TEMPO systems' self-organization and physicochemical

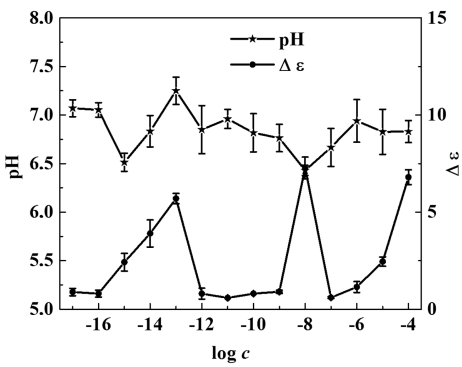

Fig. 3 – Dependence of pH and Δε in DS solutions on concentration (c, mol/L), 25°C.

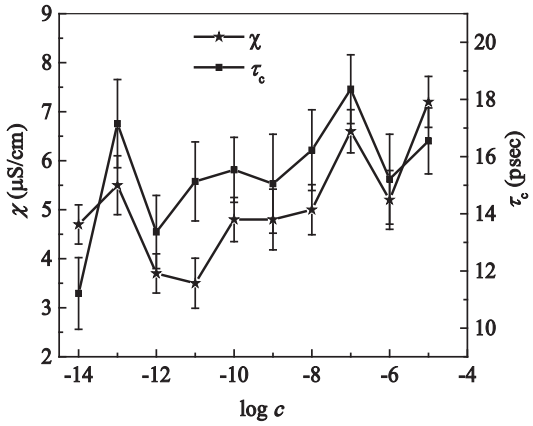

Fig. 4 – Dependence of specific conductivity (χ) and rotational diffusion correlation time (τ_c) of the TEMPO probe in the mixed DS/TEMPO system on concentration (c, mol/L) of DS ($c_{TEMPO} = 5 \times 10^{-4}$ mol/L).

properties were studied, in which the TEMPO concentration was 5×10^{-4} mol/L and the DS concentration varied in the range of 1×10^{-4}–1×10^{-14} mol/L.

No particles were detected in an aqueous TEMPO solution using the DLS method. Unlike DS systems, in DS/TEMPO systems in the concentration range of 1×10^{-4}–1×10^{-7} mol/L, dispersed phase with sizes of several nm and hundreds of nm were formed (Appendix A Fig. S4) similarly to the CTAB/TEMPO system (Konovalov and Ryzhkina, 2014; Ryzhkina et al., 2015). With DS concentrations below 1×10^{-7} mol/L, i.e., in the range of the silent zone and nanoassociate formation, particles could not be detected in the mixed DS/TEMPO system by the DLS method. This may be due to the fact that binding to TEMPO results in rearrangement and partial destruction of nanoassociates, i.e., decrease in their number, to a value that is probably insufficient for detection by the DLS method.

A nonmonotonic concentration dependence of χ in the DS/TEMPO system shows maxima at 1×10^{-13} mol/L and 1×10^{-7} mol/L, a plateau in the range 1×10^{-10}–1×10^{-8} mol/L and a silent zone at 1×10^{-12}–1×10^{-11} mol/L (Fig. 4 and Appendix A Fig. S5). Comparing the concentration dependences of χ in the DS/TEMPO and DS systems (Appendix A Fig. S5) suggests that the dependences are similar; however, below 1×10^{-7} mol/L the interval of concentrations for maximum values of χ and the silent zone are different by 1–2 orders of magnitude. As shown in Konovalov and Ryzhkina (2014) and Ryzhkina et al. (2015), the formation of the mixed dispersed phase, such as DS/TEMPO, can lead to a change of system properties and to a shift of concentration intervals, with maximum values of χ, and pH compared to the initial systems.

Fig. 4 shows the concentration dependence of τ_c in the DS/TEMPO system, at which the τ_c values drop nonmonotonically from 18 to 11 psec as the DS concentration decreases. The fact that the patterns of the concentration dependences of the τ_c values and the specific electric conductivity of the DS/TEMPO system were identical (Fig. 4) provides indirect evidence that the changes of τ_c, as well as of χ, are connected with the DS/TEMPO dispersed phase rearrangement, similarly to the DS systems (Fig. 2).

The existence of the same maximum τ_c value of 18 psec in both the domains (1×10^{-7} mol/L) and nanoassociate (1×10^{-13} mol/L) areas indicates that the mobility of the spin probe in these dispersed phases and the physicochemical properties of its microenvironment are practically the same, although the interval of their formation differs by six orders of magnitude in DS concentration. Similar results were obtained in the CTAB/TEMPO system (Konovalov and Ryzhkina, 2014; Ryzhkina et al., 2015).

Besides that, comparison of the data of Figs. 2 and 3 indicates that the maximum τ_c values occur around the concentrations at which a dispersed phase is produced that causes maximum changes in the physicochemical properties of the system and, probably, is able to exert the most pronounced effect on the biosystems. Analogous results were observed in Konovalov and Ryzhkina (2014) and Ryzhkina et al. (2014, 2015).

To test this assumption, we studied the effect of systems based on DS in a wide range of concentrations from 1×10^{-19} to 1×10^{-5} mol/L on the growth and development of the following representative hydrobionts: *C. affinis* cladocerans, *P. caudatum* infusoria and *C. vulgaris* unicellular green algae, as well as on the growth of the roots of *Triticum vulgare* wheat seeds.

The toxicity of the DS systems was determined from the death rate of cladocerans (as shown as Eq. (1)). Acute toxic action of DS systems on the *C. affinis* cladocerans was observed at concentration 1×10^{-5} mol/L (60% death rate of the tested object), ill effects (20% and 30% death rate) being found at 1×10^{-7} and 1×10^{-13} mol/L, respectively. The DS systems had no action on *C. affinis* cladocerans at concentrations of 1×10^{-9}, 1×10^{-11} and lower than 1×10^{-13} mol/L. Hence, the experiment on *C. affinis* at DS concentration below c_{thr} revealed the existence of a silent zone (1×10^{-9}–1×10^{-11} mol/L) corresponding to the absence of toxic action, whereas it appeared again at the DS concentration of 1×10^{-13} mol/L.

Similarly, the experiment using *P. caudatum* infusoria as the test object revealed biological effects of the DS systems at concentrations 1×10^{-5} and 1×10^{-7} mol/L (as shown as Eq. (2)). At those concentrations, the infusoria growth was stimulated by 32% and 20%, respectively, in response to the presence of DS, which affects this biosystem like a typical toxic pesticide, such as metaphos (Ryzhkina et al., 2017). In the range of concentrations from 1×10^{-9} to 1×10^{-11} mol/L, the toxic action on infusoria was absent (silent zone), appearing again at DS concentrations 1×10^{-13}, 1×10^{-15}, and 1×10^{-17} mol/L (stimulating the infusoria growth by 30%, 20% and 14%, respectively). At the concentration of 1×10^{-19} mol/L, the DS system did not affect the growth of *P. caudatum*.

The study of the stimulation of *P. caudatum* infusoria development revealed that the dependence of infusoria fertility (as compared to the control) on the concentration of DS in the habitat had a nonmonotonic behavior (Fig. 4), reaching a maximum at 1×10^{-5} and 1×10^{-13} mol/L (32% and 30%, respectively) and having no action at 1×10^{-9} and 1×10^{-11} mol/L (the silent zone).

The experiment using *C. vulgaris* unicellular green algae and *Triticum vulgare* wheat seed roots as test objects revealed

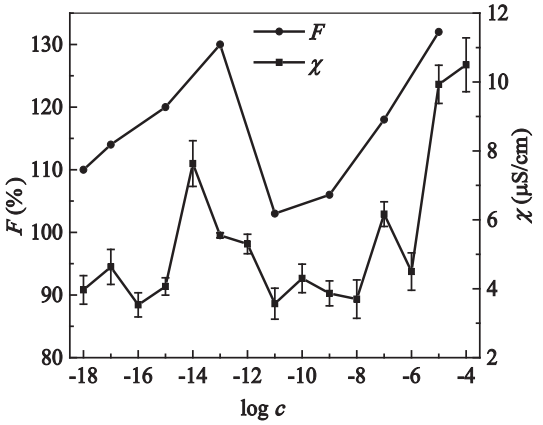

Fig. 5 – Dependence of the fertility (*F*) of infusoria and the specific conductivity (χ) of DS solutions on concentration (c, mol/L), 25°C.

virtually no negative effect of DS systems over the probed concentration range, since the change in the number of algae and the length of the roots did not exceed 20%, compared to the control (as shown as Eqs. (3) and (4)). Consequently, DS systems did not have a noticeable effect on the plant organisms, contrary to the action they had on the animal organisms.

Analysis of the results of the study of self-organization and physicochemical properties of DS-based systems and the data on the action these systems have on cladocerans and infusoria in the range of calculated concentrations from 1×10^{-19} to 1×10^{-5} mol/L indicates the relationship of these phenomena. DS systems affect the mortality of cladocerans and the fertility of infusoria in the intervals 1×10^{-5}–1×10^{-7} and 1×10^{-13}–1×10^{-17} mol/L, in which a dispersed phase of domain and nanoassociate type is formed. The greatest toxic effect on these hydrobionts in the low concentration range was observed at 1×10^{-13} mol/L, in the vicinity of which the maximum change in the size of nanoassociates and physicochemical properties of the systems occurred (Figs. 2–4).

Fig. 5 shows the non-monotonic concentration dependence of the fertility of infusoria and the specific conductivity of DS systems, which followed the same trend, with maxima at 1×10^{-5} and 1×10^{-13} mol/L and a minimum in the interval 1×10^{-11}–1×10^{-9} mol/L: in which the particle sizes could not be determined by the DLS method, probably due to a decrease in their number, as shown in Ryzhkina et al. (2015).

As shown in the work (Burlakova et al., 2003), devoted to the study of bioeffects of BACs of low calculated concentrations, one of the main ways leading to their appearance is the action on the structural and functional state of biomembranes. This in turn can change many biochemical processes and regulate the metabolism of plant and animal organisms by acting on the signal systems of the cell. It has been established (Zhigacheva et al., 2009) that low concentrations of BACs (10^{-19}–10^{-7} mol/L) can affect the level of lipid peroxidation in the mitochondrial membranes of plant and animal origin as well as the bioenergetic characteristics of these organelles, which play an important role in the body's response to stress. However, as mentioned above, there was no physicochemical substantiation of the effect of low concentrations of BACs.

Recent studies (Konovalov and Ryzhkina, 2014; Konovalov et al., 2015, 2017; Ryzhkina et al., 2011; Palmina et al., 2009) have significantly contributed to the elucidation of the mechanism of action of low concentrations of BACs on living objects.

The correlations between the parameters of nanoassociates formed in the range of BAC concentrations of about 10^{-18}–10^{-7} mol/L, the properties of the medium (pH, conductivity) and their in vitro effect on the structure (order parameter and microviscosity) of the lipid bilayer of various biomembranes were established. It was noted that a change in the structural state of membranes most clearly correlates with a change in the pH of the medium associated with the restructuring of nanoassociates. As mentioned above, modification of the membrane structure under the influence of external factors leads to a cascade of changes in cell metabolism and, ultimately, to a change in the response of the biosystem, which may explain the occurrence of bioeffects in the interval of low concentrations of BACs.

This data are consistent with the results of the work (Munari et al., 2018), in which the combined effects of seawater acidification and DS presence (6×10^{-7}–6×10^{-6} mol/L) on oxidative stress-related parameters were investigated in the Mediterranean mussel *Mytilus galloprovincialis* and the Manila clam *Ruditapes philippinarum*. The activities of superoxide dismutase, catalase and cyclooxygenase, and lipid peroxidation and DNA strand-break formation were measured in both the gills and digestive gland. The results show that the biochemical parameters measured in both the mussels and clams were influenced by the reduced pH and the contaminant, and the biomarker variation patterns differed depending on the species and tissues analyzed.

Similarly to studies (Ryzhkina et al., 2011, 2017, 2018, 2019; Konovalov and Ryzhkina, 2014; Konovalov et al., 2015, 2017; Palmina et al., 2009), the obtained data could be interpreted as showing that the general factor leading to the nonmonotonic concentration profile of the biological effects is the consistent change in the parameters of the nanoassociates and physicochemical properties of the medium, which could significantly modify cladocerans mortality and infusoria fertility under the action of the DS systems in the low range of calculated concentrations.

3. Conclusions

Summing up the experimental evidence presented above, using a set of physicochemical methods it was shown for the first time that water solutions of the widely used non-steroidalanti-inflammatory drug DS (10^{-3}–10^{-18} mol/L) are dispersed systems in which the dispersed phase undergoes a rearrangement with dilution, accompanied by changes in its size and characteristic properties. This affects the nonmonotonic dependences of the system's physicochemical properties and nonmonotonic changes in bioeffects and the presence of a silent zone in the low concentration range. It was shown that DS solutions did not have a noticeable effect on the studied plant organisms,

contrary to the action they had on the animal organisms. Given the negative effects DS has on the aquatic environment, establishing the ability of DS solutions to form a dispersed phase can be the key to explaining their toxic action towards hydrobionts in the low calculated concentration range.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jes.2019.08.013.

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