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Application of a zebrafish embryo toxicity assay for the study of surface water toxicity in the Lower Ile river

Abstract: The quality of surface waters of Lower Ile river, Kapchagay and Kutry reservoirs was assessed in zebrafish (*Danio rerio*) embriotoxicity test. The test was performed according to OECD guideline test No. 236, the exposure period was 5-72 h post fertilization, direct mutagen methylmethanesulfonate (MMS) was used as positive control to assess test system response. The standard visual mortality criteria of the test were applied for evaluation of possible lethal or teratogenic effect of surface waters. Exposure to MMS in concentration of 3.4 mg/L resulted in coagulation of 33.3% of embryos ($p \leq 0.01$) and almost 90% ($p \leq 0.01$) of survived embryos displayed various kinds of malformations to 72 hours post fertilization, which indicates test system susceptibility to the mutagens. It was established that none of surface water samples possess significant embryo toxic effects but all induce the spectrum of malformations related to axial skeleton (scoliosis, end tail malformation), water/salt balance and chorion permeability (oedema) and growth patterns (growth retardation) in different incidences. The lowest rate of teratogenicity was observed in embryos incubated in samples from Kapchagay bay (28.2%, $p \leq 0.05$) and Ile river at Bakanas region (site 2, 27.2%, $p \leq 0.05$). The teratogenic effect of water samples from Kurty reservoir and Ile river (site 1) was commensurable – 33.3% ($p \leq 0.05$) and 36.0% ($p \leq 0.01$) respectively. Among all tested sites only the samples of surface waters form Kurty pond produced multiple phenotypic effects in zebrafish *Danio rerio* embryos congruous to MMS exposure and especially, relatively high level of growth retardation, proposing the presence of disrupting or alkylating compounds in surface water samples.
Key words: bioassay, zebrafish embryos, teratogenicity, mutagen, malformations.

Introduction

The assessment of the surface water quality is of highest importance for a great deal of potentially hazardous contaminants like heavy metals, polycyclic aromatic hydrocarbons (PAH) or polychlorinated biphenyls (PCBs) or polychlorinated dibenzodioxins and furans (PCDD/PCDF), and many other compounds unwanted in the environment may present in aqueous environment. Chemical analysis is very expensive and has the main disadvantage that exclusively the target compounds are detected thus the method is blind for unexpected chemicals or even unknown compounds. Bioassays allow analyzing unwanted effects on organisms of all contaminants present in the water sample within an integrated process, addition of toxic effects and even possible synergistic effects of multiple compounds are taken into account [1].

Current approaches for water quality assessment apply a battery of standardized bioassays using all kind of aquatic organisms such as algae (*Desmodesmus subspicatus*), bacteria (*Vibrio fischeri, Arthrobacter globiformis*), invertebrates (*Daphnia magna, Caenorhabditis elegans, Lumbriculus variegates, Diporeia spp.; Hyalella azteca, Chironomus riparius, Potamopyrgus antipodarum*), yeast (*Saccharomyces cerevisiae*), plants (*Myriophyllum aquaticum*) and zebrafish embryos (*Danio rerio*) [2]. In order to better address potential toxicity of aqueous contaminants, the German joint research project DanTox – Development and application of a method for the measurement of specific toxicity and molecular effect mechanisms of sediment-bound environmental pollutants using the zebrafish (*Danio rerio*) – was realized for estimation of teratogenicity, neurotoxicity, genotoxicity, mutagenicity, and subcellular mechanistic effects in embryos of the zebrafish [3]. Use of

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zebrafish embryos in bioassays is beyond any doubt as this test object has numerous advantages that make it excellent alternative method to the *in vivo* embryotoxicity and teratogenicity assays: significant number of objects obtained (one female produces up to 300 eggs); transparent chorion greatly facilitates visual investigation; rapid development (hatching takes place at 27 hours post fertilization); embryos are small and easily incubated in Petri dishes or cultural plates, thus combining the advantages of cell culture and embryo culture systems; in addition, vast literature on zebrafish experiments is available [4-7].

The standard ISO 15088:2007 "Water quality – Estimation of the acute toxicity of waste water to zebrafish eggs (*Danio rerio*)" specifies a method for the determination of degrees as a measure of the acute toxic effect of waste water and industrial effluents to fish eggs within 48 h. This provide an inimitable tool for bioassay of surface waters in rivers and reservoirs of Republic of Kazakhstan. Among them Lower Ile river and two big reservoirs – Kapshagay and Kurty are of great interest for Almaty region since they support agricultural life in adjacent areas and are actually a stock for a many polluted streams. In order to investigate the potential toxic effect of surface waters on early stages of fish life the bioassay based on *Danio rerio* embryotoxicity test was performed in the Laboratory of mutagenesis, Department of molecular biology and genetics, al-Farabi Kazakh National University. The goal of this study was the assessment of embryotoxicity (acute lethal effect) and teratogenicity of surface water of Lower Ile river and adjacent reservoirs in *Danio rerio* embryo test.

Materials and methods

Water samples collection. Surface water samples collection, filtration and conservation were performed according to the GOST (State Standard) 31861-2012 "Interstate standard. Water. General Sampling Requirements" [8]. Composite samples were taken at the following sites: 1) Kapchagay bay, 43054'45.90'' 7705'41.79''; Kurty pond, 43051'27.11'' 76020'9.84''; Ile river, site 1, 43055'7.38'' 7705'49.99''; Ile river; 2), Bakanas, 44047'43.95'' 76016'34.89''.

Animal care or egg production. A breeding stock of unexposed and healthy mature zebrafish was used for the egg production. Mature fish were maintained in aquaria at 22-24°C with a loading capacity of a minimum of 1L per fish and natural light/dark cycle. Dry flake food or live food was fed once a day. Before spawning fish were kept hungry for a day. For egg production a nest of spawners (male : female ratio 1:2) was placed for the night in a sterile spawning aquaria preliminary filled with fresh filtrated drinking water heated up to 26ºC [9]. To prevent egg predation bottom was covered with neutral plastic mesh with a grid size of 2 mm. Spawning took place at the early morning and may be recognized by the decrease of female belly. Spawners and separate grid were than immediately removed and eggs were collected in sterile flask. Fertilized eggs were separated of unfertilized one using stereomicroscope Motic DM-143 (Motic, China) and used for the further assay.

Embryo exposure. Two controls were used for each experiment: 1) a positive control consisting of the aqueous solution of direct teratogen methylmethanesulfonate (3.4 mg/L MMS); 2) negative control of pure filtered water. Fish embryos were randomly transferred in sterile Petri dishes with medium volume of 25 ml. For bioassay water samples preheated up to 26ºC were used as incubation media and experiments were continuous throughout all period of embryonic development. The incubation was performed at incubator Binder at 26ºC for the 72 hours post fertilization (hpf). The exposure was stopped at 72 hpf when the hatching take place and it is easy to distinguish the malformations of spinal cord. Developmental parameters were monitored and documented according to OECD recommendations for acute embryotoxicity and teratogenicity assays [10].

Scoring. At different time points (24, 48 and 76 hpf) fish embryos were evaluated and scored for lethal or teratogenic effects using stereomicroscope Motic DM-143 (Motic, China). All embryos were staged according to Kimmel et al. [11]. Different lethal or teratogenic endpoints are summarized in Table 1.

Validity parameters and statistics. The fertilizing rate of the fish eggs should be higher than 50%. The assay is considered to be valid if the viability of the negative control eggs exceeds or is equal to 90% after 48 hpf (no lethal or teratogenic effects). The experiments were performed in triple sequence. Thus, typically 25 fish embryos per Petri dish and 75 fish embryos per group were used. Data was processed and visualized using MS Excel 2010 (Microsoft Office Professional Plus 2010 software). The standard error for each parameter is the result of three independent experiments.

Table 1 – Lethal and teratogenic effects observed in zebrafish *Danio rerio* embryos depending on the observation time, according to Busquet et al. [12]

Category	Physiological/dismorphogenic effect	24 hpf	48 hpf	72 hpf
Lethal effect	Coagulated eggs	+	+	+
Teratogenic effects	Malformation of head		+	+
	Malformation of tail		+	+
	Malformation of end tail		+	+
	Malformation of heart		+	+
	Scoliosis		+	+
	Deformity of yolk	+	+	+
	Growth retardation	+	+	+

Ethical considerations. All animal experimental procedures were conducted in accordance with the international regulations. Within the chorion, fish embryos are not subject to Directive 2010/63/EU, which regulates the use of animals in scientific experiments. As Article 1.3 of the Directive declares, independently feeding larval forms are subjected to Directive 2010/63/EU, the experiments stopped at hatching. In the current version of the test protocol the Fish embryotoxicity test is limited to two or three days and is classified as a non-animal test in legal terms.

Results and discussion

Zebrafish embryogenesis is a powerful *in vivo* model system to assess the quality of surface water and rapid developmental progression compared to mammals makes it an ideal for the embryo toxicity assay. We performed a set of experiment to assess the potential toxic and/or teratogenic profile of surface water of lower reach of Ile river and adjacent reservoirs. Experiments were started at 4-6 hpf and stopped at hatching that generally takes place at 72 hpf; by this time a larva has completed most of its morphogenesis. Actually the exposure began since early gastrula period of 50% epiboly when blastoderm remains unifrom in thickness and germ ring is visible from the animal pole. The assessment for lethal and teratogenic effects took place on 24, 48 and 72 hpf. Figure 1 summarizes the lethal and teratogenic effects of controls and water samples collected at Ile river basin in *Danio rerio* embryos at 72 hpf considered to be acute embryotoxicity effect.

As shown in Figure 1 the negative control triggered no lethal effects in the fish embryos with 2.7% of eggs coagulated and only 4% of eggs displayed malformations within three experiments. The total percentage of teratogenic and/or lethal eggs in each individual control group experiment consisting of 25 eggs each was < 10% after 48 hpf and thus all experiments were considered to be valid. The ability of the test object to response to direct teratogen was assessed in experiment with MMS, which is known to be highly toxic, teratogenic and mutagenic for vertebrates and thus was chosen as positive control. Exposure to MMS in concentration 3.4 mg/L resulted in coagulation of 33.3% of embryos ($p \leq 0.01$) and almost 90% ($p \leq 0.01$) of survived embryos displayed various kinds of malformations to 72 hpf so that test system is susceptible for the mutagens.

For the surface water samples only a very slight toxic effect (visual mortality criteria) was observed that is below required embryo toxicity level of 10% - mortality in Kurty pond was only 3.8%, 4.0% and 2.5% in Ile river (site 2 and site 1, respectively) and at Kapchagay bay was 8.9%(differences are not significant).

One may conclude that exposure of *Danio rerio* embryos to surface water of Ile river region did not lead to acute embryotoxic effect. However, when studying teratogenic effects the significant increase in number of embryos displaying various kinds of malformations has been detected (Figure 1, B). That is the lowest rate of teratogenicity was observed in embryos incubated in samples from Kapchagay bay (28,2%, $p \leq 0.05$) and Ile river at Bakanas region (site 2, 27.2%, $p \leq 0.05$). The teratogenic effect of water samples from Kurty reservoir and Ile river at bridge region (site 1) was commensurable – 33.3% ($p \leq 0.05$) and 36.0% ($p \leq 0.01$), respectively.

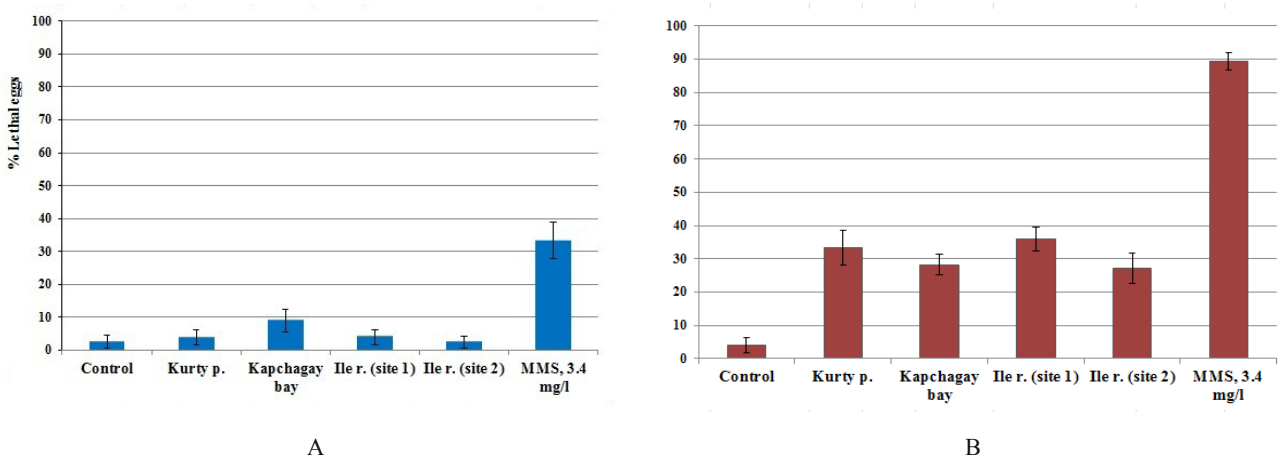

Figure 1 – Overview of the lethal (A) and teratogenic (B) effect in zebrafish embryos for 72 hours incubation in natural water samples
Note: the standard error for each parameter is the result of three independent experiments

It is very important to assess the individual morphologic malformations in zebrafish embryos exposed to surface water samples. The absolute incidences of the different induced teratogenic endpoints in zebrafish are summarized in Figure 2. The main malformations observed in fish embryos exposed to surface water were scoliosis, malformation of endtail, growth retardation and oedemas. A clear incidences of tail curvatures and endtail absence were only observed in the group exposed to direct mutagen MMS (data not shown). Teratogenic effects are considered as fingerprint endpoints, if the endpoint is observed in ≥ 50% of all teratogenic fish eggs in the test groups. Some malformations such as effects on the spinal cord, occured more frequently than others. The scoliosis defined as S-shapes curvature of the embryo trunk occurred in all groups of tested surface water with frequency more than 50%. Despite the level of teratogenic eggs in control group is only 4.0%, the gross was of scoliosis – 66.7%, whereas other 33.3% made up by malformations of tail (data not shown) and there were observed no such alterations as growth retardation, malformations of tail tip and oedema. Scoliosis incidences were as well observed in all tested groups: 84.5% it constituted in Kurty pond samples, 85.2% and 86.4% in Ile river (sites 1 and 2 respectively) and least in Kapchagay bay – 68.2%. Scoliosis leads to the defects in somitogenesis and influence the formation of excretory system. When transition to exogenous feeding, this spinal cord malformation is actually the precondition for the excessive larvae loss.

In our study the pericardial and yolk sac oedema were summarized to facilitate scoring and observed in three groups only: in water samples from Kurty p., Kapchagay bay and in case of embryo exposure to direct mutagen MMS, that is 53.9%, 22.7% and 20.9% of all malformation incidences, respectively. Pericardial oedema was occasionally so strong that the heart was prolonged into the narrrow tube, the yolk sac oedema was accompanied by local yolk destruction and accumulation of the liquid in the cavity. The accumulation of the liquid in the pericardium and/or yolk sac is usually referred to the disruption of water and salt balance in embryo.

It is very important to mention the role of chorion in zebrafish embryos resistance and susceptibility to xenobitics and maintenance of water balance in embryo. The chorion is the acellular, highly structured envelope enclosing a developing embryo, separating it from the external environment. The outer chorion membrane complex with a thickness of 1.5-2.5 μm consists of three layers: electron-dense outer and innermost layers with a thickness of 0.2-0.3 μm and 1.0-1.6 μm, respectively, separated by an electron-lucent middle layer (0.3-0.6 μm in thickness); the middle and inner layers are pierced by pore canals while the outermost layer is covered with projections of 2.0-3.0 μm in diameter [13]. The glycoproteins, which constitute the outer layer of the chorion, participate in creation of flexible membrane to which particles and microorganisms adhere. Chorion provides protection against microorganisms and protozoans accomplishes mechanical protection of the embryo and is assumed to offer an undefined protection against pollutants. Nevertheless, since in natural conditions the embryo is developed inside chorion and its barrier function must be considered in developmental ef-

fects of surface water the removal of chorion in case of bioassay is completely unjustified. It should also be taken into consideration that chorion permeability is not equal at all embryonic stages and for all group of substances. Although the pore canals are closed or obliterated, the chorion seems to be freely permeable to water, electrolytes and small molecules. It is suspected, that the chorion pores potentially restrict the uptake of compounds depending on their size. This was found for fluorescent dextrans exceeding 3 kDa as well as for polymers, higher molecular weight surfactants and nanoparticles [14]. As for lipophilic substances, most of which can penetrate membranes easily, it seems rather applicable that with increasing lipophilicity the substance is accumulated in the yolk and becomes available slowly at the beginning of yolk consumption, providing delayed toxic effect [15].

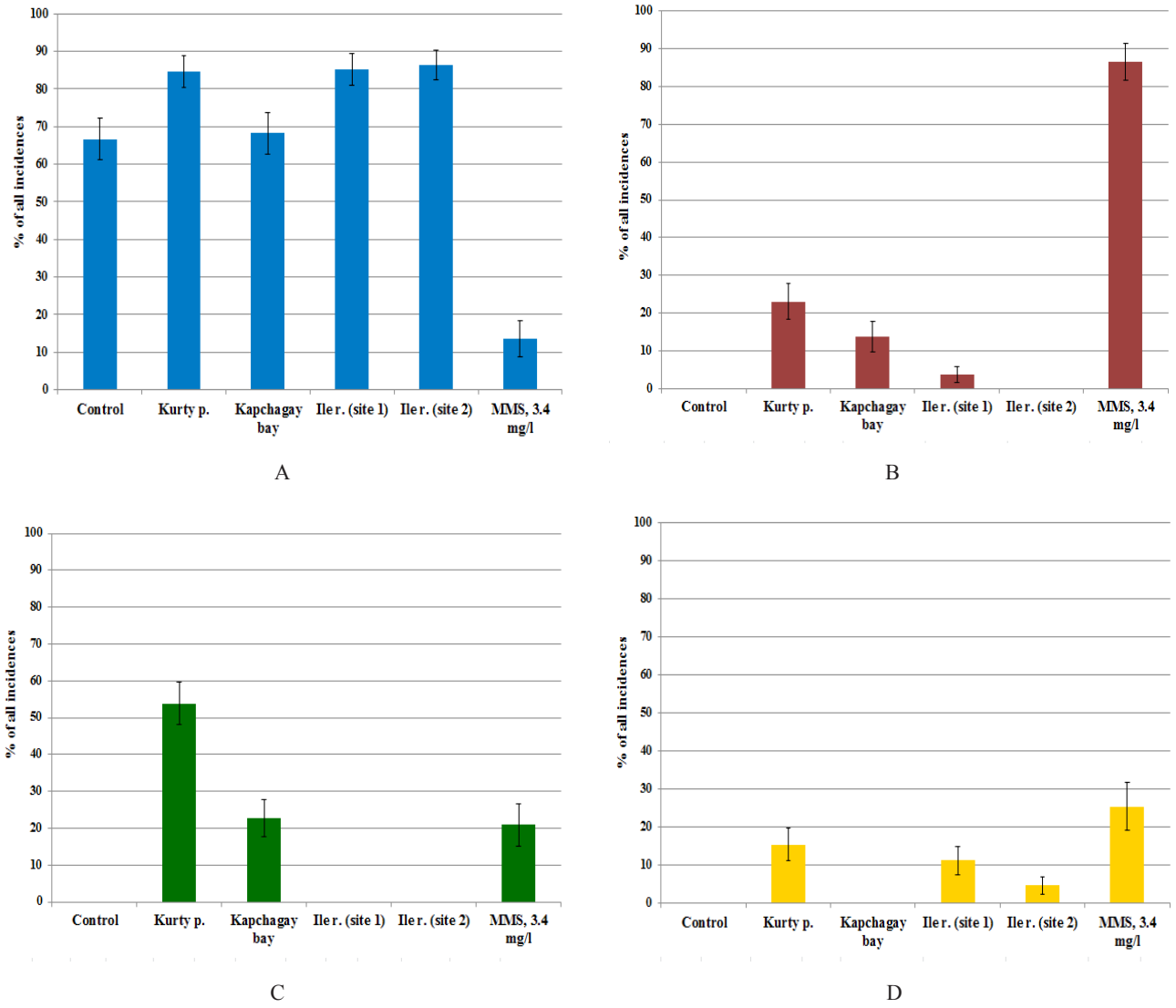

Figure 2 – Summary of individual morphologic malformations in zebrafish *Danio rerio* embryos observed for 72 hours incubation in natural water samples.
Note: A – scoliosis; B – growth retardation; C – oedema;
D – endtail malformation; the standard error for each parameter is the result of three independent experiments.

On the other hand, some studies showed that dechorionated embryos were less susceptible to the toxic effect of heavy metals than embryos with intact chorions, possibly because of the Donnan equilibrium: cations with negative standard electrode potentials (e.g. Zn^{2+}, Cd^{2+}, Pb^{2+}) would easily pass the

chorion (which acts as an ion exchanger) and would be accumulated in the perivitelline fluid. Thus, embryos with a chorion were more susceptible to these ions than those without. In contrast, cations with positive standard electrode potentials (e.g. Hg^{2+}, Cu^{2+}, Ag^{2+}) with high affinities to sulfhydryl groups would bind to the chorion, which thus would acts as a barrier [13]. Metal accumulation in the chorion seems to be strongly pH dependant – the lower the pH is, the more metal is bound to the chorion. In our experiments MMS promoted the excessive dye (methylene blue) accumulation in perivitelline space and further capture by embryo macrophages. In normal conditions the zebrafish chorion is impermeable for the dye but the exposure leads to disruption of its permeability resulting besides in excessive water and ions influx in embryo tissues and finally oedema. The penetration of toxic substances may also be facilitated. Dye accumulation was also revealed for the samples from Kapchagay bay and Kurty pond indirectly supporting the role of chorion permeability in oedema development.

Malformations of endtail in their way did not occur in water samples from Kapchagay bay but instead were registered in Ile river samples - both point 1 and point 2 (11.1% and 4.5% of all malformations), but the share is quite beyond fingerprint endpoint. Incubation of zebrafish embryos in surface water samples from Kurty p. produced 15.4% of end tail malformations being only in 1.6 times less than exposure to MMS (25.4%). There was recorded no growth retardation in negative control and Ile r. point 2 groups, minor share of 3.4% in Ile r. point 1 group and above required 10% limit observed only in Kurty p. and Kapchagay bay samples that is 23.1% and 13.6%, respectively. Growth retardation is considered to be one of the most noticeable teratogenic effect of chemical compounds and hardly resulted from environmental changes e.g. water temperature, osmolarity or hardness being the outcome of chemical exposure whether to cytotoxic or alkylating agent. In support a clear incidence of growth retardation was only observed in the MMS group – 86.6% of all malformations. Methylmethansulphonate was shown to significantly increase DNA damage in zebrafish embryo cells that was assessed in vitro and in vivo using the comet assay [16]. Reproductive and genotoxic effects in zebrafish after chronic exposure to methyl methanesulfonate was also determined in a multigeneration study [17]. It is also proved that MMS produces toxicity mainly by damaging cell membranes, induce cell death by activating the apoptotic pathway triggered by the activation of the mitochondrial damage pathway, cause sister chromatid exchanges and chromosomal aberrations [18; 19].

In our experiments in response to MMS embryos displayed multiple effects such as growth retardation, oedema, scoliosis, malformation or absence (occasionally) of tail tip characteristic for direct mutagen/teratogen. It should be noted that MMS as direct teratogen did not induce gross incidences of spinal cord malformation – in positive control scoliosis constituted only 13.4% whereas growth retardation in opposite shares about 85% of all incidences. Among all tested sites only the samples of surface water form Kurty pond produced multiple phenotypic effects in zebrafish embryos and especially, relatively high level of growth retardation, proposing the presence of disrupting or alkylating compounds in surface water samples. Other samples did not show significant effect, nevertheless induced malformations may be due to exposure to heavy metals (oedema), surface active agents or aromatic carbohydrates (scoliosis) and others. Taken together, the surface water samples from lower rich of Ile river, Kapchagay and Kurty reservoirs did not possess significant embryo toxic effects but did induce the spectrum of malformations related to axial skeleton (scoliosis, end tail malformation), water/salt balance and chorion permeability (oedema) and growth patterns (growth retardation) in different incidences. One may predict that dwelling fish species at the early stages of their life cycle, that is considered to be the most susceptible and vulnerable stage, would be exposed to potent teratogen(s) resulting in fish population deterioration. Anyway, it could be argued that zebrafish embryos are the very susceptible, effective and informative tool for bioassay especially in terms of embryonic toxicity for vertebrates and further investigations directed towards clarification of toxic substances and their toxic effects for vertebrates in active surface water samples.

Conclusion

In the present study, we assessed the quality of surface waters of Lower Ile river, Kapchagay and Kutry reservoirs using zebrafish (*Danio rerio*) embriotoxicity test. It was established that none of surface water samples possess significant embryo toxic effects but all induce the spectrum of malformations related to axial skeleton (scoliosis, end tail malformation), water/salt balance and chorion permeability (oedema) and growth patterns (growth retardation) in different incidences. The lowest rate of teratogenicity was observed in embryos incubated in samples from Kapchagay bay (28.2%, $p \leq 0.05$) and Ile river at

Bakanas region (site 2, 27.2%, p≤0.05). The obtained knowledge of the embryotoxic effects of water will allow using them in further research on improving sanitary and hygienic standards and the developing medical and preventive measures.

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