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IN VITRO CYTOTOXIC AND TERATOGENIC POTENTIAL OF SEDIMENT EXTRACTS FROM SKADAR LAKE USING FISH CELL LINE RTL-W1 AND DANIO RERIO EMBRYOS

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Abstract - As a part of Sediment Quality Triad (SQT), organic extracts of sediment from Skadar Lake (a Mediterranean lake and the largest freshwater reservoir in southeastern Europe) were investigated in order to evaluate possible ecotoxicological contamination by organic pollutants and to obtain a comprehensive insight into the ecotoxicological hazard. Sediments were investigated for toxicity by two different bioassays. Acute cytotoxicity was investigated using the fibroblast-like cell line RTL-W1 (*Oncorhynchus mykiss*) in combination with the neutral red retention assay. The embryos of zebrafish (*Danio rerio)* were used to assess the toxic and teratogenic potential of organic extracts of the sediment. Preliminary results point to the presence of a cytotoxic and teratogenic potential in Skadar Lake sediment extracts in certain locations.

Key words: In vitro toxicity testing, sediment cytotoxicity, embryo toxicity, zebrafish (*Danio rerio),* Skadar Lake

INTRODUCTION

Sediments are the natural and ultimate reservoir for numerous potentially toxic chemical and biological contaminants. Many persistent organic pollutants (POPs) such as polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs) and organochlorine pesticides (OCPs) can be taken up by living organisms through contact with sediment, interstitial water and from food (Viganó et al., 2001). Persistent and ubiquitous chemicals accumulated in sediments can be responsible for multiple effects on living organisms at different ecosystem levels, affecting the DNA structure, viability of cells, organ function, reproductive status, population size, and ultimately species survival and biodiversity (Bolognesi and Hayashi, 2011). In addition, contaminated sediments in rivers, streams, lakes and coastal ports represent a potential threat and hazard to the ecosystem and human health. Contaminated sediments are known to have various adverse effects on organisms even when contaminant levels in the overlying water are low (Chapman 1989). The sediment contamination can have many detrimental effects on an aquatic ecosystem, some of which may be readily evident and others more subtle or unknown (Apitz et al., 2005). In most receiving waters the effects on an ecosystem are difficult to observe and require the use of a variety of investigation and risk assessment tools, such as benthic macroinvertebrate community analyses and bacterial community structure analyses using 16S rDNA-based fingerprinting methods such as TTGE (Kostanjsek et al., 2005). An alternative methodology to determine the toxicity of complex sediments

are biological tests, which produce a global response to the complex mixture of chemicals without any prior knowledge of the composition or its chemical properties (Zegura et al., 2009; Hollert et al., 2002).

Sustainable environmental development depends to a great extent on our capacity to monitor the effects of chemicals on ecosystems. Consequently, several *in vitro* bioassays have been used in assessing the potential toxicity of water sediments. For example, cell cultures, in particular those derived from fish, have been successfully employed as a biological alternative to living fish to assess the toxic effects of sediment extracts (Landolt and Kocan, 1984; Gagne et al., 1996; Kohlpoth et al., 1999; Kammann et al., 2001, 2004; Davoren et al., 2005; Woo et al., 2006; Traven et al., 2008). In addition, a large number of biomarkers have been identified over the last couple of decades that are now used to monitor the environmental effects of pollutants (Castano and Becerril, 2004).

The combination of different risk assessment tools is necessary as an instrument in the comprehensive process of ecological risk assessment (ERA) and weight of evidence approaches (WOE). The main objective of the Water Framework Directive (WFD) is to ensure a good ecological, chemical and hydro-morphological status in all water bodies, and it is another process that is expected to have a significant impact on the waters of the Balkan region. The implementation of the Water Framework Directive (COM, 2000) is changing the scope of water management from the local scale to basin (watershed or catchment) scale (often transboundary). Lake Skadar/Shkodra (Fig. 1) is a large and shallow Mediterranean lake shared between Montenegro and Albania in the western Balkans. It is the largest freshwater reservoir in southeastern Europe. Lakes larger than 100 km^2 are referred to as 'very large' lakes in the Water Framework Directive (WFD) (EU, 2000). The lake was promoted as a National Park in 1983 (Official Gazette SRCG, 1991) and as a wetland site of international significance and importance, a so-called Ramsar site (Site 3YU003, 1995). The lake's only surface outlet is the Buna River, which flows into the Adriatic Sea. This region of the Balkans is in a transitional phase, both politically and economically. As a result of political instabilities, water and sediment management have not progressed within a program of monitoring. Montenegro's program for monitoring aquatic ecosystems to date has been poor and in the last decades focused only on random chemical analysis. In this context, data on this lake are relatively limited concerning the ecotoxicological status of the water and sediment, and environment risk assessment. The research group from the EULIMNOS project has reported on the influence of contaminants on the Skadar Lake ecosystem. In 2004, the first measurements of SPMD water samples of the Lake were published and showed significant EROD-inducing and estrogenic potential, indicating that toxicologically relevant compounds were readily available for uptake by resident aquatic biota (Rastal et al, 2004). The phytoplankton community structure and level of chlorophyll *a* indicated moderate pollution of the Lake with organic compounds (Rakocevic-Nedovic and Hollert, 2005).

The aim of the present study was to investigate the toxic potential of the Skadar Lake sediment extracts using *in vitro* biotesting systems with the fish cell line RTL-W1 and zebrafish embryos (*Danio rerio*), and to propose future Lake monitoring for early detection of changes in the environment incurred by the pollution.

MATERIALS AND METHODS

Sample collection and extraction

Sediment samples were collected by means of an Ekman sampler (surface 225 cm^2) at three locations on Skadar Lake (Fig. 1). Radus (T1) is the lake site where waters from a strong underground spring mix with streams caused by the influx of two River Morača branches into the lake; it is also an important winter fish habitat. The middle lake site (T2) is located in the pelagic zone, distant from potential pollutant-discharging sites. The mouth of the River Morača site (T3) was chosen due to the fact that this river is a major source of the lake pollution by industrial

waters, mainly from the Podgorica Aluminum Plant (KAP) and municipal wastewaters of Podgorica city entering the river 10 to 20 km upstream (Fig. 1.)

After homogenization, samples were shock-frozen at -30°C and freeze-dried immediately. In a Soxhlet apparatus, the dried sediment from each site was separately extracted with acetone. Extracts were reduced in volume using a rotor evaporator and concentrated close to dryness with N_2, and the solvent was changed to dimethyl sulfoxide (DMSO). The maximum concentration of DMSO was 0.25% for the bioassays. The content of each extract was 20 g dry sediment in 1 ml of solvent. Analyses of toxicity of sediment organic extracts were performed at the Laboratory for Aquatic Toxicology, Institute of Zoology, University of Heidelberg, Germany.

Neutral red retention assay with fibroblast-like cell line RTL-W1

The acute cytotoxicity of the sediment extracts was determined with the neutral red retention assay as detailed by Babich and Borenfreund (1992), with modifications by Klee et al. (2004). Permanent cell lines RTL-W1 (Lee et al., 1993) from rainbow trout (*Oncorhynchus mykiss*) were used for biotesting. Extracts of sediments, dissolved in DMSO, were diluted with two times concentrated nutrient media L15 (to which 18% bovine serum albumin and 2% solution of penicillin/streptomycin were added). Eighty mg 3,4-dichlorphenol/ml. served as a control. Sediment extracts were serially diluted in L15 medium in microtiter plates and incubated for 48 h at 20°C, after which 0.4% solution of neutral red (2-methyl-3-amino-7-dimethylaminophenanzine) was added. Then again, cultures were incubated for 3 h at 20°C. Neutral red was extracted using a solution containing 1% glacial acid and 50% ethanol for 15 min at room temperature. Retention of the neutral line was determined photometrically at 540 and 690 nm (Borenfreund and Pürner, 1985) using a multiwell plate reader. The cytotoxic potential of individual extracts was subsequently calculated as NR50 values (effective concentration for 50% cell death in the neutral red test compared to the negative controls with non-exposed cells).

Early life-stage toxicity test with Danio rerio eggs

Adult zebrafish were bred at the Department of Zoology, University of Heidelberg, and kept in 160 L tanks under flow-through conditions at a water temperature of 27.0 ± 0.5°C (Hollert et al., 2003). Fish were fed twice a day with TetraMin dry flakes (Tetra, Melle, Germany) and *Artemia* nauplii. *Danio rerio* embryos (Fig. 2) were exposed to different concentrations of the sediment organic extracts and after 24 h and 48 h of exposure embryo development was observed using an inverted microscope to determine mortality. Criteria for the lethal effect (Table 1) are the presence of somites, coagulation of embryos, detachment of tail, development of eyes, developmental retardation, heart functioning, blood circulation, pigmentation, formation of edema, spinal deformations and hatching, etc. Mortality was determined through EC_{50} (effective concentration that kills 50% of cells) (Lange et al., 1995; Ensenbach, 1998; Nagel, 2002; Hollert et al., 2003). Percent mortality was calculated using Prisma 2.01, Graph Pad Inc., USA (Laale, 1977; Westerfield, 1987). According to the German DIN regulation it is considered that if a trial shows a 20% mortality compared to negative controls, then the test is positive, indicating substrate toxicity. The higher the percentage of mortality, the greater the toxicity of the substrate. For the experiment, 300-1000 fertilized eggs were exposed to sediment extracts in 24-well microtiter plates (in 2 ml standard water pH 7.8±0.1 (ISO 7346/3 according to DIN 38415-6-v). Negative controls were 10 eggs in 1 % solution of DMSO. Eggs in a 0.37 mg 3.4-dihloranilin (DCA)/l solution served as a positive control. The 24-well microtiter plates with eggs were incubated at 25°C and after 24 and 48 h exposure the development of embryos using microscopic technique was observed.

RESULTS AND DISCUSSION

Acute cytotoxicity tests with the fish cell line RLT-W1 are widely utilized in *in vitro* assays in ecotoxicology (Segner, 1998). In the growth medium, sediment extracts were diluted 7 times in concentrations

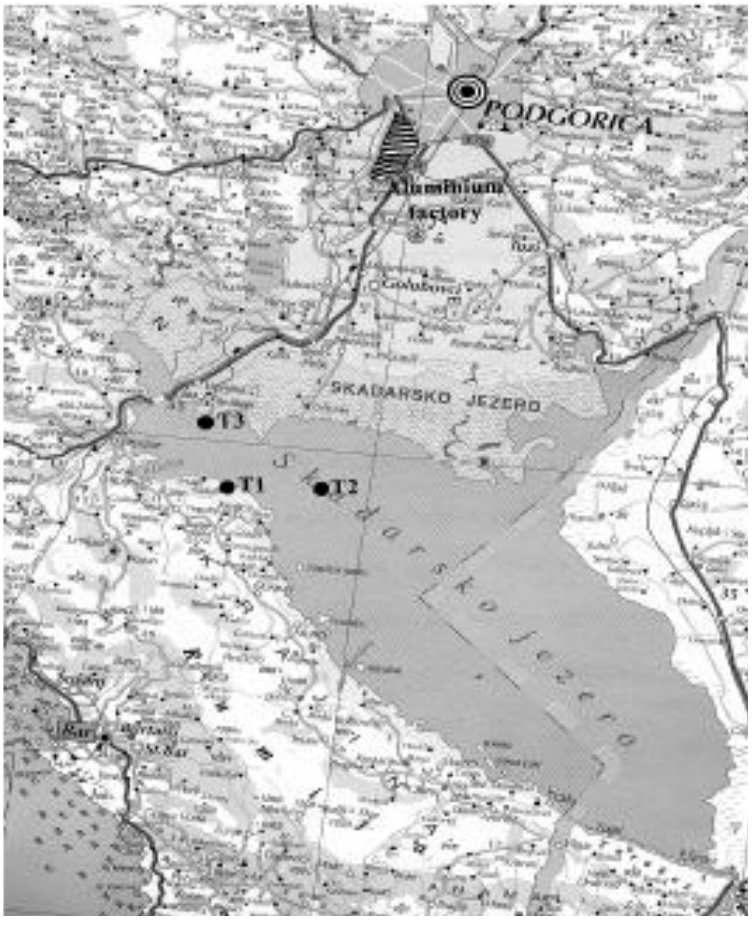

Fig. 1. Map of the locations of the various sampling sites on Skadar Lake.

ranging from 1:1 to 1:1000. The viability of the cells is expressed in relation to the viability of cells that were not exposed to samples. The results obtained on the RTL-W1 cells showed a potential cytotoxic effect of the sediments. Recorded test results ranged in values: at the Radus site (T1) NR50 was 5 mg/ml (Fig. 3); at the middle lake site (T2) NR50 was 9 mg/ml (Fig. 4) and at the mouth of the river Morača (T3) NR50 was 6.5 mg/ml (Fig. 5). Comparing the obtained results, the lowest cytotoxicity was found in sediments from sampling site T2 and the highest cytotoxicity was recorded from T1 and T3.

To assess the toxicity of sediments, early-life stage zebrafish embryos were used. The embryos were treated with different concentrations of sedi-

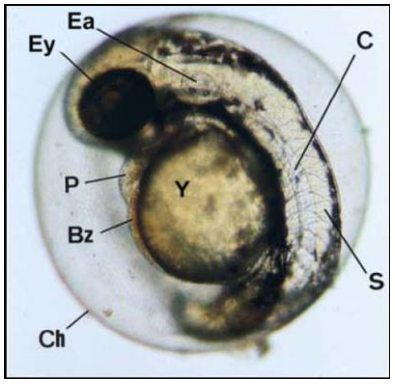

Fig. 2. Zebrafish embryo 48 h post fertilization, illustrating the transparency of egg and embryo which enabled us to examine major structures. Ey – eye anlage; Ea – ear; S – somites; C – chorda; Y – yolk sac; P – pericardium. Egg diameter was about 0.6 mm.

Table 1. Toxicological endpoints for the embryos recorded in the fish egg assay to determine the mortality of zebrafish embryos or larvae. ● = considered as a lethal effect, ○ = recorded but not considered lethal (according to Nagel 2002, modified (cf. Hollert et al., 2003c)).

Endpoint	24 h	48 h	72 h	96 h	144 h
Lack of somite formation	●	●	●	●	●
Coagulation of embryos or larvae	●	●	●	●	●
Non-detachment of tail	●	●	●	●	●
Non-development of eyes	○	●	●	●	●
Lack of heart function		●	●	●	●
Lack of blood circulation		●	●	●	●
Developemental retardation	○	○	●	●	●
Lack of pigmentation		○	●	●	●
Edema formation		○	●	●	●
Spinal deformations			●	●	●
Lack of Hatch		○	○	●	●

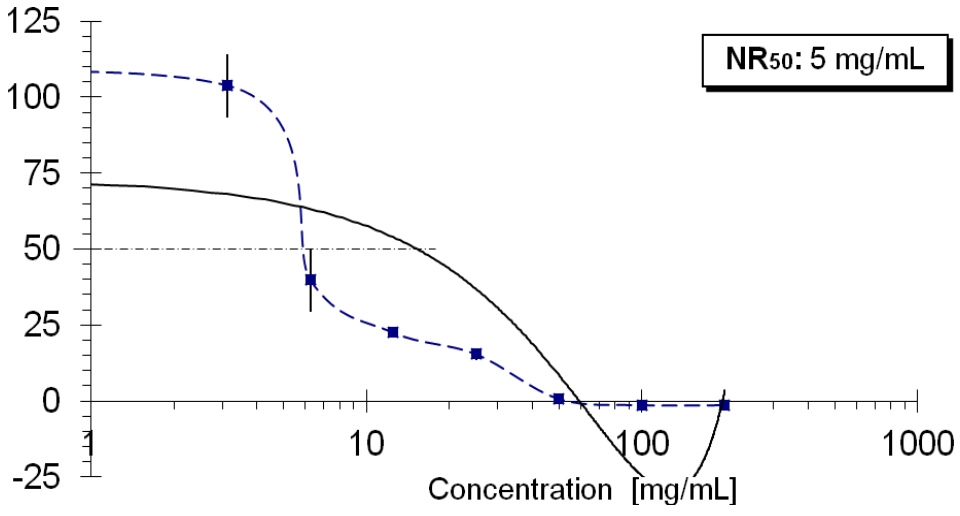

Fig. 3. Concentration response curves of the life cells RTL-W1 in culture according to concentration of sediment extract T1. The concentration of extract needed to destroy 50% of the cells was calculated.

ment extract (Fig. 6). After 24 h of treatment, the embryonic development and survival were observed using an inverted microscope. Based on the criteria for determining mortality (*D. rerio*; Ensenbach, 1998; modified, Hollert et al., 2003), the percentage of embryo mortality was calculated and the minimal effective concentration EC_{50} was determined. After 24 h of exposure, only the Radus, site (T1) sediment showed an increased mortality rate and EC_{50} 34mg/ml. After 48 h of exposure, the mortality of embryos increased, and in T1 EC_{50} was 33 mg/ml, in T3 it was 50 mg/ml and T2 60 mg/ml (Fig. 6).

The most prominent changes occurred in the embryos during development after an incubation period of 48 h with organic extracts of sediments, and were related to the occurrence of edema, coagulation of embryos and, less frequently, retardation, deformation of the spine and lack of pigmentation (Table 1).

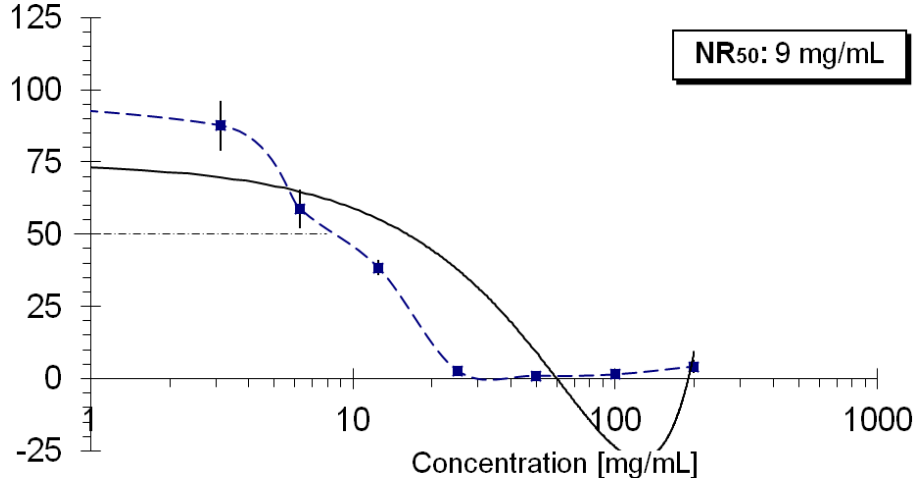

Fig. 4. Concentration response curves of the life cells RTL-W1 in culture according to concentration of sediment extract T2. The concentration of extract T2 needed to destroy 50% of the cells was calculated.

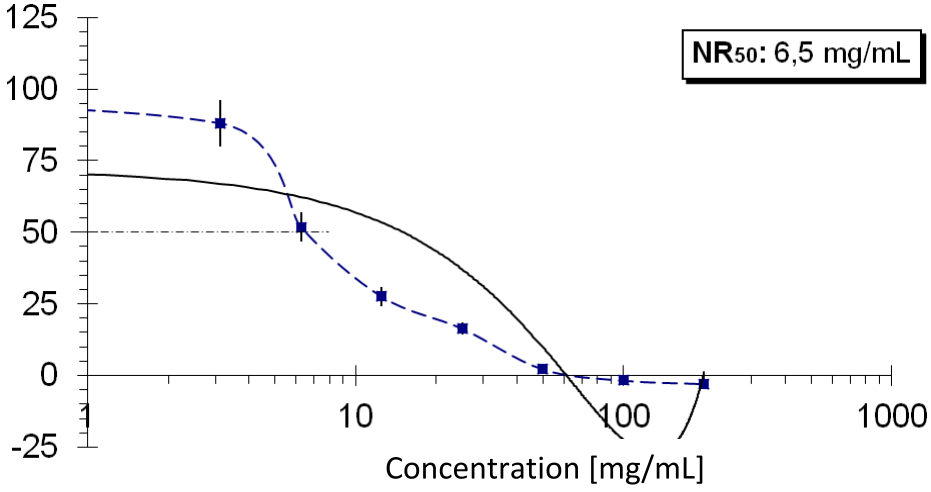

Fig. 5. Concentration response curves of the life cells RTL-W1 in culture according to concentration of sediment extract T3. The concentration of extract T3 needed to destroy 50% of the cells was calculated.

These results are consistent with our results for the sediment toxicity biotests on bacterial cells of *A. globiformis* (Perovic et al., 2007). A very similar toxic effect at the sampling sites was observed in the *Danio rerio* embryo test (Fig. 4) and the bacterial *A. globiformis* acute-cytotoxic test. However on the basis of these investigations, the second point by the intensity of toxicity, the mouth of the River Morača (T3), is the only approved site where in previous studies chemical measurements (Filipovic, Stesevic, Rastall) and analysis of the structure of microbial communities (Kostanjsek et al., 2005) found contamination that measurably affects the structures of living communities.

The toxic effect in the sampling site Radus can be linked to several possible causes. One is the possible influence of contaminated ground water origi-

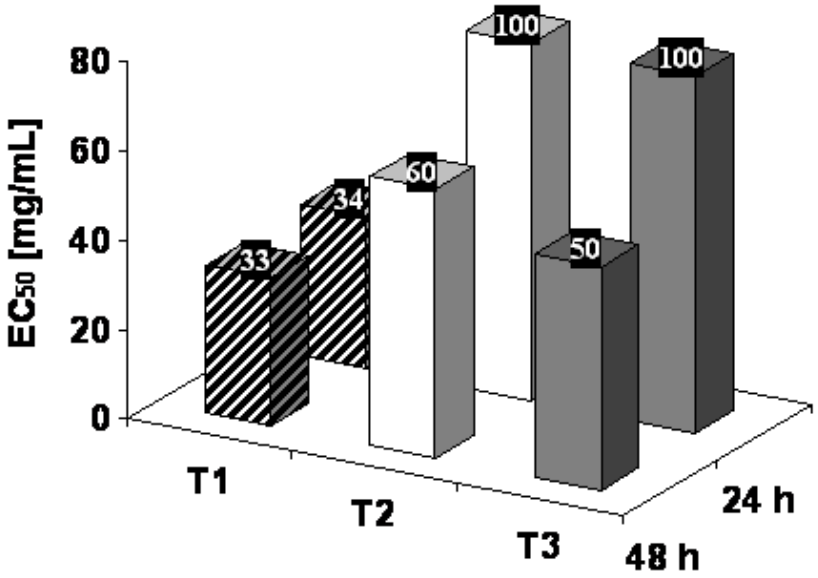

Fig. 6. Effective concentrations of sediment extract for 50% mortality of *Danio rerio* embryos (EC50) presented in bars for three investigated sites after 24 h and 48 h.

nating from Zeta valley and the influence of the aluminum plant and agriculture in the Zeta valley. Another more likely influence, in our opinion, could be streams carrying pollution from the Morača River and Virpazar village along the coastal base of Mt. Rumija. Moreover, we should also consider the possibility that the Radus is inhabited by large schools of fish during annual migration. The enrichment of the sediment with organic matter from fish feces may contribute to the increased accumulation of pollutants originating from the food chain.

CONCLUSION

The applied combination of biotests used for the determination of acute cytotoxicity and specific effects such as teratogenicity may prove to be a suitable tool for the assessment of sediment toxicity. It was shown that the neutral red assay with the RTL-W1 cell line and zebrafish embryo test are applicable to the Lake sediments. The toxic potential of sediment extracts from Skadar Lake was observed in both *in vitro* toxicity tests.

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