

ASSESSMENT OF THE GENOTOXIC POTENTIAL OF LAKE SKADAR SEDIMENTS USING AMES TEST AND COMET ASSAY ON THE FISH CELL LINE RTL-W1

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Abstract - In this study we evaluated the genotoxic potential of surface sediment extracts of Lake Skadar using a combination of two *in vitro* tests: the Comet assay on the fibroblast-like permanent cell line RTL-W1, and the Ames test on the strain *Salmonella typhimurium* TA98. The obtained results show that both tests were successful in determining the genotoxic potential in the sediment organic fractions. They possess enough sensitivity to detect early warning signals and evaluate the genotoxic potential in sediments of the Lake. The genotoxic potential was recorded and compared in the sediment samples from different locations on the Lake Skadar.

Key words: Genotoxic potential, sediments, Ames test, Comet assay, RTL-W1, Lake Skadar, Montenegro

INTRODUCTION

Sediments represent material created by the deposit of suspended particles from the water profile at the bottom of an aquatic ecosystem. Adsorbed particles in the sediments may be remobilized in the water profile and affect living organisms and aquatic ecosystems (Stronckhorst et al., 2004). Some particles may be remobilized via bioturbation or flood events (Hollert et al., 2000). Many pollutants, potential genotoxic agents such as heavy metals, polyaromatic and chlorinated substances and others, accumulate in the sediments (Hellmann, 1996; Neumann-Hensel, 2000; den Besten et al., 2003). Therefore, sediments may represent a persistent source of pollutants and a major emitter of toxic substances in aquatic ecosystems. A large number of pollutants accumulated in sediments can be responsible for multiple effects on

the organisms at different ecosystem levels, by affecting organ function, reproductive status, population size, and ultimately species survival and biodiversity (Bolognesi and Hayashi, 2011).

The investigation of sediments as a long-term secondary source of contamination, bioaccumulation and possible cause of adverse acute and chronic effects on living species, is of special interest in aquatic ecotoxicology and required by the European Water Framework Directive.

Montenegro's program for monitoring aquatic ecosystems up to now was generally poor and in the last decades focused only on random data of chemical analysis. In this study, we consider the possibility of the implementation and development of more specific biological endpoints by following toxicity bi-

omarkers with the goal of improving program strategy for the early detection of changes in the environment incurred by pollution.

Genotoxic chemicals induce DNA damage and mutations, and chronic exposure to some of these, even at low doses, may have an effect on the living community structure (biodiversity) and increase, among other things, the risk for cancer development in certain species. Several studies have shown a high association between fish liver tumors, neoplastic lesions in different organs of fish and polluted sediments containing PAHs and other organic genotoxins (Metcalf et al., 1990; Balch et al., 1995). Using only physico-chemical analysis it is difficult to quantify the risk associated with such chemical pollutants since they can occur at concentrations too low to allow precise analytical determination but still exert significant toxic effects on different species through chronic exposure and accumulation in tissues and organs. They can enter the food chain in much higher concentrations. Moreover, it can be very difficult to assume the synergistic and antagonistic effects present between compounds (Zegura et al., 2009), especially when many genotoxins and their metabolites in water and sediments have not been identified yet.

An alternative methodology to determine the genotoxicity of water samples and sediments are bioassays that generally produce a global response to a complex mixture of chemicals without the need for precise chemical characterization.

The aim of this study was to investigate the genotoxic potential of the Skadar Lake sediment extracts using *in vitro* bio-testing systems and to propose future monitoring of early changes in the environment incurred by pollution in the Lake.

MATERIALS AND METHODS:

Sampling locations

The investigated region was Lake Skadar (Montenegrin side). This area is classified as an internationally important Ramsar site (Skadar Lake, Site,

3YU003, Ramsar Convention, 2002) and promoted as a National Park IUCN Category II (Eco-net, 2002).

The sediment samples were collected in September 2003 on three locations on the Skadar Lake: Radus (T1) is a lake site where the waters from strong underground springs, mix with streams caused by influx of two branches of the River Morača into the lake; it is an important winter habitat for fish; the middle lake site (T2) is located in the pelagic zone, far from potential pollutant-discharging sites; the third site (T3) is at the mouth of the River Morača, chosen because the river is a major source of lake pollution of industrial waters, mainly from the Podgorica Aluminum Plant (KAP) and municipal wastewaters of Podgorica city, which enter the river 10-20 km upstream (Fig 1.)



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

Sediment extraction procedure

The sediments were taken from the bottom surface (0-5 cm) by Eckman's sampler (surface 225 cm²) and

held in the freezer after homogenization at -20°C (Hollert et al., 2002). The sediment samples were then dried in the laboratory freeze dryer (beta 1-8 K; Martin Christ, Germany) at -50°C. The samples were kept in bottles made from glass in darkness at 4°C (Kosmehl et al., 2004). Extraction of organic fractions was done from 20 g of drained sediments with 400 ml of acetone as solvent, over the course of 24 h by Soxhlet extraction (Kosmehl et al., 2004; Hollert et al., 2000). The obtained extracts were reduced in a rotor evaporator at 400 mbar, 36-38°C, to 10 ml and were additionally dried in a stream of nitrogen gas and dissolved in 1 ml of dimethyl sulfoxide (DMSO) (Hollert et al., 2000). The resulting concentration of extracts was 20 g dry sediment-equivalent per 1 ml of DMSO.

The process of sediment extraction and analyses of genotoxic potential were completed in the Laboratory for Aquatic Ecology and Toxicology at the Institute for Zoology, University of Heidelberg, Germany.

Cell cultures

The fibroblast-like permanent cell line RTL-W1, isolated from the liver of female trout *Oncorhynchus mykiss* (Lee et al., 1993), has a high biotransformation capacity when exposed to cytochrome P4501 (CYP1A) inducing substances, such as polycyclic aromatic hydrocarbons (Kosmehl et al., 2004; Behrens et al., 2001), di-benzo-dioxins (Lee et al., 1993) and other organic compounds (Segner et al., 2000; Behrens et al., 2001; Clemons et al., 1998). The cells were cultivated in Leibovitz's L15 medium according to the method by Klee et al. (Klee et al., 2004). They were trypsinized using 0.05% trypsin/0.02% EDTA and washed twice with PBS before being used in the Comet assay (Kosmehl et al., 2004).

Salmonella typhimurium TA98 *hisD3052 rfa* Δ *uvrB* *bio*-*pKM101* were used for mutagenicity assay (Maron and Ames, 1983). TA98 is excision repair deficient and carries chromosomal frameshift mutations *hisD3052*. This cell strain contains *rfa* mutation conferring increased permeability to large

molecules, and carries the mutated plasmid pKM101 (Maron and Ames, 1983). The bacteria were grown in LB medium (5 g NaCl, 10 g bacto-tryptone, 5 g yeast-extract, in 1,000 ml distilled water) at 37°C with aeration.

Comet assay

UV light (240-280 nm for 5 min) was used as positive control. For exposure to sediments, extract cells were transferred to 6-well plates and incubated for 12 h in medium to allow cell attachment to complete.

The Comet assay was performed according to Kosmehl and co-workers (Kosmehl et al., 2004). Cells were embedded in 0.7% low melting agarose (LMA) layers on the precoated slides and again coated on ice for 3 min and dried at 37°C for 5 min. Cell lyses was performed in 100mM EDTA, 2.5M NaCl, 1% Triton X-100 and 10% DMSO (pH 13.0) for 1.5 h in the dark at 4°C. After electrophoresis in the buffer at 25 V and 310 mA for 20 min, the samples were neutralized by incubation in 400 mM Tris at pH 7.4 for 2 min. Slides were directly analyzed or stored in PBS in a humid box for not more than 8 days at 4°C. Immediately before scoring, the DNA was stained with 75 ml of 20 mM ethidium bromide (Sigma-Aldrich) and cover-slipped.

Slides were examined using a fluorescent microscope (Leica, Germany). For each concentration, images of 100 randomly taken nuclei per experimental point were analyzed with the image analysis Comet 3.0 software (Kinetic Images, Liverpool, UK).

For statistical analysis, data were analyzed with the H-test according to Kruskal and Wallis (SigmaStat 2.03; SPSS-Jander, Erkrarh, Germany). The induction factor was calculated by dividing the median of each concentration by the median of the corresponding control group.

Ames test

The mutagenicity of the sediment samples was assessed by the *Salmonella* plate incorporation assay

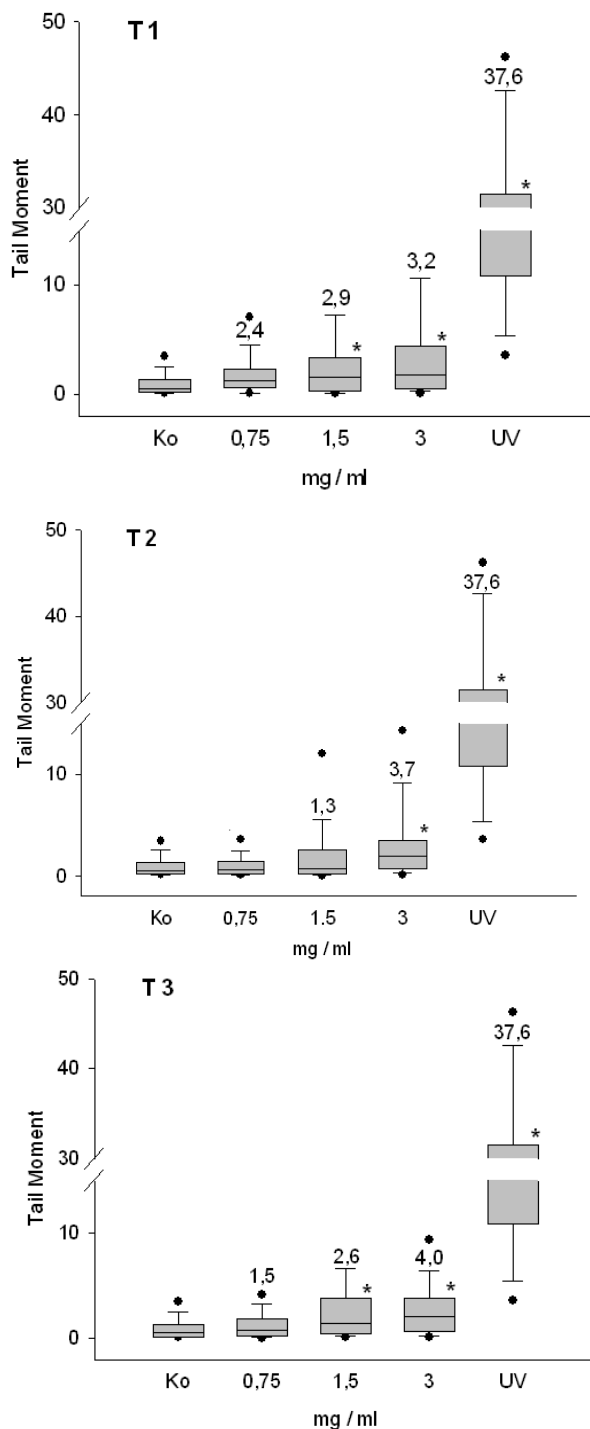


Fig. 2. DNA damage RTL-W1 cells according to the concentration of sediments (extract of sediments). Location, a) Radus (T1); Middle Lake (T2); Mouth of River Morača (T3).

* Significant genotoxicity (Dunnnett test with $p < 0.05$)

according to the standard test protocol of Maron and Ames (1983) with and without exogenous metabolic activation (S9 induction by β -naphthoflavone-phenobarbital).

The highest concentration tested in the assay was 2,000 mg/plate and the lowest was 62.5 mg/plate. Ten replicates for negative control and three replicates per each concentration were tested. Data were analyzed with the H-test according to Kruskal and Wallis (SigmaStat 2.03; SPSS-Jander, Erkrarh, Germany). Revertant colonies were counted and the induction factors were calculated by dividing the number of revertants of the extract concentrations by the mean of revertants of the negative control. A significant mutagenic potential was assumed if induction factor (IF) > 1.5 .

RESULTS AND DISCUSSION

This study presents the first investigation into the genotoxic and mutagenic potential of sediments in Lake Skadar. In order to detect potential genotoxic compounds from the sediments of Lake Skadar, we used a combination of the Ames test (prokaryotic reverse mutation test) and Comet assay (eukaryotic genotoxicity test). At present these tests are increasingly used to assess the genotoxic and mutagenic potential of wastewater (Stahl, 1991) and sediments (Marvin et al., 2000). The combination of different tests is recommended because all genotoxic substances do not lead to the emergence of mutations (Grummt, 2000; Reifferscheid and Grummt, 2000; Kosmehl et al., 2004).

In the Comet assay we used the fibroblast-like permanent cell line RTL-W1 isolated from the liver of female trout *Oncorhynchus mykiss* (Lee et al., 1993). These cells have a high biotransformation capacity when exposed to cytochrome P4501 (CYP1A) inducing substances, such as organic pollutants (Segner et al., 2000; Brack et al., 2002). Furthermore, RTL-W1 cells have relatively high biotransformation capacities, if compared to other fish cell lines such as RTG-2 cells (Kosmehl et al., 2004; Rocha et al., 2009). In this investigation, five concentrations of

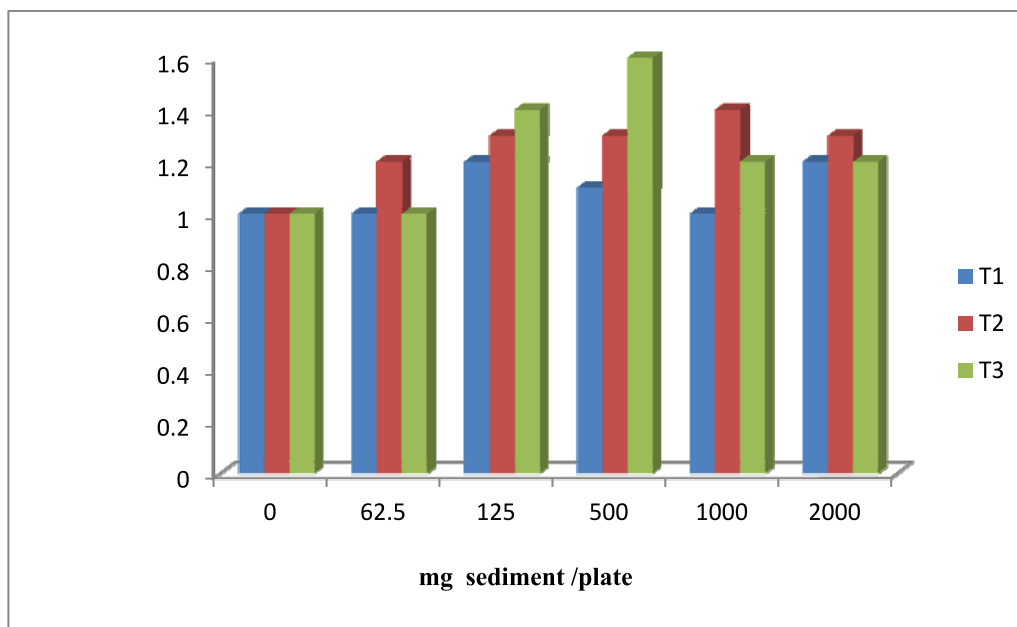


Fig. 3. Induction factor for mutagenicity of extract sediments in strain *S. typhimurium* TA98 without (S9) supplementation

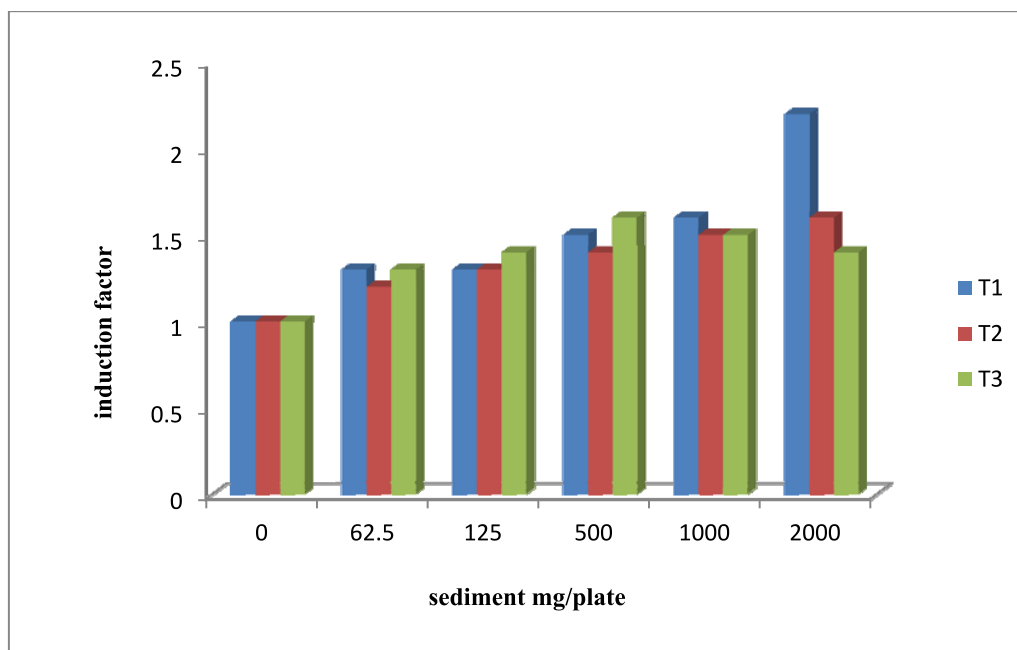


Fig. 4. Induction factor for mutagenicity of extract sediments in strain *S. typhimurium* TA98 with (S9) supplementation

sediment extracts were tested per sample. The highest used concentration per sample was adjusted to be below the level of toxicity (5 mg/ml) to the cell line RTL-W1, determined by using the neutral red cell toxicity assay (Perovic et al., 2004).

For sediment sampling locations, the Comet assay with RTL-W1 cells documented the genotoxic potential with a positive dose-response relationship (Fig 2a, 2b and 2c, showing data from the three sites). The genotoxicity was detected in sediments from the mouth of the River Morača (T3) and Radus (T1), starting from concentrations of 1.5 mg/ml, whereas the middle lake site (T2) as the reference site, had significant effects only at concentrations 3 mg/ml. In all samples, increasing the concentration increased the genotoxic potential. Therefore, the calculated maximal induction factors of the sediment samples ranged from 2.6 to 4.0. The highest genotoxicity was detected in sediments from mouth of the River Morača (induction factor was 4.0 at the concentration of 3 mg/ml).

According to water quality investigation using SPMD membranes (performed by Rastall and co-workers, 2004), the SPMD samples from the mouth of the River Morača collected during 30 days of SPMD membrane exposure induced significant EROD activity in RTL-W1 cells, normally triggered by various dioxin-like toxicants. This investigation proves the presence and entry of dioxin-like toxicants into Lake Skadar by the Morača water.

In the Ames test system, where the number of *his*⁻ → *His*⁺ revertants on strain TA98 without metabolic bioactivation was detected, only the sediment extracts from the mouth of the River Morača showed increased induction (Fig 3). The mutagenic potential of the sediments was observed with the concentration 500 mg/plate (Fig. 3) with an induction factor of 1.6. This result shows that components that could have potential mutagenic effects are present in the samples. In the test system of *S. typhimurium* TA98 with metabolic bioactivation (added S9 fraction), the enzyme system had no influence on the increased mutagenic activity of sediments from the site at the

mouth of the River Morača (Fig 4). An increasing number of *his*⁻ → *His*⁺ revertants was observed in the sediments from Radus, where the induction factor measured was 1.6 at a concentration of 1,000 mg/plate and 2.2 for the concentration of 2,000 mg/plate (Fig 4). A lower mutagenic activity was observed in the sediments from the middle of the Lake, where the induction factor was 1.5. We assume that components that require metabolic processing (it is pro-mutagenic compounds) are present in the sediments from the Radus and middle Lake sites. The results obtained in the test *S. typhimurium* TA98 with and without S9-induced metabolic activation indicated the possible presence of various contaminants in sediments of the Lake.

The identification of readily bioavailable pollutants in Lake Skadar using semipermeable membrane devices (SPMDs) and chemical analysis, showed the presence of hydrophobic organic compounds (Rastall et al., 2004). The most frequent class of organic pollutants, which were detected using GC-MS analysis (Rastall et al., 2004) in SPMD water samples, were alkylated PAHs and other PAH derivatives, sterols and sterol derivatives, and these compounds was measured in the samples taken from the mouth of the River Morača. These PP-PAHs certainly may contribute to the effect observed in the Ames test and Comet assay. Of the measured polycyclic hydrocarbons, the most present representatives are fluoranthene, benzo[a]anthracene and chrysene, known as mutagenic compounds. These compounds are available to the aquatic biota of Lake Skadar and pose a health risk to the environment and humans through the food chain.

Examination of potentially genotoxic compounds from the sediment samples revealed that the three selected locations could be considered as sites that do not show significant pollution degradation, but which indicate the presence of negative ecotoxicological impact of particle-bound pollutants transported presumably by the River Morača.

According to Kosmehl et al. (2004), the testing of sediments and suspended particulate matters for

genotoxicity should become a standard requirement for routine aquatic toxicity and wastewater tests, since aquatic organisms exposed to wastewater discharges and particle-bound substances suffer an increased risk of genetic damage. Aquatic organisms such as fish accumulate pollutants directly from contaminated water or indirectly through the ingestion of contaminated aquatic organisms. Genotoxic pollutants may lead to the contamination not only of the aquatic organisms themselves, but also of the entire ecosystem and, finally, of humans via the food chain (Matsumoto et al., 2006).

CONCLUSIONS

Differences in the genotoxic potentials of the different locations could be identified by both the comet assay and the Ames test, with a comparison and correlation with results from other research approaches such as bacteria community structure analyses (Kostanjsek et al., 2005) and chemical analysis of samples collected using SPMDs as a sampler (Rastall et al., 2004), and others. In this study, genotoxic potentials could especially be detected in the samples from the mouth of the River Morača and less so in those from Radus. However, in any conclusion on the state of the environment, these results should be combined with chemical measurements and hydrological data.

To get a clearer picture of the influence of anthropogenic factors on the state of the environment and natural habitats in Lake Skadar, it is necessary to make a wider study with a larger number of samples. It is also necessary to make an integrated study, which will combine in real time the results of bioassays with chemical analysis of water and sediments and the state of living species communities.

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