LOCAL ASSESSMENT OF WATER AND SEDIMENT QUALITY

AS A PREREQUISITE FOR WATER MANAGEMENT STRATEGIES IN JORDAN

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Local assessment of water and sediment quality

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Meinen Eltern.

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Contents

Summary

In order to clarify questions related to adequate and sustainable water management strategies in Jordan, the present thesis was initiated as an interdisciplinary project under the superordinate topic *"Water in sensitive regions – Handling limited water resources in sensitive regions of the Near East"* within the scope of the corporate project *"Global Change and Globalization"* of Heidelberg University within the scope of the Excellence Initiative II of the German Research Foundation, which was then integrated into the Heidelberg Center for the Environment. The objective of this dissertation was to elucidate the ecotoxicological hazard and risk of the main Jordanian surface waters (Jordan River, King Abdullah Canal, Yarmouk River, Wadi Mujib, Zarqa River) based on sediment (eco)toxicity assessment of a total of 20 sampling sites. To the best of knowledge, this study is the first to apply ecotoxicological bioassays to address water quality in terms of surface water sediment contamination in Jordan. The *in vitro* test battery included (a) general toxicity (cytotoxicity in the neutral red assay with RTL-W1 cells), (b) genotoxicity (DNA damage to RTL-W1 and V79 cells), (c) embryo toxicity (*Danio rerio*), and (d) dioxin-like activity (EROD assay with RTL-W1 cells), all of which were conducted with acetonic Soxhlet extracts of sediments. It was complemented by assessment of geomorphological parameters and measurement of nutrients and salts.

The result of the *in vitro* bioassays document that sediments from all surface waters were differentially polluted by contaminants that induced mainly genotoxic effects, but also cytotoxicity, embryo toxicity and elevated dioxin-like toxicity. Toxic potentials of the extracts were generally higher in the neutral red assay than in the fish embryo toxicity test. In most sediment samples, the comet assay proved to be more sensitive; however, for four sampling sites, the micronucleus showed stronger effects. The recently developed test design of a novel EROD assay including the use of β-naphthoflavone as a reference substance and the normalization of EROD activity against MTT reduction proved to be a most promising alternative to conventional protein-based normalization in EROD determination. Based on the differential results, a stepwise processing of toxicity assessment cannot be recommended, since a relationship between the different bioassays for toxicity assessment in terms of "if-then" or "if not-then not" could not be established in this study.

For the comprehensive classification of Jordanian surface waters, the results were rated according to toxicity threshold values based on a fuzzy logic-classification approach or according to a rank-sum based classification, resulting in three generalized toxicity levels. The bioassays were also rated according to ecological relevance, and the results were transferred into quality classes in accordance with the EU Water Framework Directive 2000/60/EC. Although results for the single rivers led to a heterogeneous pollution scenario, contamination hot spots could clearly be identified. In conclusion, the northern part of the Jordan River at Baqura, the outlet of Mujib Dam, the outlet of the Unity Dam of the Yarmouk River and the outlet of the wastewater treatment plant Khirbet As Samra discharging into the Zarqa River showed strong to moderate effects in at least four of the five tests applied and were thus rated quality class V indicating very high contamination. Results imply that sewage water treatment is not yet sufficient, particularly regarding mutagenic and dioxin-like compounds, and that non-point sources add to the overall pollution situation.

Since the results of this study suggested a certain discrepancy between conventional routine monitoring programs conducted by local authorities, which assign an overall good water quality to Jordanian surface waters, it is strongly recommend to include sediment toxicity assessment and effect-driven specific chemical analyses into regular monitoring programs and considerations for integrated water management.

Zusammenfassung

Die vorliegende Arbeit entstand im Rahmen eines interdisziplinären Projekts mit dem Titel *„Water in sensitive regions – Handling limited water resources in sensitive regions of the Near East"* als Teil der Exzellenzinitiative II *„Global Change and Globalization"* der Universität Heidelberg und der Deutschen Forschungsgemeinschaft, um Fragen nach einem nachhaltigen Wassermanagement in Jordanien zu klären. Das Projekt wurde in das *Heidelberg Center for the Environment* integriert. Ziel dieser Dissertation war es, das ökotoxikologische Gefahrenpotenzial jordanischer Oberflächengewässer (Jordan, King Abdullah Kanal, Yarmouk, Wadi Mujib, Zarqa) über die Beurteilung der Belastung der Sedimente von insgesamt 20 Probenstellen zu ermitteln. Es ist dies die erste Studie, die sich diesem Ansatz der Wasserqualität in Jordanien widmet. Mit Hilfe einer *In vitro*-Testbatterie wurden in acetonischen Soxhlet Extrakten folgende Parameter erfasst: (a) allgemeine Toxizität (Zytotoxizität im Neutralrottest mit RTL-W1 Zellen), (b) Gentoxizität (DNA-Schäden bei RTL-W1 und V79 Zellen), (c) Embryotoxizität (*Danio rerio*) und (d) dioxinähnliche Wirksamkeit (EROD-Bioassay mit RTL-W1 Zellen). Die biologischen Wirktests wurden durch die Erfassung von geomorphologischen Parametern, Nährstoffen und Ionen ergänzt.

Die *In vitro*-Tests ergaben, dass die Sedimente aus *allen* Gewässern mit diversen Substanzen belastet sind, wobei vor allem gentoxische Effekte gehäuft auftraten; aber auch Zyto- und Embryotoxizität sowie dioxinähnliche Effekte traten regelmäßig auf. Unerwarteterweise überstieg das toxische Potenzial im Neutralrottest in vielen Fällen die Effekte im Fischembryotest. Mit Ausnahme von vier Sedimentextrakten erwies sich der Comet-Assay als sensitiver verglichen mit dem Mikrokerntest. Ein neu entwickelter Bioassay zur Bestimmung der dioxinähnlichen Wirksamkeit von Umweltproben, der β-Naphthoflavon als Referenzsubstanz nutzt und die EROD-Aktivität auf die Zellvitalität im MMT-Test normiert, erwies sich als vielversprechende Alternativmethode zu herkömmlichen Assays mit Normalisierung der EROD-Aktivität auf Protein. Auf Grund der sehr differenzierten Effektmuster in den einzelnen Biotests erwies sich ein schrittweises Vorgehen bei der Ermittlung der Sedimenttoxizität als nicht praktikabel, da die einzelnen Tests funktionell nicht miteinander zusammenhängen und daher kein kausaler Zusammenhang zwischen einzelnen Tests im Sinne von „wenn – dann" oder „wenn nicht – dann nicht" hergestellt werden konnte.

Um die Charakterisierung jordanischer Oberflächengewässer zusammenzufassen, wurden die Befunde in einem Fuzzy-Logic-Ansatz unscharfen Toxizitätsklassen zugeordnet oder nach einem rangsummenbasierten Verfahren in drei Toxizitätsstufen eingeordnet. Hierbei wurden die Ergebnisse entsprechend ihrer ökologischen Relevanz gewichtet und in Qualitätsklassen eingestuft, die sich an der Europäischen Wasserrahmenrichtlinie 2000/60/EC orientierten. Trotz der heterogenen Belastungssituation der einzelnen Flussläufe konnten so Schwerpunkte der Kontamination ermittelt werden. So zeigten der nördliche Teil des unteren Jordans bei Baqura, der Auslauf des Mujib Reservoirs, der Ausfauf des Unity Damms am Yarmouk und die Mündung der Kläranlage Khribet As Samra in den Zarqa in mindestens vier der fünf Testsysteme wenigstens mäßige Effekte und konnten somit der Qualitätsklasse V mit hoher Kontamination zugeordnet werden. Die Ergebnisse zeigen, dass die Abwasserbehandlung in Jordanien vor allem hinsichtlich der Elimination gentoxischer und dioxinähnlich wirksamer Substanzen noch nicht ausreichend ist, und dass diffuse Schadstoffquellen einen wesentlichen Beitrag zur Belastung jordanischer Gewässer leisten.

Da die Ergebnisse dieser Studie durchaus Diskrepanzen zu den Monitoringprogrammen lokaler Behörden aufweisen, die den jordanischen Gewässern eine insgesamt gute Wasserqualität zuschreiben, wird dringend die Integration toxikologischer Untersuchungen und effektdirigierter chemischer Analysen in Routineüberwachungsprogramme empfohlen.

1. Introduction

1.1 Background of the thesis

In areas of water shortage and in view of scenarios predicting a modified water distribution as a consequence of climate change, limited access to adequate water supplies may have severe impact not only on ecosystems but also on the development of human activities. Appropriate distribution and usage of available water supplies is an essential prerequisite for the maintenance of human health and a sustainable development in such regions (Falkenmark and Widstrand 1992, Haines et al. 2006). Besides a minimum required quantity of water, water quality issues receive increasing attention (Bartram and Cairncross 2010, Hunter et al. 2010).

To elucidate questions of adequate and sustainable water management strategies in Jordan, the present thesis was initiated as an interdisciplinary work under the superordinate topic *"Water in sensitive regions – Handling limited water resources in sensitive regions of the Near East"* within the scope of the project *"Global Change and Globalization"* of Heidelberg University for the Excellence Initiative II of the German Research Foundation (DFG). As global change and globalization are very complex and extensive topics, the interdisciplinary project was subdivided into four groups of expertise:

Group I: Water in sensitive regions – Handling limited water resources in sensitive regions of the Near East (Jordan).
Group II: Global change and the energy system: Assessing options and their impacts.
Group III: Element cycles and socioeconomic dynamics – Understanding global processes on a local scale (Canary Islands).
Group IV: The psychology and neuroeconomics of ageing societies managing complex climatic systems: Hotter and greyer.

The present study is part of group I, together with the following projects:

- "Age and recharge rate of groundwater reserves in the Nubian and Disi Aquifers", Department of Environmental Physics,
- "Palaeodrainage systems, hydroclimatic changes and traditional water use in Egypt and Jordan", Department of Geography,
- "Water in the Middle East as an instrument of power – water conflicts, actors and discourses", Department of Geography,
- "Water Management in arid regions – a comparative legal study with a specific focus on groundwater utilization", Department for German and European Administrative Law and
- "Water and economic development – assessing contributions and constraints to growth in the Near East", Research Centre for Environmental Economics.

With the foundation of the Heidelberg Center for the Environment (HCE) in July 2011, "*Global Change and Globalization*" was integrated into this institutional strategy of the Excellence Initiative II.

1.2 Water shortage in Jordan

> "Our water situation forms a strategic challenge that cannot be ignored. We have to balance between drinking water needs and industrial and irrigation water requirements. Drinking water remains the most essential and the highest priority issue ".
>
> H.M. King Abdullah II, 1999

The Hashemite Kingdom of Jordan is a semi-arid to arid country in the north-western part of the Arabian Peninsula. It is bordered by Israel and the West Bank in the west, Syria in the north, Iraq in the east and Saudi Arabia in the south. The climate in Jordan is partly influenced by the Mediterranean Sea and is characterized by hot, dry summers and cool winters. Practically all precipitation occurs in winter and is centered in the western highlands. Rainfall diminishes towards the east, with large parts of the country receiving less than 100 mm a year, rendering two thirds of the country to be semi-arid to arid (Fig. 1). The aridity is also even more severe with high evaporation rates: about 92 % of the total rainfall of 8215 MCM (million cubic meters) evaporates, only 5 % recharges the groundwater reserves (Ministry for Water and Irrigation 2009).

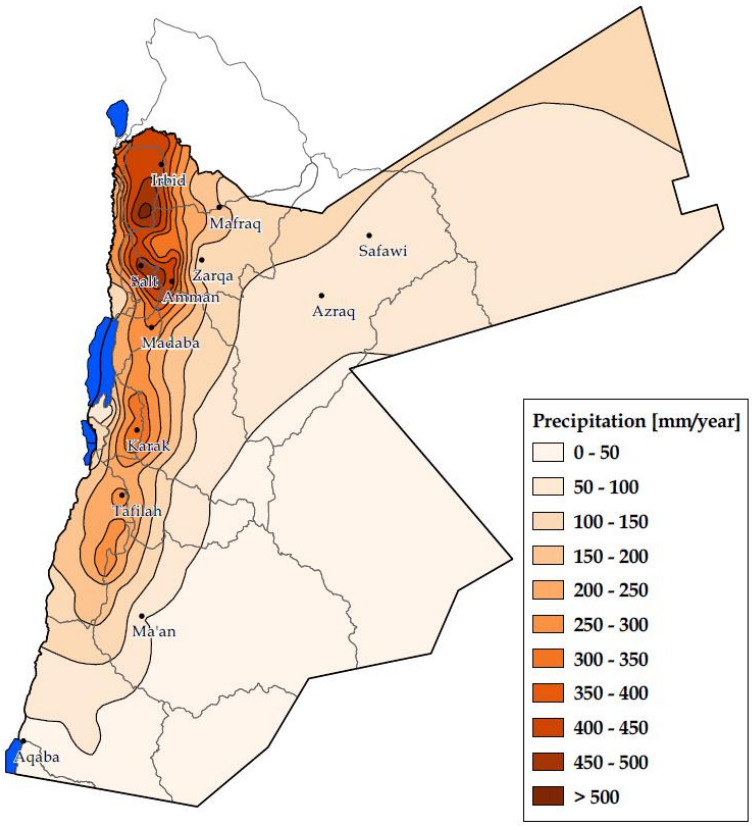

Fig. 1: Precipitation pattern and surface water basins in Jordan. While the highlands receive considerable amounts of rainfall, most of the country is characterized by semi-arid to arid climate. Data WAJ (2010), cartography by Thomas Bonn (2013).

Due to very limited freshwater resources, Jordan is one of the water poorest countries in the world, having an annual per capita renewable water availability of less than 145 m^3/year in 2007 (Hashemite Kingdom of Jordan and GTZ 2008a), which is far below the 500 m^3/year limit for absolute water scarcity according to the Falkenmark water stress index (Falkenmark et al. 1989). The pressure on water is particularly marked in the Jordanian capital, Amman, where the vast majority of households receives water only once or twice per week. Jordan's renewable water resources add to a total amount of 867 MCM per year, whereas the demand exceeds 1505 MCM/a, leaving a deficit of 638 MCM/a (Hashemite Kingdom of Jordan and GTZ 2008b). Currently, about 63 % of the water used are allotted to agriculture, 30 % to domestic usage, 5 % to industrial usage, and 1 % to tourism (Bonn 2013).

The situation is about to deteriorate further due to climate change (Abu-Taleb 2000) and due to a demographic boom caused by decreasing infant mortality and the large influx of refugees from Palestine in the 1960s and 70s, from Iraq in the last decade and today from people fleeing the civil wars in Syria and the Iraq (Manasreh 2010). In the Za'atari refugee camp in northern Jordan alone, 80,000 people find shelter at the moment. To address current and future water demand scenarios and to optimize future water resource management, Jordan has adopted a National Water Strategy. This document is a comprehensive set of guidelines applying a dual approach of demand management and supply management (Ministry for Water and Irrigation 2009). It gives for example priority to municipal and industrial needs and aims to cap agricultural use of water by regulating the amount of irrigated agriculture and to promote water efficiency in irrigation by appropriate water tariffs or by increased wastewater reuse. To create a comprehensive awareness among Jordanians is regarded as prerequisite to reduce water demand.

Water shortage has become a permanent issue in Jordan, and water managing is one of the most important topics in the country´s policies. Management and monitoring programs, however, are far from being centralized. General water quality control, for example, are under the guidance of the Ministry of Water and Irrigation, the actual tests and monitoring programs, however, are conducted by the laboratories of the Water Authority of Jordan. Furthermore, the Ministry of Health and the Royal Scientific Society apply their own quality monitoring (Royal Scientific Society 2000), and the Ministry of Agriculture surveys the suitability of water for irrigational purposes. The Jordan Valley Authority supervises mixing of treated wastewater and fresh water for irrigation of the Jordan valley. Irregularities concerning water issues are avenged by the Ministry of Environment. Besides national public authorities, many national and international Non-Governmental Organizations such as the Friends of the Earth of Middle East, the USAID or the GTZ have conducted studies on improving water resources management (Bartels 2011, Gafny et al. 2010, IUCN et al. 2006, USAID 2013). The coordination and consolidation of

knowledge and insights remain a challenge and are often complicated by differing expectations (Bonn 2013).

Water resources are not only limited, but also vulnerable in terms of quality (Hashemite Kingdom of Jordan and GTZ 2008a). A major problem is the still insufficiently developed access to sewerage of households and especially industries. The current status of sewage connection for the different regions of Jordan is shown in (Fig. 2). Far too often, untreated sewage has access to surface water or threatens the quality of groundwater resources (Abu-Rukah and Al-Kofahi 2001).

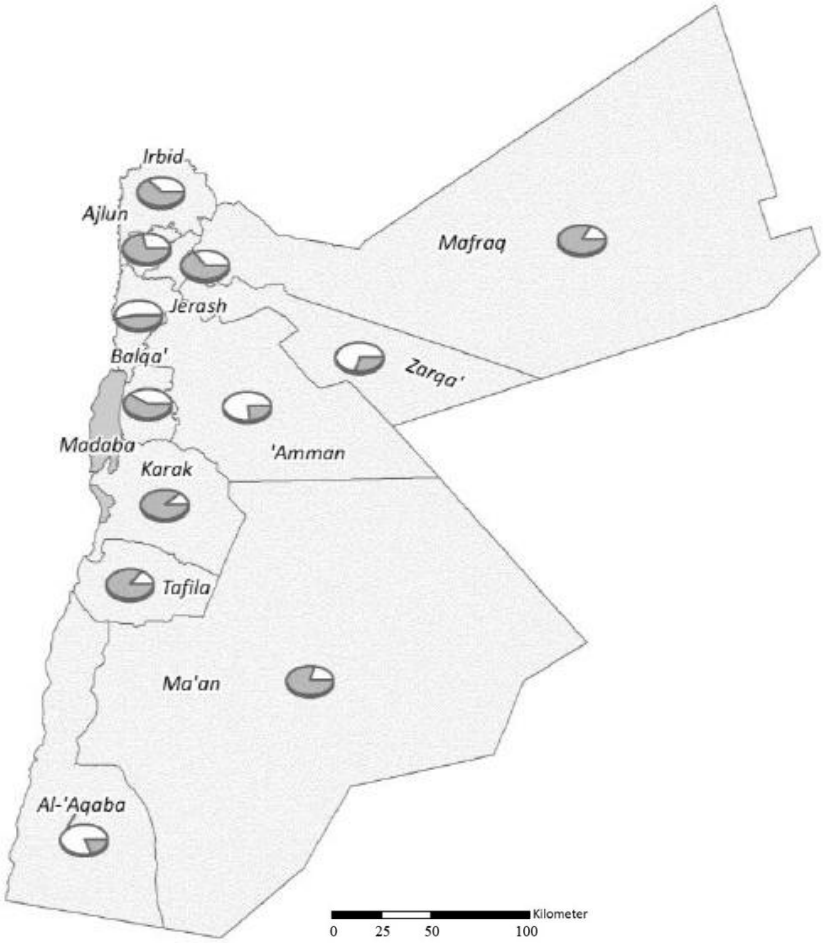

Fig. 2: Coverage to sewer connection in Jordanian governorates (dark grey: connected share, light grey: not connected share). Cartography Thomas Bonn (2013).

An overwhelming amount of literature has focused on water issues especially in the Jordan Valley. However, most of it is characterized by a mere technical (i. a. Al-Weshah 2000) or social approach (i. a. Al-Weshah 2000, United Nations 2002), disregarding toxicological and ecotoxicological considerations (Alawi et al. 1996, Ghrefat and Yusuf 2006, Shahin 2004). Toxicological and ecotoxicological risk assessment is an indispensable tool for comprehensive

classification, management and recovery procedures of water bodies. A basic test-battery often involves *Daphnia* Acute Immobilization Test according to the OECD guideline 202 for the assessment of acute toxicity to filtrating water organisms, the Luminescent Bacteria Inhibition test according to ISO 11348 for the assessment of acute bacteria toxicity, the *umu*-test with *Salmonella typhimurium* according to ISO 13829 for the assessment of genotoxicity and the Alga Growth Inhibition Test after the OECD guideline 201 for the assessment of acute and chronic toxicity to algae. An extensive test-battery especially for the evaluation of drinking water, however, should also involve testing of acute toxicity to vertebrates as assessed in the Zebrafish Embryo Toxicity Test with *Danio rerio* (OECD TG 236), of direct genotoxic effects e.g. in the Micronucleus Assay with hamster lung cells (OECD TG 487), and of bioavailability as assed in contact assay with e.g. *Arthrobacter globiformis* (DIN 38412 – 48).

1.3 The study area: Jordanian surface waters

Surface water in Jordan is very limited due to low precipitation and high evaporation levels (Fig. 1). Water bodies are either fed directly by runoff from rainfall (e.g. Wadi Mujib), by groundwater springs (e.g. Yarmouk River, Jordan River) or by discharge from waste water treatment plants (e.g. Zarqa River, Yarmouk River, parts of the Jordan River). The total average surface flow adds up to 693 MCM per year (Royal Scientific Society 2000). With increasing distance to the Mediterranean Sea from west to east and furthermore from north to south, the annual average regimen generally declines due to less influence of moisture sources. Most streams are adversely affected either by wastewater discharges or water supply abstraction. Apart from the Jordan River, few streams are fed enough by groundwater or springs to flow permanently throughout the year. However, nearly all available (fresh or treated) surface water is used and supplies up to almost 35 % of the total water used in Jordan (EXACT 1998). With the exception of the King Abdullah Canal, Wadi Mujib and spring discharge, however, surface water is said to be exclusively used for irrigation or watering of animals (Afonso et al. 2004).

Fig. 3: Major streams and wadis of Jordan (source: UNEP/DEWA/GRID-Geneva, 2001).

1.3.1 Jordan River

Although, by international standards, the Jordan River is a small river, it is the third largest perennial river in the Middle East. Culturally, it is known for its historical and political function as border between Israel and the West Bank on the one side and Lebanon, Syria and Jordan on the other (Gleick 1993). This rich mix of antagonistic nations, societies, cultures, religions, politics, ethnicities and languages is partially reflected in an amalgam of conflicts and complexities. As the Jordan River constitutes one of the most important water resource to its surrounding dry lands, all neighboring countries enforce their claim to the usage of its water. Though Jordan and Israel settled the abstraction quantities in the Peace Treaty of 1994 (The Hashemite Kingdom of Jordan and the State of Israel 1994), the political situation between Israel and Syria is still tense as reflected by the occupation of the Golan Heights.

The upper Jordan River originates from three main springs: the Hasbani and Dan in Lebanon and the Banias in the northern Golan Heights (Howari and Banat 2002). Leaving Lake Tiberias, the largest surface fresh water reservoir in the region, as a small stream, the Lower Jordan River commences its way through the agriculturally used Jordan Valley, which is part of the large tectonic structure of the Great African Rift Valley. Historically, the largest tributary to the Lower Jordan River was the Yarmouk River (EXACT 1998). However, due to water supply projects in Israel, Syria and Jordan, this fresh water source has been drastically reduced, and

additional fresh water enters the river mainly during floods and negligible contributions from small springs (Shavit et al. 2003). The Bitania wastewater treatment plant (10 MCM/a) and the Saline Water Carrier (15 MCM/a) are currently the main water sources (Farber et al. 2005). Annual average precipitation ranges from 1600 mm in the north at the slopes of the Mount Lebanon to 250 mm around Lake Tiberias and to 100 mm in the south (Comair et al. 2012, Hassan and Klein 2002). After 251 km in total and 190 km in the Jordan Valley, the river discharges into the Dead Sea.

Massive abstractions and water utilization in the upper regions have led to a significant reduction of the inflow to the Dead Sea. Originally, the Jordan River once carried about 1.3 billion cubic meters of fresh water to the Dead Sea per annum (Gafny et al. 2010, Salameh and Naser 1999). Nowadays, it is no more than 100 to 200 millions of cubic meters per year (Salameh and Naser 1999). In consequence, the level of the Dead Sea has dropped by more than 25 m, and its length has shortened for more than 20 km over the last 20 years (Ben-Avraham et al. 2008).

A well-known problem of the Lower Jordan River is its high salinization due to hyper saline springs that are discharged into the river *via* the Saline Water Carrier and due to high evaporation levels (Farber et al. 2004, 2005). Furthermore, about 16.5 MCM/a of saline water are pumped into the Jordan River artificially (Farber et al. 2005). The groundwater discharging into the Jordan River is also highly influenced by agricultural wastewaters and composed of varying proportions of brines and sulfate- and nitrate-rich saline waters. It, thus, constitutes a non-point source of contamination (Holtzman et al. 2005, Vengosh 2003). Although increasing abstraction to up to 95 % of the water (Salameh and Naser 1999), intense agricultural usage, discharges form extensive fishponds in the upper Jordan Valley (Gat and Dansgaard 1972), and run-off from winter rainfall may further reduce water quality, only limited literature is available on water and sediment quality of the Jordan River such as contamination with heavy metals and pharmaceuticals (Banat and Howari 2003, Gafny et al. 2010, Howari and Banat 2001, Howari and Banat 2002, Pankrotov et al. 2005, Tiehm et al. 2011). However, due to its prominent role as fresh water provider, it is indispensable to investigate the quality situation and its effects on the environment.

1.3.2 King Abdullah Canal

Formerly known as the East Ghor Canal, the King Abdullah Canal (KAC) is the largest artificial water conveyor in Jordan with a length of 110 km and a discharge capacity of 20 m^3/s (Jordan Valley Authority 2009). Construction was primarily financed by the USAID as part of the Johnston Plan and it was completed in 1987 after three different construction phases since 1959. The northern end of the KAC receives water diverted from the Yarmouk River *via* a 900 m long

tunnel. It plays a major role in agriculture of the Jordan Valley, as it irrigates 23,000 ha of land. Furthermore, shortly after completion, it was decided that the KAC water should also be used for the drinking water supply of the capital Amman after proper treatment mainly based on chlorination. Today, about 60 MCM are pumped annually from the KAC to Amman after being treated at the Zai treatment plant, which is located between Deir Alla as the water intake site in the Jordan Valley and Amman (Alkhoury et al. 2010). Since the water of KAC is an essential source for drinking water supply, water quality and human health effects have become crucial. However, especially during the first 10 years of pumping, water quality has been affected by strong odor and bad taste mainly caused by eutrophication problems of the KAC (Alkhoury et al. 2010). However, quality control is basically restricted to chemical analysis *via* chromatography, but not surveyed by toxicological or ecotoxicological studies.

1.3.3 Wadi Mujib

The Wadi Mujib is the largest contributing stream to the Dead Sea on its eastern side (EXACT 1998). Construction of the Mujib reservoir started in 1999 and was finished in 2002 for the purpose of drinking water and recharging groundwater aquifers. The reservoir has a catchment area of 4,380 km^2 and was designed to store about 217 MCM of rainwater. From the dam, water flows through the wadi to its mouth into the Dead Sea, where surface runoff is collected by a conveyor to be transferred to the water treatment station of Sweimeh (Margane et al. 2008). It provides irrigation water for the southern farmlands of the Jordan Valley, for the Arab Potash Company at the eastern shore of the Dead Sea, and drinking water for the hotels at the northern shore of the Dead Sea. Furthermore, it supplies the capital Amman with potable water, which makes water quality a primary concern. The water is collected from surface runoff, which flows during the winter season through the Al-Lajoun valley and Wadi Wala and which receives various kinds of effluents such as domestic, industrial, municipal wastewater, and agricultural wastewater. Although eutrophication seems to be a minor problem in the Wadi Mujib (Al-Harahsheh and Al-Amoush 2010), water quality is threatened, since Manasreh et al. (2010) showed that sediments of the Wadi Mujib are polluted by Cd and, to a lesser extent, by Zn, Ni and Cu; in contrast, contamination by Mn and Pb was low. At least, Cd and Zn originate from anthropogenic sources such as the wastewater effluents of the treatment plant Al-Lajoun.

1.3.4 Yarmouk River

As the Yarmouk River is the main source of water for the King Abdullah canal and the main tributary of the Jordan River in the Jordan Valley, it is the most important surface water resource of Jordan. It also constitutes the border to Syria and the Golan Heights in the north and to Israel in the Jordan valley shortly before it opens into the Jordan River. The catchment area of the

Yarmouk River is mainly agrarian with small-scale industries located in Jordan and Syria. With the completion of the Al Wahda Dam/Unity Dam in 2011, further rainwater and surface runoff can be harvested at the upstream of the river. The distribution of the water is regulated in the Peace Treaty between Jordan and Israel (1994), whereby Israel is allowed to pump 12 MCM during summer period and another 13 MCM in winter. Thus, Jordan is left to use the rest of the water which naturally varies due to changes in precipitation. Critical and yet unsettled remains the Syrian share of the waters of the Yarmouk River.

During flood events in winter, untreated effluents of two water treatment plants are discharged into the river. Furthermore, the effluents of two stabilization ponds are discharged into the river, mainly during floods. Besides, leachates of the Akader solid waste disposal reach the river directly on days when liquid loads exceed evaporation and infiltration potential. In general, the water quality of the Yarmouk River is believed to be good (EXACT 1998), however, its sediments are known to be contaminated with heavy metals, especially Hg and Cd, due to anthropogenic sources in the catchment area (Abu-Rukah and Ghrefat 2001).

1.3.5 Zarqa River

Following the Jordan and Yarmouk Rivers, the Zarqa River is the third largest stream in Jordan in terms of annual discharge. Its spring lies east of Amman, and the Wadi Dulheil is its largest tributary after the effluent of Jordan's largest water treatment plant, Khirbet As-Samra. This plant treats about 80 % of the wastewater generated in Jordan (Shatanawi and Fayyad 1996). Wastewater composes nearly all of the Zarqa River flow during summer, degrading water quality (EXACT 1998, Shatanawi and Fayyad 1996). Two more wastewater treatment plants discharge their effluents into the river: Jerash and Almirad. The King Talal Dam regulates the river, before its water is released into the KAC for irrigational purposes in the Jordan Valley.

The Zarqa River's watershed encompasses Jordan's most densely populated and industrialized area. About 3000 industries are registered in the governorate, making up more than 52 % of the country's total industry (Mrayyan and Hamdi 2006). Decreased discharge of fresh water combined with increased discharges of organic load from domestic and industrial waste disposals have led to environmentally relevant concentrations of organic pollutants in the river sediments (Abderahman and Abu-Rukah 2006a, Batarseh 2003, IUCN et al. 2006, Scott and Abumoghli 1995, Shatanawi and Fayyad 1996). The main source for heavy metals are textile and paint plants (Al-Jundi 2000). Biodiversity is threatened and contamination of the water is a cause of disease in humans and livestock (IUCN et al. 2006). Several restoration projects have been ventured to improve water quality (IUCN et al. 2006, Mohsen 2007), and the assessment of water quality and sediment contamination of the Zarqa River is an urgent issue (Al-Wer 2009).

1.4 Assessment of sediments in ecotoxicology

As sediments serve as sinks for xenobiotics that are transported in the water (Ahlf et al. 2002, Calmano 2001, Chapman et al. 2002, Chapman et al. 1998), they are often referred to as 'the memory of water'. There is a general agreement that sediment-bound substances are important to understand the fate and effect of contaminants as well as water quality (Wölz et al. 2009). Substantial sediment contamination often persists even after the discharge of chemicals has been terminated. Investigations of sediment contamination are therefore indispensable for a holistic evaluation of the quality of water courses, especially as sediment bound contaminants can be resuspended and remobilized through flood events, dredging or a change in the pH value, salinity or redox potential of the water (Calmano et al. 1992, Hollert et al. 2003, Spencer et al. 2006, Wölz et al. 2009) and, thereby, can become a source of contamination. Furthermore, sediment-associated substances may have direct adverse effects on sediment-dwelling organisms and result in a disruption of the aquatic ecosystem (European Sediment Research Network 2004) or may lead to bioaccumulation in organisms. In cases contaminants are released from sediments they can enrich in the food chain (Ankley et al. 1992, Ingersoll et al. 1995), and humans might be adversely affected through the consumption of contaminated fish or mussels as well (Matsumoto et al. 2006).

Relations between aquatic sediments, organisms and ecosystems are multilayered and complex, and their monitoring should, therefore, be an integral part of environmental risk assessment. However, the role of sediments in water quality assessment has been neglected by, e.g., the EU water framework for a long time. Only in 2012, a recommendation has been made to add sediment assessment into water quality monitoring (Europäische Kommission 2012). By now, there has been a paradigm shift since sediments are no longer only viewed as troubling compartment of water bodies, but are recognized as an important resource, as a habitat for organisms and as a source for nutrients in agriculture. Sustainable sediment management has, thus, received increasing attention (Apitz and Power 2002, MacDonald 1994, Netzband 2007).

Chemical analysis can serve as a useful tool to assess the occurrence of chemicals such as persistent organic pollutants (POPs) as polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs). However, they do not give information about their bioavailability, effects of interactions, combined or additive effects, or their actual dose-dependent effects on organisms (Carlsson et al. 2014, O'Connor and Paul 2000). Bioassays, on the other hand, offer the opportunity to test the influence of whole sediments, extracts or eluates on organisms *via* various exposure paths, such as food, direct contact, or pore-water, without knowing the exact mixture of chemicals.

Model organisms for the assessment of sediments in ecotoxicology are diverse ranging from arthropods (*Chironimus tentans*), annelids (*Lumbriculus variegates, Tubifex tubifex*), crustaceans (*Hyalella azteca*) to fish (e.g., *Danio rerio*(Borgmann and Munawar 1989, Dermott and Munawar 1992, Hallare et al. 2005, Ingersoll et al. 1995, Kosmehl et al. 2008b, Leppänen and Kukkonen 1998). As European legislation requires that non-animal alternative approaches of testing should be used in the place of animal procedures wherever possible (REACH 2006) and as the principal EU directive for the protection of animals used in scientific studies states as final goal the "full replacement of procedures on live animals for scientific and educational purposes as soon as scientifically possible to do so" (European Parliament and the Council 2010), a lot of research has been devoted to the development of *in vitro* alternative test systems to reduce animal tests. Among them are, for example, acute cytotoxicity tests (Castaño et al. 1996, Fent 2007b, Lange et al. 1995), genotoxicity tests with permanent cell cultures (Fenech 2007, Hartmann et al. 2001a), endocrine screening tests with *Salmonella* and fish embryo tests (Bachmann 2002b, Braunbeck et al. 2005, DIN 2001, Embry et al. 2010, Lange et al. 1995, OECD 2011, 2013).

1.5 Objectives of the present thesis

The challenge of water contamination is very high in areas of water shortage, i.e. under conditions when any available source of water including the reuse of wastewater is essential for human survival. Within the interdisciplinary project described above, the key issue of this dissertation was to elucidate the ecotoxicological hazard and risk caused by Jordanian surface waters based on solid phase extraction and sediment (eco)toxicity assessment. According to the current state of research, no ecotoxicological study assessing sediment quality or effects of sediment contamination on organisms has ever been conducted in Jordan so far. Thus, to the best of knowledge, this study is the first to apply ecotoxicological bioassays to assess water quality in terms of surface water sediment contamination in Jordan.

As the principal prerequisite for sampling and authorized export of samples, the main goal of the first working period was to establish contacts to and collaborations with local authorities and partners in Jordan. A cooperation agreement could be signed between the Department for Environmental Physics, the Department of Zoology and the Water Authority of Jordan, allowing the German partners to have access to restricted areas, obtain export permissions and use the local laboratories. A promising scientific exchange involving visits of Jordanian partners to Heidelberg was accomplished. Although there was already a rough idea about potential sampling sites, namely to follow the drinking and wastewater paths of the capital Amman, precise sampling sites could only be defined through extensive disucssions with authorities and stakeholders and through expeditions into the field. Originally planned overlaps of sampling sites with the collaborating groups (Ch. 1.1) in order to design a comprehensive

strategy for water use and re-use could not be realized due to local situations and circumstances affecting all projects and requiring a reorganization of areas of investigation. Limited access to the border rivers Jordan and Yarmouk due to militarily restricted areas or floating mines, or the complex terrain of e.g. Wadi Mujib basically determined sampling of this study, covering a sufficiently broad area of investigation for each surface water body.

In this thesis, efforts were directed to promote rapid and cost-effective biological response parameters for recognition and effects of potentially hazardous contaminants. For the determination of sediment and water contamination, a battery of toxicological tests is required. In the context of the overall scope of the project, this test battery had to be economically feasible and also needed to cover a multitude of potential biological effects. The *in vitro* test battery included a) general toxicity (cytotoxicity), b) genotoxicity (DNA damage), c) embryo toxicity and d) dioxin-like activity. It was complemented by assessment of morphological and physical parameters and measurement of nutrients and salts. All biotests were conducted with acetonic sediment extracts and water extracts based on solid phase extraction with C18 cartridges. Given the strong trend to non-animal testing in toxicology and ecotoxicology under the regulations of the new European chemical legislation (REACH – Registration, Evaluation, Authorization and Restriction of Chemicals), the project exclusively applied methods relying on non-animal testing such as cell cultures with rainbow trout liver cells (Boettcher et al. 2010, Bols et al. 1999, Kosmehl et al. 2004) and Chinese hamster lung cells (OECD TG 487) and evaluations with early embryonic stages of fish (*Danio rerio*). Fish have been selected as major test organisms, since they represent *the* vertebrate model for the evaluation of toxic effects in/from the aquatic environment (OECD TG 236).

After data acquisition, the wealth of toxicological information had to be transformed into a simple classification of toxicity in order to make results accessible to local authorities and decision makers and to facilitate a scientifically well-based strategy for integrated water resources management. The most important aim of this dissertation is, therefore, to provide easy-to-use and substantial science-based information on water quality as a prerequisite to arrive at an optimized (re-)use of water. Therefore, the results of the bioassays were classified according to toxicity threshold values established within the framework of a fuzzy logic-classification approach by Keiter et al. (2009b) or by a rank-sum-based analysis in cases comparison to the values determined by these authors did not apply due to methodological differences. The results obtained from the rank-sum analysis and allocation to toxicity levels *via* threshold values were then transformed into quality classes in accordance with the classification criteria for physical and chemical parameters after Graw and Borchardt (1999), which complies with the EU Water Framework Directive 2000/60/EC (EU-WRRL 2000)

2. Materials and Methods

2.1 Sampling

In October 2009 and October 2010, sediment samples were taken at 20 different sites of the five main surface water bodies of Jordan: Jordan River, King Abdullah Canal, Wadi Mujib, Yarmouk River and Zarqa River. All sampling was conducted in cooperation and close collaboration with the Water Authority of Jordan. Sediment samples were taken with a stainless steel shuffle near to the riverbank at depths of 1-10 cm and transferred directly into 2 liter PE wide-mouth bottles. Samples were cooled and stored protected from light for transport in the field and then stored at -20°C as soon as possible until further processing as described in Ch. 2.2. Additionally in 2009, water samples at a volume of one liter were taken at 8 sampling sites at the Zarqa River and King Abdullah Canal. The water was filtered with borosilicate glassfibre filters (Typ MN 85/70, Machery & Nagel, Düren, Germany) and stored and cooled in brown glass bottles (Fa. Schott, Mainz) until processed for further usage in the biotests as described in chapter 2.3.3. Since no effects were recorded for the water extracts in the bioassays, water sampling was not further conducted in 2010.

2.1.1 Jordan River

Alongside the Lower Jordan River, 5 sites were sampled within an air line distance of 93 km and approximately 160 km of actual flow distance. Accessibility limited the selection of sampling sites. Thus, sites were selected for close proximity to bridges to ensure reasonably safe access to the river segment, as several regions of the Lower Jordan River are considered hazardous due to the potential presence of landmines. All sampling was conducted on the eastern bank of the river and further cooperation with the Jordan Valley Authority, the Jordanian Military and the Intelligence Agency of Israel was inevitable due to the still tense situation in the border district. Sampling locations are shown in Fig. 4 and were selected to include sites spanning from the north to the south of the lower river to ensure adequate representation of the river course. Further information and GPS data of the sampling sites are summarized in (Tab. 1).

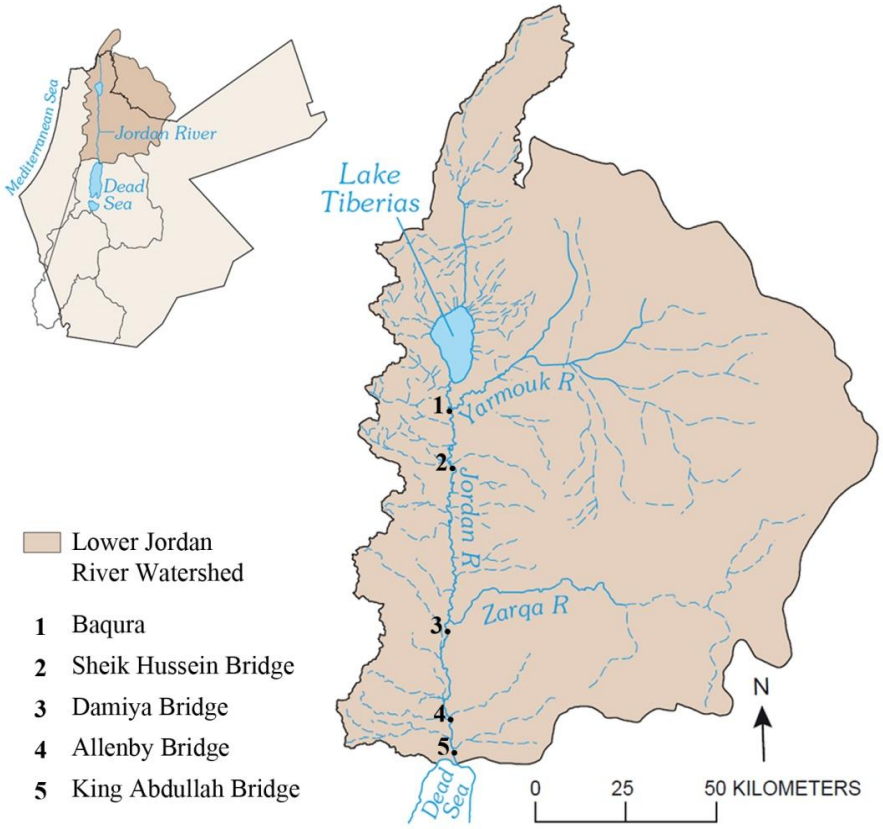

Fig. 4: Sampling sites at the Jordan River; map modified according to EXACT (1998).

Tab. 1: Overview of the sampling sites at the Jordan River.

Sampling Site	Synonym	GPS data	Date of sampling
Gesher	Jordan 1	32°38'07.5" N 35°33'56.7" E	11.10.2010
Sheik Hussein Bridge	Jordan 2	32°29'48.97" N 35°34'32.68" E	11.10.2010
Damiya Bridge	Jordan 3	32° 6'9.45" N 35°32'6.19" E	20.10.2010
Allenby/King Hussein Bridge	Jordan 4	31°52'27.00" N 35°32'27.00" E	10.10.2010
King Abdullah Bridge	Jordan 5	31°48'3.77" N 35°32'47.89" E	10.10.2010

2.1.2 King Abdullah Canal

Water distribution systems such as the Jordanian King Abdullah Canal (KAC) distribute water from areas of water affluence to areas of water shortage. The northern end of the KAC receives water diverted from the Yarmouk River via tunnel of 900 m lenght. Despite surveillance and

fencing, water quality may be altered during the canal's flow through the Jordan Valley through discharge of agricultural sewage water, livestock farming or waste. For this reason, one sampling site was chosen with considerable distance to the Yarmouk River being also the water intake site for later drinking water treatment at Zai treatment station and being, thus, of crucial importance. The second sampling site was chosen after the confluence of KAC and the Zarqa River (Fig. 5, Tab. 2)

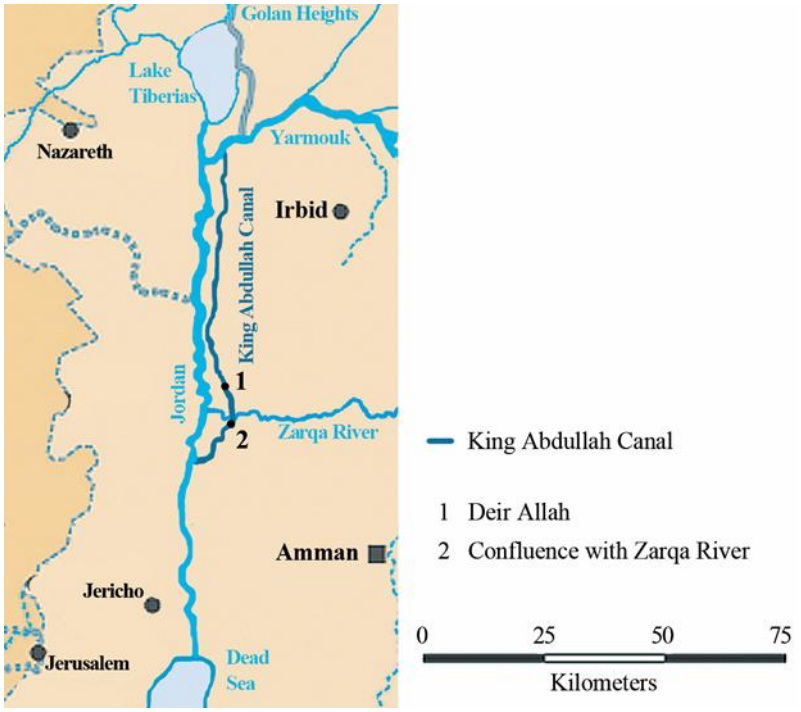

Fig. 5: Sites at the King Abdullah Canal, modified after UNEP/DEWA/GRID-Geneva, 2001

Tab. 2: Overview of the sampling sites at the King Abdullah Canal.

Sampling Site	Synonym	GPS data	Date of sampling
Deir Allah	KAC 1	32°11'45.2" N 35°37'06.8" E	21.10.2009 11.10.2010
Confluence with Zarqa River	KAC 2	32°10'59" N 35°37'06.8" E	11.10.2010

2.1.3 Wadi Mujib

At the Mujib reservoir, two sites were sampled (Fig. 6, Tab. 3): the southern shore of the reservoir at the height of the dam and the outlet stream below the dam. Furthermore, at the mouth to the Dead Sea after joining with the Wadi Wala, another sample was taken, which is of high relevance for toxicity assessment since this is where water is abstracted for treatment and further usage as drinking, industrial, and irrigational water.

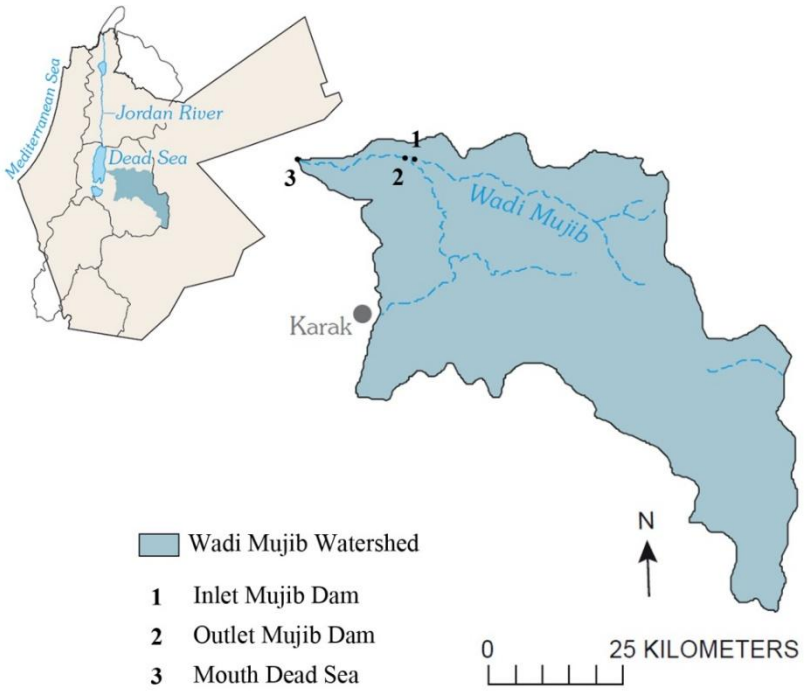

Fig. 6: Sampling Sites at Wadi Mujib, modified according to EXACT (1998)

Tab. 3: Overview of the sampling sites at the Wadi Mujib.

Sampling Site	Synonym	GPS data	Date of sampling
Mujib reservoir inlet	Mujib 1	31°26'35.94" N 35°49'03.66" E	05.10.2010
Mujib reservoir outlet	Mujib 2	31°26'48.6" N 35°49'26.6" E	05.10.2010
Mujib mouth to Dead Sea	Mujib 3	31°34'29.7" N 35°33'04.4" E	05.10.2010

2.1.4 Yarmouk River

As for the Jordan River, accessibility limited the selection of sampling sites at the Yarmouk River, being the border river between Jordan, Syria and the Golan Heights. To access sampling sites, further authorization was needed from the Jordanian and Syrian military. Locations were selected for easy accessibility and to cover a preferably wide area. Thus, four sites were sampled which are shown in Fig. 7 and Tab. 4.

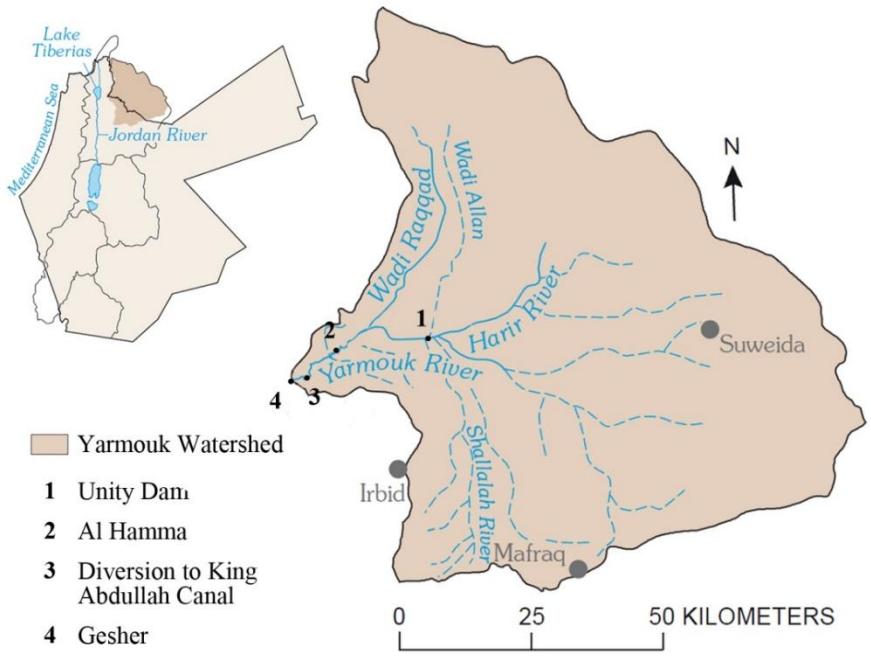

Fig. 7: Sampling Sites at the Yarmouk River, modified according to EXACT (1998)

Tab. 4: Overview of the sampling sites at the Yarmouk River.

Sampling Site	Synonym	GPS data	Date of sampling
Unity Dam	Yarmouk 1	32°44'00.6" N 35°51'50.3" E	11.10.2010
Wadi Raqab	Yarmouk 2	32°43'03.4" N 35°42'49.0" E	01.10.2010
Diversion to KAC	Yarmouk 3	32°10'55.0" N 35°38'16.2" E	01.10.2010
Gesher	Yarmouk 4	32°40'35.3" N 35°37'29.8" E	02.10.2010

2.1.5 Zarqa River

As the Zarqa River is known to be strongly contaminated by urban and industrial effluents (Abderahman and Abu-Rukah 2006, Batarseh 2003, Shatanawi and Fayyad 1996), six samples were taken at various crucial sites to cover an extensive part of the river. As the Zarqa River was totally dry during the time of sampling, the first sample was taken in the Wadi Dulheil from the effluent of the wastewater treatment plant Khirbet As-Samra. Wadi Dulheil flows into the Zarqa River after approximately 10 kilometers. The geography of the sampling sites is shown in Fig. 8 and further data can be obtained from Tab. 5.

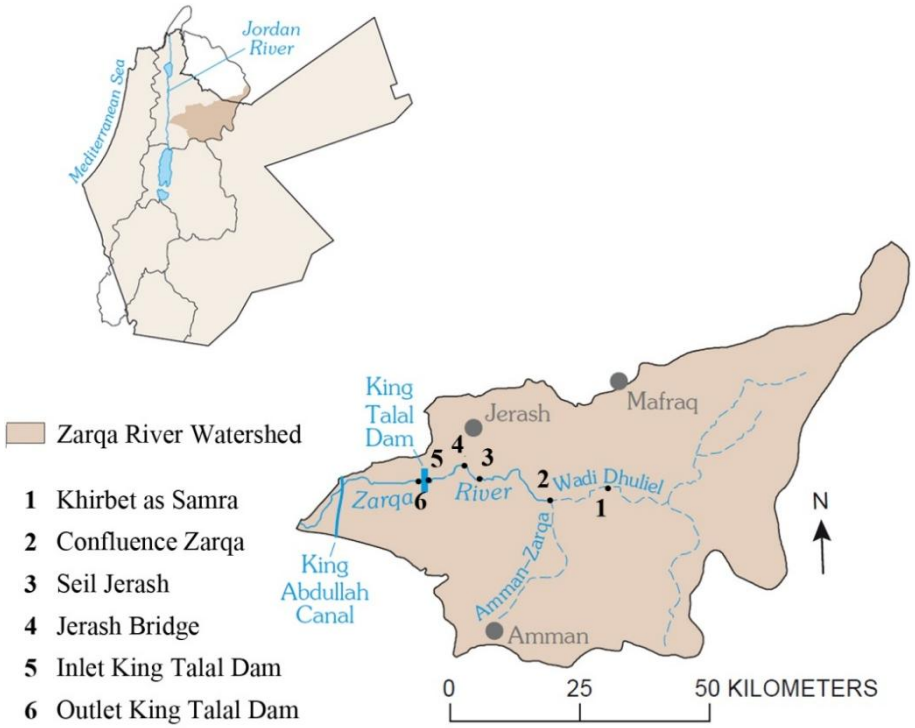

Fig. 8: Sampling Sites at the Zarqa River, modified according to EXACT (1998).

Tab. 5: Overview of the sampling sites at the Zarqa River.

Sampling Site	Synonym	GPS data	Date of sampling
Khirbet As Samra	Zarqa 1	32° 8'58.22" N 36° 8'49.69" E	18.10.2009 07.10.2010
Confluence Zarqa	Zarqa 2	32° 8'52.01" N 36° 3'6.68" E	18.10.2009 07.10.2010
Seil Jerash	Zarqa 3	32°12'2.79" N 35°54'2.85" E	20.10.2009 02.10.2010
Jerash Bridge	Zarqa 4	32°12'58.30" N 35°52'59.19" E	02.10.2010
Inlet King Talal Dam	Zarqa 5	32°11'29.84" N 35°49'54.48" E	19.10.2009 02.10.2010
Outlet King Talal Dam	Zarqa 6	32°11'28.04" N 35°47'48.80" E	19.10.2009 02.10.2010

2.2 Morphological and physical parameters, nutrients and salts

For each sampling site, the following physical parameters were recorded: width, depth, current velocity, depth of visibility; temperature, oxygen, biological oxygen demand in five days (BOD_5), pH value and electrical conductivity (Multi 350i electrode, WTW, Weilheim, Germany). Furthermore, ammonia, nitrite, nitrate, phosphate and chloride (Aquamerk test kits, Merck, Darmstadt, Germany) were measured. For assessing the BOD_5, a two liter brown glass bottle was filled completely with water, measured for oxygen content and stored protected from light at 20 °C for five days. It was then measured again for oxygen content. The BOD_5 is the difference between the two values expressed in mg O_2/L. Results were classified according to the guidance of the LAWA (1998) as shown in Tab. 6.

Tab. 6: Substance-based categorization into quality classes according to LAWA (1998).

		Quality class						
	Unity	no contamination I	low cnotamination I - II	moderate contamination II	considerable contamination II - III	increased contamination III	high contamination III - IV	very high contamination IV
Nitrate	mg/L	<= 1	<= 1.5	<= 2.5	<= 5	<= 10	<= 20	> 20
Nitrite	mg/L	<= 0.01	<= 0.05	<= 0.1	<= 0.2	<= 0.4	<= 0.8	> 0.8
Ammonium	mg/L	<= 0.04	<= 0.1	<= 0.3	<= 0.6	<= 1.2	<= 2.4	> 2.4
Total phophate	mg/L	<= 0.05	<= 0.08	<= 0.15	<= 0.3	<= 0.6	<= 1.2	> 1.2
Oxygen	mg/L	> 8	> 8	> 6	> 5	> 4	> 2	<= 2
Chloride	mg/L	<= 25	<= 50	<= 100	<= 200	<= 400	<= 800	> 800

For the remaining parameters, classification was conducted according to criteria of Graw and Borchardt (1999) which comply with the EU Water Framework Directive 2000/60/EC (EU-WRRL 2000) as shown in Tab. 7. Here, the LAWA categories I and I-II are merged to class 1, II remains class 2, II-III equals class 3, III is classified 4 and III-IV and IV reflect class 5.

Tab. 7: Categorization according to Graw and Borchardt (1999).

		Quality class				
	Unity	1	2	3	4	5
Temperature	°C	< 18	18 - 20	20 - 22	22 - 24	> 24
pH-value		6.5 - 8	6 - 6.4 8.1 - 8.5	5.5 - 6.9 8.6 - 9	5 - 5.4 9.1 - 9.5	< 5 > 9.5
electrical conductivity	µS/cm	300	301 - 500	5001 - 700	7001 - 900	900
BOD_5	mg O_2/L	< 1	1 - 3	3.1 - 5	5.1 - 10	> 10

2.3 Sample processing

2.3.1 Freeze drying of sediments

For conservation and stable storage, all sediments were freeze dried. Freeze drying is a gentle method to remove water from the samples to be able to use them for later extraction (Hjorth 2004, McClymont et al. 2007). 250 ml of each sampling site were transferred into a 500 ml round bottom flask (Duran, Schott, Mainz, Germany) and shock-frozen for 20 minutes under constant rotation in a - 30 °C isopropanol bath (N6, C41, Haake, Vreden, Germany). The rotation ensures freezing of the sediments in a thin layer, thus, resulting in a maximum surface area which facilitates later freeze drying. With a freeze drying machine (Alpha 1-4, Christ, Osterode, Germany), sediments were dried through sublimation at -1.4 bars for 48 to 72 hours. Sieving with a mesh size of 1.2 mm (stainless steel, Haver & Boecker, Oelde, Germany) removed leaves, twigs and larger particles.

2.3.2 Organic extraction of sediments

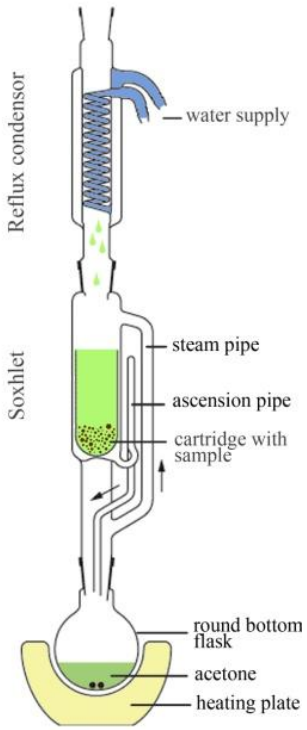

Fig. 9: Soxhlet.apparatus.

In sediment toxicology, organic extraction is used to solve organic compounds ligated to particles (Ahlf 1995, Erdinger et al. 2004, Hollert et al. 2009, Raynie 2006). Of each freeze-dried sediment sample, 20 g were transferred into a cellulose extraction cartridge (Whatman, Dassel, Germany), covered with glaswool and put into a soxhlet apparatus (Fig. 9). Acetone was used as organic solvent since it is ecologically compatible and at the same time has a broad spectrum of dissolving power, resulting in high effects in the bioassays (Erdinger et al. 1997, Seiler et al. 2006). Besides, its boiling point of 56 °C reduces the risk of degrading heat-sensitive substances. For the extraction process, 250 ml acetone are evaporated through a heating plate. Condensation at a reflux condenser ensured steady wetting of the sediment samples. As soon as the acetone reached the vertex of the ascension pipe, it was transferred back into the round bottom flask and the cycle repeated. Thus, com-pounds dissolved by acetone accumulated in the flask. The cycle repeated approximately ten times per hour, and the system ran for 14 hrs over night. Subsequently, the organic extract was reduced to a volume of 5-7 ml with a rotary evaporator (300 - 500 mbar, 38 °C; Heidolph, Kehlheim). Extracts were then transferred into 8 ml glass vials and dried almost completely under a constant nitrogen stream. For usage in biotests, extracts were resolved in 1 ml DMSO.

2.3.3 Solid-phase extraction of water samples

Solid-phase extraction is a chromatographic technique used to prepare samples for subsequent analysis by concentrating and purifying analytes from solutions by sorption onto a disposable solid-phase cartridge, followed by elution (Thurman and Mills 1998) (Fig. 10). As the analysis of the water samples from 2009 (Ch. 2.1) was non-target-oriented, an extraction cartridge with a wide adsorption spectrum, the Oasis™ HLB cartridge (Cheng et al. 1997, Parkerton et al. 2000, Snow et al. 2002), was used. It contains a resin made from a co-polymer of divinylbenzene and vinyl pyrrolidinone. The pyrrolidinone functionally acts as an imbedded hydrophilic group and thus provides enhanced retention for polar analytes and of non-polar analytes. In order to concentrate and enrich contaminants potentially dissolved in the water s, cartridges were fitted to a vacuum block (Baker) which was connected to a vacuum pump. Prior to sample application, the cartridges were conditioned with 6 ml of methanol and subsequently washed with 6 ml of distilled water each. 1 L of previously filtered water from each sampling site was then applied through the cartridges with a flow rate of 10 ml/min (Fig. 10). Afterwards, cartridges were dried under a constant nitrogen stream, and the samples were eluted with two times 3 ml of acetone (Sigma Aldrich, suprasolve). For further usage in biotests, the acetone was evaporated under constant nitrogen stream, and extracts were resolved in 1 ml of DMSO resulting in a final concentration of 1 L extracted water per ml DMSO.

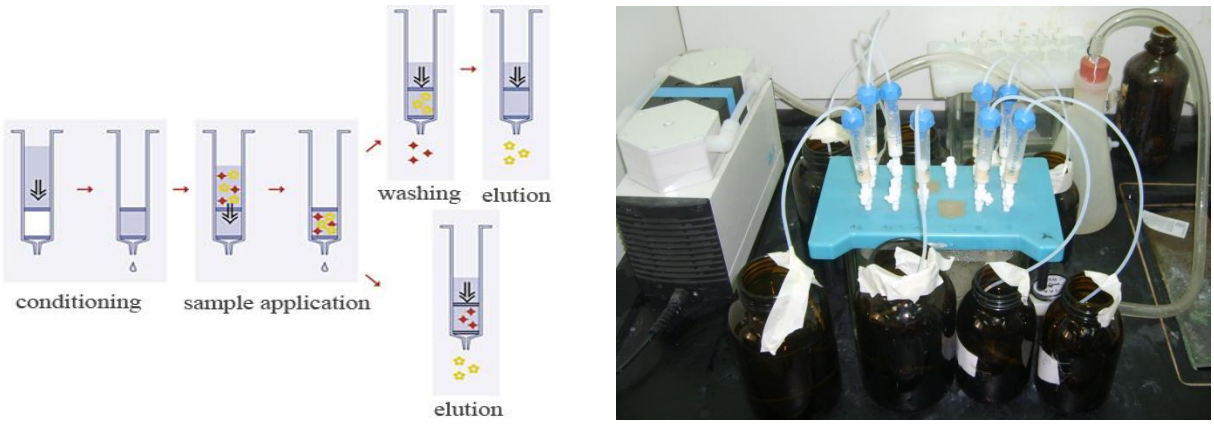

Fig. 10: Principle of solid-phase extraction, modified after Chromabond (left) and applied solid-phase extraction of Jordanian water samples with Oasis™ HLB cartridges (right).

2.4 *In vitro* assays

2.4.1 Cell culture

Rainbow trout liver cell line RTL-W1

In 1984, the permanent cell line RTL-W1 was developed by Bols and co-workers from the liver of a male adult rainbow trout *(Oncorhynchus mykiss).* Lee et al. (1993) could show that this cell line can be cultured under *in vitro* conditions. Furthermore, it can be used in several biotest systems to assess, e.g., the cytotoxic and genotoxic potentials of chemicals or complex environmental samples (Keiter et al. 2006, Rocha et al. 2009, Schirmer et al. 2000, Seitz et al. 2008).

RTL-W1 cells were obtained from Drs. Niels Bols and Lucy Lee (University of Waterloo, Canada). Culture conditions according to Lee and co-workers (1993) were adopted: RTL-W1 cells were cultured at 20 °C in L15 medium (Sigma), supplemented with 10 % fetal bovine serum (Biochrom AG, Berlin), 1 % l-glutamine-solution (200 mM, Sigma), and 1 % penicilline-streptomycine solution (SIGMA Aldrich). The cells were incubated in 75 cm^2 plastic culture flasks (Greiner, Frickenhausen, Germany) and could be passaged once a week for usage in biotests approximately 100 times. 4 - 5 days after passaging, they formed a confluent monolayer and were ready to be used in a biotest. For passaging, the old medium was discarded and the cells were washed with 5 ml of phosphate buffered saline (PBS without calcium and magnesium, Sigma). In order to detach the cells from the culture flask, 2 ml of a trypsin solution were used over 3 minutes. Proteoylsis was inhibited by adding 5 ml of supplemented L15-medium containing protease inhibitors in fetal bovine serum. Cells were resuspended in the supplemented L15-medium and equally distributed in two new flasks containing 10 ml of supplemented medium or used in biotests. In case of the later, antibiotic-free medium was used to avoid interactions with any possible compounds of the extracts.

V79 cells (Chinese hamster lung)

For the assessment of cytotoxicity (Ch. 2.4.2) and genotoxicity in the micronucleus assay (Ch. 2.4.3), the permanent mammalian cell line V79 from Chinese hamster lung cells was used. The cells were obtained in 2006 from RCC (Roßdorf, Germany). Cells were cultured at 37 °C under 5 % CO_2 in Minimum Essential Medium (SIGMA Aldrich) supplemented with 10 % fetal bovine serum (Biochrom), 1 % l-glutamine-solution (200 mM, Sigma), and 1 % penicilline-streptomycine solution (Sigma). The cells were incubated in 25 cm^2 plastic culture flasks (Greiner) and were passaged as described above twice a week and used for the biotests until passage 15.

Examination of mycoplasma contamination

Mycoplasma contamination still remains one of the major problems in cell culture. These bacteria can induce an unlimited variety of effects in the cell cultures they infect (Butler and Leach 1964, Collier et al. 1969, Fogh et al. 1971, Sokolova et al. 1998). Therefore, it is indispensable prerequisite to guarantee that all cells used in bioassays are not contaminated by mycoplasmas. To this end, the RTL-W1 as well as the V79 cells were screened at least every four weeks, during experiments every two weeks, for contamination by mycoplasmas through the polymerase chain reaction according to Tang et al. (2000) with slight modifications by Uphoff and Drexler (2002) and Hopert et al. (1993). In case contamination could be observed, all affected cells, media and solutions used were discarded, and uninfected cryo stocks were thawed and used instead.

2.4.2 Acute cytotoxicity

Neutral red assay with RTL-W1 cells

Preliminary to other *in vitro* tests with cell cultures, sediment extracts had to be tested for acute cytotoxicity to avoid false positive results (Choucroun et al. 2001, Hartmann et al. 2001c, Hartmann and Speit 1997, Henderson et al. 1998). The method used in this assay followed Borenfreund and Puerner (1985) with slight modifications (Kosmehl et al. 2004). Exposure was conducted in 96-well plates (TTP, Renner, Dannstadt, Germany) with a cell density of 4-5 x10^4 cells/well and dilution of the sediment extracts was carried out directly in the wells (Fig. 11). According to Keiter et al. (2006), the DMSO concentration used in the assay should not exceed 1 % to exclude solvent specific effects on the cells. Thus, the highest concentration was 200 mg SEQ/ml. Negative, solvent and process controls were applied to exclude any non extract specific effects. 3,5-Dichlorphenol (Aldrich, Steinheim, Germany) at a final concentration of 4 mg/ml was used as positive control.

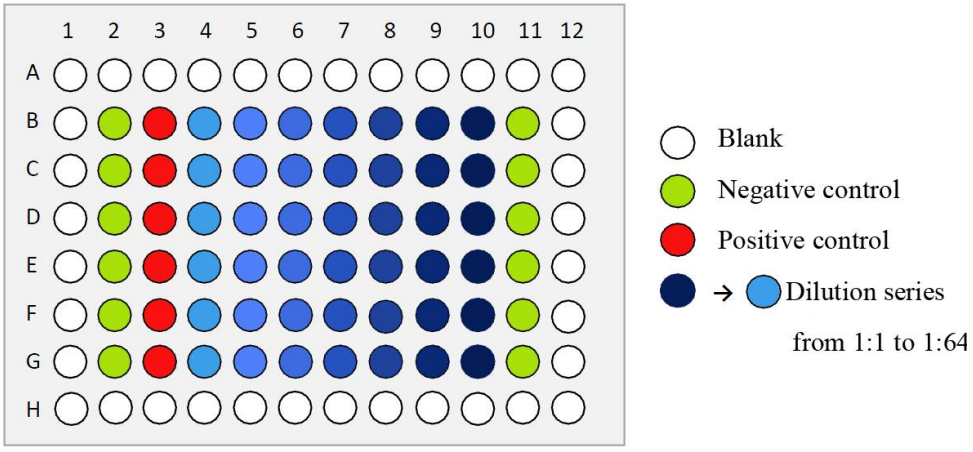

Fig. 11: Scheme of a 96-well plate for the neutral red assay.

After 48 hours of exposure, the test medium was replaced by a 1:80 dilution of 0.4 % neutral red solution with medium, and cells were incubated for another 3 hours at 20°C to ensure uptake of the dye into intact cells (Barile 1994, Segner 1998b). After discarding surplus dye solution and duplicate thorough washing done twice with PBS, 100 µl of the neutral red extraction solution (1 % glacial acetic acid [Merck, Darmstadt, Germany], 50 % ethanol p.a. [Riedel-de Haën, Seelze, Germany] in Aqua bidest) were added to each well and shaken for 30 min. The retention of neutral red was measured photometrically at 540 nm against a reference wavelength of 690 nm using a multiwell plate reader (Spectra™ III; Tecan, Crailsheim, Germany). The assay was considered valid if the medians of the two negative controls did not differ from each other by more than 20 %. The percentage of the values for each well was calculated on the median of the negative controls in column 2. In the next step, for each concentration the medians (in percent of the negative control) and the standard deviations were calculated. Finally, dose-response curves were plotted using SigmaPlot 11.0 (Jandel SPSS, Erkrath, Germany). Each extract was tested in three independent replicates and if possible, NR_{50} and NR_{80} values were stated. At these concentrations, the neutral red uptake of the exposed cells corresponds to 50 and 80 % of the negative control or, in other words, to 50 and 20 % lethality of the cells.

Neutral red assay with V79 cells

Acute cytotoxicity to V79 cells was tested with the neutral red assay as explained for the RTL-W1 cells above. However, 20 hours prior to exposure, 100 µL with approximately 10^4 cells (10 x 10^4 cells/ml) were seeded into each well to ensure secure attachment to the wells' surface. Dilution of the extracts was then conducted in a separate well plate and later transferred carefully onto the cells from which the old medium had been removed before. Furthermore, for exogenous metabolic activation, S9-mix (Mp Biomedicals, Eschwege, Germany) was added to the test medium to gain concentration ranges for the micronucleus assay. The S9-fraction had been extracted from livers of rats exposed to Aroclor 1254 (Ames et al. 1975); its final concentration in the mix was 10 % which in turn was used at a concentration of 20 % in the test system (Tab. 8). S-9 exposure was only conducted for four hrs due to the inherent toxicity of the S9-Mix. Measurement of neutral red retention was performed as explained above.

Tab. 8: Composition and concentrations of the S9-Mix.

Substance	Concentration of stock solution	Concentration in S9-mix	Volume for 2.5 ml
Isocitrate	1 M	5 nM	12.5 µl
NADP-solution	0.1 M	4 mM	100 µl
KCl-$MgCl_2$-solution	1.65 M KCl 0.4 M $MgCl_2$	33 mM KCl, 8 mM $MgCl_2$	50 µl
Phosphat buffer pH 7,4	0.2 M	15 mM	187.5 µl
S9-fraction		10%	250 µl
Sterile water			1900 µl

2.4.3 Genotoxicity

Genotoxicity is the interaction of DNA at the level of genes, chromosomes or whole genomes caused by chemical or physical agents, which can also lead to permanent mutations (Fent 2007a). Mutagens cause DNA lesions, strand breaks, modified bases and DNA crosslinks (Farmer et al. 2003, Kim and Hyun 2006). These alterations cause a change of the DNA sequence during the next cell cycle. Most mutagens are also known to cause cancer (Fent 2007a). Directly genotoxic chemicals are of electrophilic character and do not require metabolic activation to interact with the DNA (Marquardt 1994). Indirect genotoxic substances, on the other hand, require metabolic activation to cause adverse effects. Due to the differences in their metabolic capabilities, these effects may differ between species, individuals, and organs (Marquardt 1994, Nehls and Segner 2001). The role of genotoxins in the environment is still unsettled (Fent 2007a), however, the study and research of genotoxically contaminated environmental samples has received special attention, because mutagenesis and carcinogenesis can threaten the survival of individuals, entire populations, and species, especially when effecting gametes.(Connell et al. 1999).

Comet assay with RTL-W1 cells

The comet assay is a sensitive, rapid and simple technique for evaluating DNA breakage at the level of single eukaryotic cells (Mitchelmore and Chipman 1998, Nehls and Segner 2005, Singh et al. 1988, Tice 2000). The comet assay was performed under alkaline conditions according to Singh et al. (1988) with modifications detailed in Schnurstein and Braunbeck (2001) and Boettcher et al. (2010) using the cell line RTL-W1 without exogenous metabolic activation. In order to avoid false positive results, Henderson and coworkers (1998) showed that no concentration of a substance should be tested in the comet assay affecting the cell vitality more than 25 %. Hence, the results of the cytotoxicity test were used to determine the highest applicable test concentrations for the comet assay, which ensured 80 % vitality of the cells. Exposure was conducted in antibiotic-free medium for 24 hours and negative controls with the test medium, solvent controls with 1 % DMSO, and a process control of the Soxhlet extraction were also tested. For the positive control (Green et al. 1993, Klee et al. 2004, Kosmehl et al. 2004, McKelvey-Martin et al. 1993, Schnurstein and Braunbeck 2001), RTL-W1 cells were irradiated for 5 min at 252 nm using a crosslinker (8W, UV Stratalinker 1800, Stratagene, La Jolla, California).

All slides were examined at a magnification of 320 X using a fluorescence microscope (Aristoplan, Leica, FRG) equipped with an excitation filter of 518 nm and an image-analysis system (Optilas, Munich, Germany) with a grey-scale CCD camera (JAI Pulnix TM-765E Kinetic, Glostrup, Denmark) and Comet 3.0 software (Kinetic Images, Liverpool, UK). For each concentration, the tail moments of 100 randomly selected cells were analyzed by

multiplying length and fluorescence intensity of the tail (Schnurstein and Braunbeck 2001). The data was compiled in box plots showing median, 25 and 75 percentiles as a box and 5 and 95 percentiles as dots (Sigma Plot 11.0, SPSS - Jandel, Erkrath, FRG). For statistical analysis, data were analyzed with the H-test according to Kruskal and Wallis. In case of significant differences, a post hoc test according to Dunn was used to identify groups that differed significantly, which were then marked by an asterisk. The induction factor (IF) was calculated by dividing the median of each concentration by the median of the corresponding control group. To simplify the comparisons, data were converted into a "concentration dependent induction factor" (CDI) according to Seitz et al. (2008). The CDI is a simple index that integrates all important information, providing a basis for a general comparison of the genotoxic potential in the comet assay. The CDI is calculated as follows:

$$\mathrm{CDI} = \sum_{i=1}^{n} \frac{\mathrm{IF_i}}{\mathrm{C_i}}$$

IF_i = induction factor of the concentration i; c_i = concentration i; and n = n concentrations.

Micronucleus assay with RTL-W1 cells

The *in vitro* micronucleus assay was performed according to ISO/DIS 21427-2 (2004) and the OECD Guideline 487 (2010) with modifications after Boettcher et al. (2010) using the cell line RTL-W1 without exogenous metabolic activation. Before exposure, cells were transferred to 6-well plates with ethanol-cleaned cover slips (Assistent, Sondheim, Germany) and incubated for 24 h in pure medium to ensure complete cell attachment to the slides. Exposure to sediment extracts was conducted in antibiotic-free medium for 24 hours with the same concentrations as used for the Comet assay. Nitroquinoline-*N*-oxide (NQO, Sigma) in a concentration of 100 mg/ml medium was used as positive control, and negative controls with the test medium, 1 % DMSO and a process control of the Soxhlet extraction were also tested.

Following treatment, cells were incubated with pure medium for another 72 h to ensure cell division after exposure. Cells were then fixed for 10 min with methanol/acetic acid (4:1) diluted in PBS (1:1). A second fixation was performed with methanol/acetic acid (4:1). Air-dried slides were kept in the well plates until visual examination under a fluorescence microscope (Aristoplan, Leitz). Staining was conducted with acridine orange (40 mg/L PBS; Sigma), resulting in nuclei and micronuclei appearing green and cytoplasm appearing red. Criteria for micronuclei and anomalies in RTL-W1 cells were set according to ISO 21427-2 (2004).

Micronucleus assay with V79 cells with metabolic activation

The *in vitro* micronucleus assay with V79 cells was performed according to ISO/DIS 21427-2 (2004) and the OECD Guideline 487 (2010) with exogenous metabolic activation through

addition of S9-mix (Mp Biomedicals). The S9-fraction was extracted from livers of rats that had been exposed to Aroclor 1254 (Ames et al. 1975) and its final concentration in the mix was 10 % which in turn was used at a concentration of 20 % in the test system (Tab. 8). 6 Hours prior to exposure, V79 cells were seeded at a density of 2 x 105 cells/ml into 1 ml chamber slides (Lab-Tek Culture Chambers, Permanox slide, Nunc, USA) to guarantee attachment to the slides. Sediment extracts were tested in serum- and antibiotic-free medium at four different concentrations, of which the highest resulted in no more than 20% lethality in the neutral red assay. 100 µM benzo[a]pyrene served as a positive control to prove metabolic activation by the S9-mix (Békaert et al. 1999). Three negative controls with the test medium only, 1 % DMSO and a process control of the Soxhlet extraction were also tested. Exposure with S9-mix was conducted for 4 hrs to avoid toxic effects caused by the S9-fraction itself. This was followed by incubation in pure complemented medium for another 20 hours to guarantee cell division after exposure. Fixation and staining was conducted in the same way as shown above for RTL-W1 cells. Criteria for micronuclei and anomalies V79 cells were set according to ISO 21427-2 (2004). The induction factor (IF) was calculated by dividing the number of micronucleated cells in the treatments with the equivalent number in the negative controls. Furthermore, the lowest effect concentration (LOEC) was determined for each sampling site.

2.4.4 EROD assay

Induction of cytochrome P450 dependent monoxygenases in fish is a well-established and well-documented biomarker to study responses to xenobiotic exposure at the molecular level (Behrens et al. 2001, Burke et al. 1985, Fent , Hahn and Stegeman 1994, Sarasquete and Segner 2000, Scholz and Segner 1999). The cytochromes P450 are a multi-gene family of heme-containing proteins (Guengerich 1988) that oxidize, hydrolyze, or reduce compounds through the insertion of atmospheric oxygen to the substrate, generally increasing the water solubility of substrates, thereby enhancing their elimination (Andersson and Förlin 1992). However, these phase I metabolites of some contaminants may be the intrinsic toxic compound and are thus bioactivated through the enzyme system (Guengerich et al. 1985). Induction of the isoenzyme cytochrome P4501A (CYPlA), that, among others, metabolizes polycyclic aromatic hydrocarbons (PAHs), is mediated through the binding of certain xenobiotics to the cytosolic aryl hydrocarbon receptor (AhR) which then translocates to the nuclei to switch partner molecules from Hsp90 to Arnt to bind as promoter complex to specific DNA sequences (Mimura and Fujii-Kuriyama 2003). Among the more than 1000 known organic compounds that bind to the AhR are environmentally critical planar polychlorinated biphenyls (PCBs), PAHs, polychlorinated dibenzodioxins (PCDDs) and polychlorinated dibenzofuranes (PCDFs) (Fent 2007a). The catalytic activity of CYP1A can be assessed by the dealkylation of the sythetic substrate 7-ethoxyresorufin (7-EXR) to fluorescent resorufin (EROD activity).

Increased EROD activity after exposure to PAHs and PCBs has been shown in RTL-W1 cells (Bols et al. 1999).

EROD assay with RTL-W1 cells

To evaluate the dioxin-like activity of the sediment samples, a new protocol of the live-cell-EROD assay for RTL-W1 cells developed in cooperation with Patrick Heinrich and Ulrike Diehl was applied (Heinrich et al. 2014). This new approach combines assessment of cell vitality through the cytotoxicity with thiazoly Blue tetrazolium bromide (MTT assay (Cole 1986, Gerlier and Thomasset 1986)) to exclude overlying cytotoxic effects of the samples and measurement of EROD activity through scaling of the results to the number of cells and EROD induction of β-napthoflavone (BNF, SIGMA Aldrich). Since maximum EROD activities tend to show natural fluctuation among experimental runs, EROD induction capabilities of substances and environmental samples are usually expressed in relation to established reference substances. BNF was used as reference substance instead of 2,3,7,8-tetrachlorodibenzo-*p*-Dioxin (TCDD), as TCDD is one of the most toxic substances known (Birnbaum and Tuomisto 2000, Cantrell et al. 1996, Ott and Zober 1996, Poland et al. 1982, Safe et al. 1991) and should, therefore be, avoided in routine laboratory use. Furthermore, it could be shown that BNF serves as an equally good inductor of EROD activity in RTL-W1 cells in even lower concentrations resulting in similar maximum inductions and time-response relationships (Fig. 11).

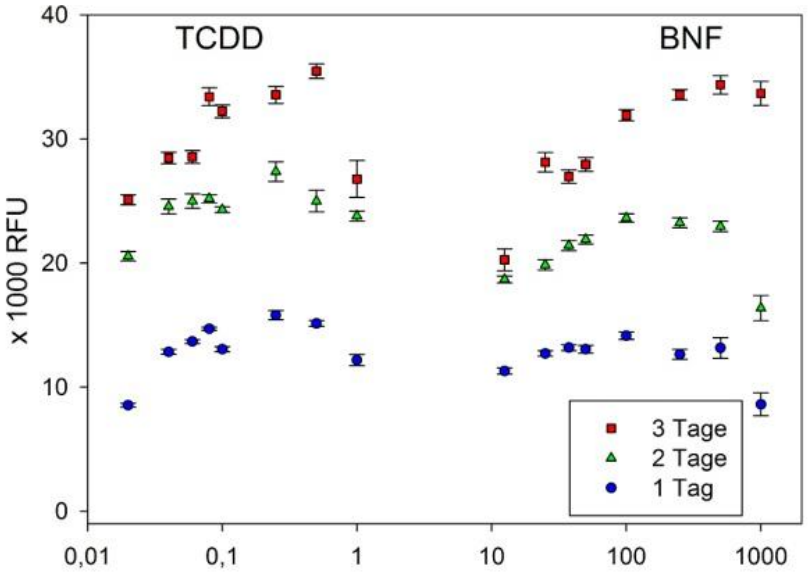

Fig. 11: Comparative EROD induction of TCDD and β-napthoflavone.

Exposure. 24 Hours prior to exposure, 100 µL of a cell suspension of RTL-W1 cells in L-15 medium (SIGMA) with a density of $4x10^4$ cells/ml was seeded into each well of 96-well plates (TTP) except for 6 wells that later served as blanks and two rows for the resorufin standards and the cell dilution row for the straight calibration line of the basal metabolic rate of

the cells. Sample dilutions were prepared in test tubes, the highest concentration being 400 mg SEQ/ml with 2 % DMSO. As reference for the induction of EROD activity, eight 1:2 dilutions of BNF was also prepared in test tubes, the highest concentration being 200 nM. 3,5-Dichlorphenol (3,5-DCP, Aldrich) at a concentration of 160 mg/ml was prepared to serve as positive control for the MTT assay. The dilutions were added to the wells (Fig. 12) and since these already contain 100 µL of cell suspension and L15-medium, the concentrations were diluted 1:2 in each case resulting in the highest concentrations of 200 mg SEQ/ml for the sediment extracts, 100 nM for BNF and 80 mg/ml for 3,5-DCP. To obtain the straight calibration line the basal metabolic rate for scaling of the results, 40,000, 30,000, 20,000 and 10,000 cells were seeded into two wells each of one row (Fig. 12). To guarantee identical conditions in all wells – positive and negative controls, extract dilutions and cell dilution – the final DMSO concentration used was always 1 %. The plates were incubated for 24 hours at 20°C.

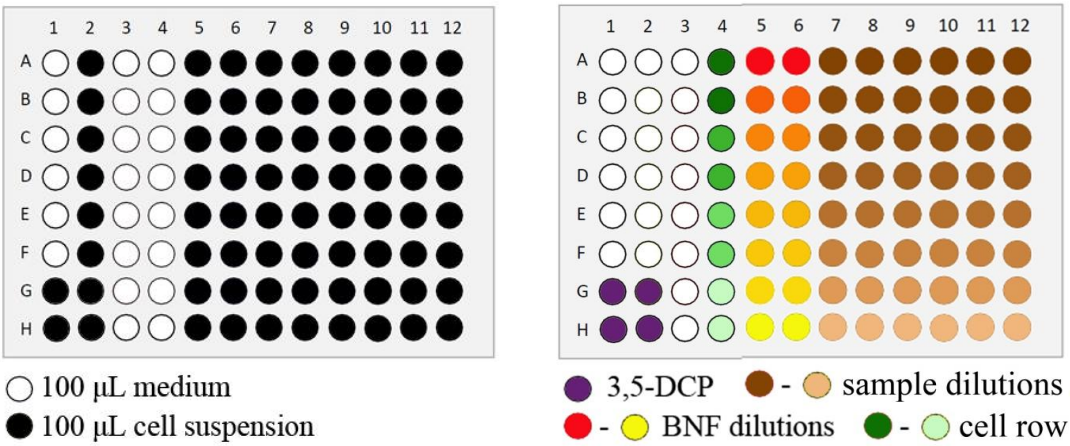

Fig. 12: Scheme for the EROD assay in 96-well-plates; left: seeding of cell suspension 24 hours prior to exposure, right: insertion of positive controls and sample dilutions and cell dilution for cell count

As reducing sulfur compounds may be present in acetonic sediment extracts and are able to react with MTT, well-plates containing only the dilution rows of the sediment extracts but no cells were also prepared and incubated for 24 hours at 20°C. These external blanks were later substracted from the results of the MTT assay.

Measurement of resorufin and MTT. After exposure, medium was discarded and replaced by DMEM-medium (Sigma) with 8 µM 7-EXR except for row 3 as this was where the resorufin standards were applied. These were prepared in test tubes directly with DMEM with 8 µM 7-EXR at concentrations of 300 nM, 150 nM, 75 nM and 37.5 nM. Incubation should always be the same, ideally for 30 minutes, but it was not to exceed 45 minutes. Afterwards, 150 µL of each well were transferred carefully without influencing the cell layer into black 96-well-plates and kept dark until measurement. To the remaining 50 µL in the original plates, 150 µL

of DMEM-medium with 588 µg/ml MTT were added and incubated for 3 hours at 20°C in the dark. The same was applied to the plates with the external blanks. Meanwhlie, the fluorescence of the resorufin of the standards and of the resorufin formed by deethylation of 7-EXR by the cells was measured with the black well-plates in the plate reader (Spectra™ III) at a wavelength of 544 nm (excitation) and 590 nm (emission). After exposure, the MTT solution was discarded carefully from the treatment and external blank plates and replaced by 200 µL DMSO with 2.5 % ammonia (Wang et al. 2012). Measurement in the plate reader was then conducted at 540 nm (absorption) and 690nm (reference).

Data interpretation. Calibration curves were generated for the results of the resorufin standards and the MTT assay of the cell dilution. R^2 should not be less that 0.98 and 0.95, respectively. The EROD activity of the sediment samples was then scaled to metabolic activity in each well. This activity was measured in metabolic cell equivalents (MCE), where the activity of 1000 unexposed cells is defined as 1 MCE:

$$\text{EROD Activity} = \text{Resorufin [pmol]} \times \text{MCE}^{-1} \times \text{incubation duration [min}^{-1}\text{]}$$

Furthermore, the dose-response of BNF towards the EROD activity was fitted to the following 2-parametrical logarithmic model:

$$f(x) = a \times \ln x + b$$

To evaluate the EROD activity induced by the sediment samples, the sediment concentration inducing the highest amount of EROD-activity was selected and the concentration of BNF inducing equal activity was determined mathematically using the equation:

$$f(x) = e\text{^}(-(b/a)+(\text{Activity [fmol Resorufin} \times \text{MCE}^{-1} \times \text{min}^{-1}\text{]}/a))$$

Results from the MTT assay can help to distinguish EROD induction or inhibition from effects caused by beginning apoptosis. All information can be depicted in one graph as shown in fig. 13. To simplify matters, however, the BNF regression will be disregarded for the presentation of the results as the BNF equivalent is obtained mathematically.

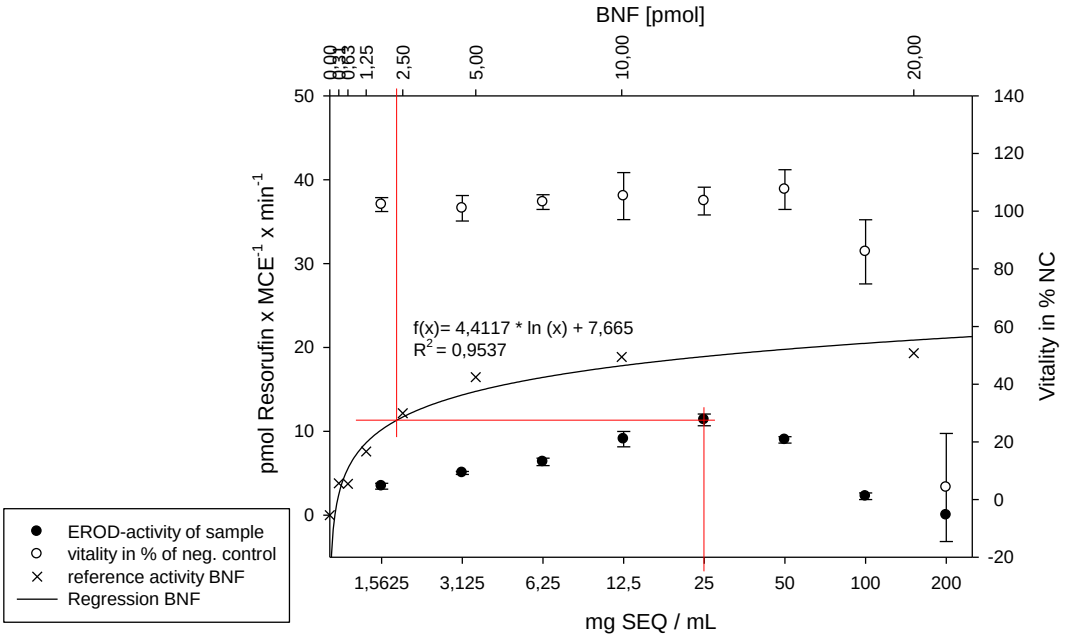

Fig. 13: Graphic illustration of the EROD activity scaled to MCE × min^{-1} induced by several concentrations of sediment samples and BNF and of the cell vitality measured through the MTT assay as vitality in 5 of the negative control.

2.5 *In vivo* assay: zebrafish embryo toxicity test with *Danio rerio*

In recent years, acute toxicity tests with fish have aroused considerable ethical concern since they are conducted with juvenile or adult animals. European legislation requires that non-animal, alternative approaches of testing should be used in the place of animal procedures wherever possible (REACH 2006). Russell and Burch (1959) originally set out the definition of the three Rs (3Rs): 'Replacement', 'Reduction' and 'Refinement'. 'Replacement' means the substitution of conscious living higher animals by insentient material, 'Reduction' means reduction in the numbers of animals used to obtain information. 'Refinement' means any decrease in the incidence or severity of inhumane procedures applied to those animals that still have to be used. Beside ethical considerations, Zebrafish Embryo Toxicity Test with *Danio rerio* test offers other advantages compared to the acute fish test. It is easy to handle, the fish are easy to keep and breed in the laboratories, and eggs can be delivered all seasons and only little volume of substances or samples is needed. Moreover, it allows an insight into toxic effects on the very early developmental stages. Investigations with other major OECD species, the fathead minnow (*Pimephales promelas*) and the Japanese Medaka (*Oryzias latipes*), revealed a high comparability between the species and a better reproducibility compared to acute toxicity tests with different adult fish species (Braunbeck et al. 2005). In addition, Ratte and Hammers-Wirtz (2003) and Lammer et al (2009) compared existing data from zebrafish embryo tests with existing data from acute in vivo fish tests and showed that there is a reliable correlation between the fish embryo test and the acute fish test.

The zebrafish *Danio rerio*

It was first described from the Kosi tributary of the Ganges River in India (*Danio rerio*, Hamilton-Buchanan 1822). It is a small benthopelagic freshwater representative of the family of cyprinids. The species has been studied extensively since the nineteenth century as it is easily obtainable, inexpensive and readily maintainable (Laale 1977). The embryonic development of *Danio rerio* is described in Kimmel et al. (1995). The fish has an elongated, slightly compressed habit and reaches a mean adult length between 3 and 5 cm. Both male and female fish are brownish-olive coloured with a yellow-white waist and five uniformly, pigmented, horizontal lateral stripes, all extending onto the end of caudal fin rays (Fig. 14). During spawn maturity, females can be distinguished easily from the male by their swollen bellies.

Male fish are more slender and show an orange to reddish tint in the silvery bands along the body. One female produces 50 - 200 eggs per day and only needs 2 to 3 days for regeneration. Thus, under appropriate conditions, a large number of non-adherent, fully transparent eggs can be obtained all-seasonally (Laale 1977). Due to a very short developmental period and the eggs' transparency, the zebrafish embryonic development has been described in detail in numerous studies (Kimmel et al. 1995, Westerfield 2000), and the species has become a major model in neurobiology and toxicology as well as in general molecular and developmental biology (Busquet et al. 2008, Hollert et al. 2003, Kimmel et al. 1995, Laale 1977, Wells et al. 2005, Westerfield 2000).

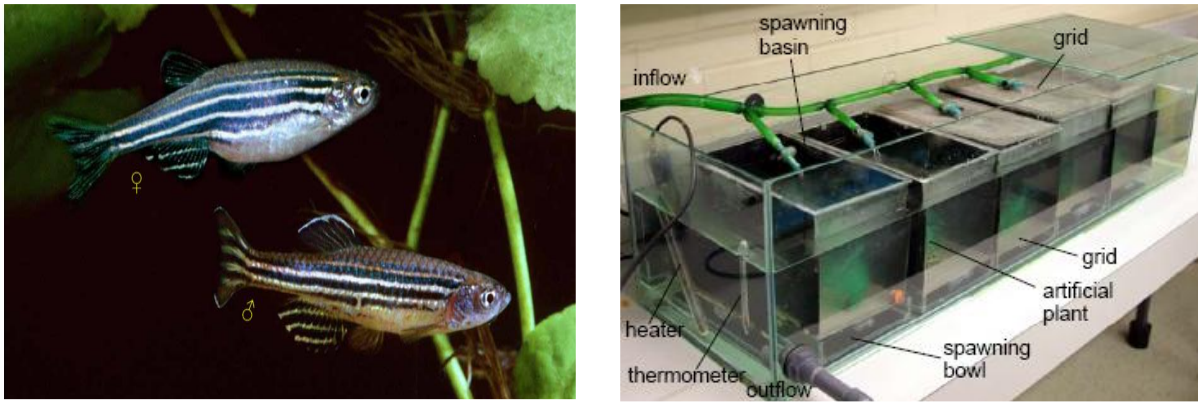

Fig. 14: Left: adult zebrafish – upper individual: female with swollen belly; lower individual: male with reddish tint in the silvery bands (Photo: Erik Leist). Right: Spawning tanks for egg production of *Danio rerio* (Photo: Nadja Seitz).

Animal husbandry and breeding of *Danio rerio*

All fish used for breeding were descendents of the "West-aquarium" strain, originally obtained from Dr. H.J. Pluta (Federal Environment Agency, Berlin, FRG). The fish were maintained in 150 L-fish tanks at 26 ± 1°C in a light-isolated room. An artificial light-dark cycle of 12 each hours was maintained. The fish tank water was purified by an internal activated-carbon filter (Eheim, Deizisau, Germany) and aerated continuously with compressed air. The animals were fed twice a day with fresh larvae from *Artemia* (Great Salt Lake Artemia Cysts, Sanders Brine Shrimp Company, Ogden, USA) and with dry flake food (TetraMinTM, Tetra-Werke, Melle) as required. For egg production, the fish were transferred into a special spawning facility (Fig. 15) at a ratio of 1:1. The fish started spawning at the latest 30 minutes after beginning of the light-period in the morning. As a special stimulant, a green plant dummy made out of plastic was used. As zebrafish tend to predate their own eggs, grids with a mesh size of 1.25 mm were adjusted at the bottom of the tanks, through which eggs fell into a separate spawning tray. Eggs were collected 1 hour after spawning.

Test procedure

For the assessment of teratogenic and embryotoxic effects of the sediment extracts, the Zebrafish Embryo Toxicity Assay was conducted according to OECD TG 236 under semistatic conditions. Using the DMSO stock solutions of the extracts, five different concentrations were tested in three independent replicates. Artificial water was used for dilution according to ISO 7346/3. The highest concentration tested was limited by the concentration of DMSO used in the assay, which was not to exceed 0.15 % due to its capability of changing the chorion's permeability (Kais 2009). Thus, extracts were tested at 50, 37.5, 25, 15.5, and 1 mg SEQ/ml and DMSO concentration was always adjusted to 0.15 % to secure constant conditions. To exclude effects caused by the dilution water, a negative control with artificial water corresponding was applied. 3,4-Dichloroaniline served as positive control at a concentration of 3.7 mg/L . Furthermore, a solvent control with o.15 % DMSO and a process control of the soxhlet extraction (ch. 2.3.2) were carried out for each replicate. As tests were conducted with 24-well-multiplates made of polystyrene (TPP; Trasladingen, Switzerland, ISO 9001, Fig 2.3.1) and adsorption of lipophilic chemicals to negative binding sites of plastic has been described in several studies (Knorr and Gätzschmann 1967, Palmgren et al. 2006, Schreiber et al. 2007), multiplates were pre-treated with the respective test concentrations 24 hours prior to the actual embryo test. A test was classified as valid according to OECD TG 236, if ≥ 90 % of the embryos in the negative and solvent controls treatments showed neither sublethal nor lethal effects, and the positive control delivered lethal effects ranging between 20% and 80% 96 hours post fertilization (hpf).

Toxicological endpoints. Embryos were examined at 24, 48, 72 and 96 hpf. Endpoints were determined according to OECD TG 236 as well as to Bachmann (2002a) and Nagel (2002) and are shown in.

Tab. **9**.

Tab. 9: Evaluation endpoints of acute toxicity and teratogenicity on the embryo of *Danio rerio*.

	hours post fertilization			
Toxicological endpoints	24	48	72	96
Coagulation	o	o	o	o
Retarded somite stage	o			
Tail not detached	o			
Lack of heartbeat		o	o	o
Failure of hatching				•
Reduced heartbeat rate		•	•	•
Lack of blood circulation		•	•	•
Reduced blood circulation		•	•	•
Affected eye development	•	•	•	•
Underdevelopment	•	•	•	•
Edema formation		•	•	•
Affected pigmentation		•	•	•
Malformation in general	•	•	•	•
Tail malformation		•	•	•
Spine malformation			•	•

o: lethal endpoint; •: sublethal endpoint

Data interpretation. The LC_{50} and EC_{50} values, the concentrations at which 50 % of the embryos were lethally affected or showed any effect, respectively, were determined with ToxRat® Professional, Version 2.10 (ToxRat Solutions, Alsdorf) using probit analysis with linear maximum likelihood regression or moving average computation after Thompson for each exposure time. The logarithmic average of all replicates was then used for toxicity assessment. Furthermore, the lowest observed effect concentration (LOEC) and the no observed effect concentration (NOEC) were determined. All graphs in this thesis were prepared with Sigma Plot 11.0 (SPSS - Jandel, Erkrath, FRG).

2.6 Classification of sediment toxicity

For the classification of the Jordanian watercourses studied within the scope of this thesis, the results of 1) the neutral red assay with RTWL-W1 cells, 2) the comet assay with RTWL-W1 cells, and 3) the fish embryo toxicity test with zebrafish were rated according to the toxicity threshold values established within the framework of a fuzzy logic-classification approach by Keiter et al. (2009b). Following this approach, the dataset of each bioassay is divided into three toxicity levels (non-toxic, moderately toxic and strongly toxic) to cover the entire response range of test systems. To gain a location-independent insight into the response ranges, data from various studies were integrated into this calculation: Danube (Keiter et al. 2009a, Keiter et al. 2008, Keiter et al. 2006, Seitz et al. 2008), Rhine (Kosmehl et al. 2004) and Neckar (Braunbeck et al. 2009, Hollert et al. 2000, Hollert et al. 2002b). Among other methods, the empirical method was used and proved to be the most suitable by Keiter et al. (2009b). It offers the possibility to consider the whole specific response range of a biotest by showing the distribution of the dataset (Ahlf and Heise 2005) and to, thus, allocate results of the bioassays to the toxicity levels. The dataset is then categorized into three equal intervals with 33.3 % each, the lower interval representing non-toxic results, the medium interval representing moderately toxic results and the upper interval representing strongly toxic results (Fig. 15). The threshold values resulting out of this classification are listed in Tab. 10 and were applied to the results obtained for Jordanian surface waters in this study. Three colors are used to facilitate orientation: red = strongly toxic, yellow = moderately toxic, green = non-toxic.

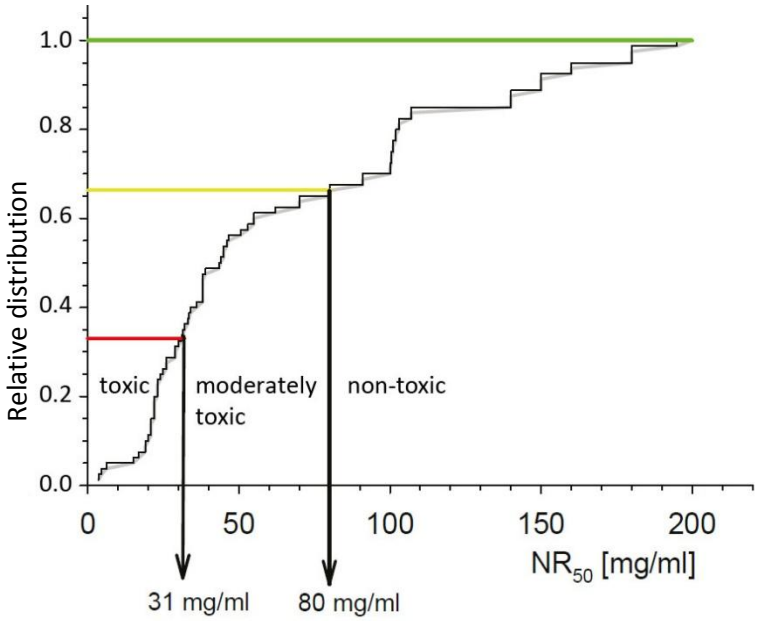

Fig. 15: Empiric method for the determination of toxicity threshold values according to Keiter et al. (2009b) as shown exemplarily for the neutral red assay. The relative distribution of NR_{50} values is divided into three equal intervals determining toxicity levels (Fig.: Keiter).

Tab. 10: Threshold values for toxicity levels according to the fuzzy logic approach by Keiter et al (2009b) based on the empirical method.

	Threshhold values for the toxicity levels		
	non-toxic	Moderatley toxic	Strongly toxic
Neutral red assay [NR_{50} mg SEQ/ml]	> 80	80-31	< 31
Comet assay [CDI]	< 0.24	0.24 -0.5	> 0.5
Fish embryo toxicity test [LC_{50} mg SEQ/ml]	> 21	21 -11	< 11

A rank-sum based classification was applied according to Canfield at al. (1994) and Hollert et al. (2002b) for the EROD assay and the Micronucleus assay as they were conducted after a different protocol than in Keiter at al. (2009b). Thus, data for each individual result was scaled proportionally between 1 % and 100 % (e.g. an IF in the Micronucleus assay of 1.3 being the lowest and an IF of 4.1 being the highest observed effect). Scaling of data results in a relative ranking of results. The ranked data is then classified into three groups equivalent to the toxicity levels of Keiter et al (2009b): non-toxic < 33.3 %, moderately toxic for 33.3 to 66.6 % and strongly toxic > 66.6 %.

3. Results

3.1 Morphological an physical parameters, nutrients and salts

3.1.1 Jordan River

Anthropogenic influence on the Lower Jordan River was apparent at each sampling site. At sites Jordan 1 and 2, the river was channeled even though it was not artificially fortified (Fig. 16). Fluvial adjustment was most striking in the anthropogenic abstraction of water during the river flow: width and depths of the river declined rather steadily from 3 m and 100 cm at Jordan 2, 10 m and 100 cm at Jordan 2, 4 m and 30 cm at Jordan 3, 3 m and 10 – 20 cm at Jordan4 to 3 m and 20 cm at Jordan 5. These changes have also been described as severe interference in other studies (Gafny et al. 2010, Hassan and Klein 2002). The water generally showed strong turbidity. Thus, visibility ranged from 5 (Jordan 1) to 30 cm (Jordan 5). Strong odor of hydrogen sulfur emerged during sampling at site Jordan 1. All other sites showed constructional modifications due to bridges (Fig. 17). At site 5, concrete slabs lay in the water due to the blasting of the King Abdullah Bridge in 1967.

Fig. 16: Sampling sites at the Jordan River: a, b) Baqura (Jordan 1), c, d) Sheik Hussein Bridge (Jordan 2).

Fig. 17: Sampling sites at the Jordan River: a, b) Damiya (Jordan 3), c, d) Allenby Bridge (Jordan 4), e, f) King Abdullah Bridge (Jordan 5).

Physical characteristics as well as the concentrations of nutrients and salts in the water of the Lower Jordan River are summarized in Tab. 11. The results were classified according to LAWA (1998) as well as Graw and Borchardt (1999) as described in Ch 2.2. Considering all allocable parameters to the same degree and calculating the mean value of the classes rated, sampling site Jordan 1 and Jordan 2 are rated as water quality class III-IV, whereas the remaining sites were rated class III and II-III. Electrical conductivity was very high at all sampling sites and thus rated class IV. It increased during the flow from 7.4 mS/cm to 13.5 mS/cm. This may be due to geogenic influence and due to the discharge of saline waters (Shavit et al. 2003), but can

also be an indicator of inorganic contamination (Buhr et al. 2001). Toxicity of substances in the water may be influenced by ph values. Due to the contents of limestone in the fluvial sediments (Rimmer and Salingar 2006), ph values were found to be slightly alkaline, but rather constant ranging from 7.7 (Jordan 2) to 8.3 (Jordan 1 and 5). Oxygen contents generally improved along the river flow. The worst situation was found at site Jordan 2, where there were only 2.0 mg/ml to be measured and, thus, was rated class IV. Oxygen contents below 3.0 mg/ml are usually considered unsuitable for fish. Only the sites Jordan 4 and 5 could be rated class I and I-II showing 11 and 8.2 mg O_2/ml, respectively. This could probably be ascribed to the turbulences due to shallow water and rather high current velocity of 1 m/s. The other two sampling sites with 6.6 and 7.8 mg O_2/ml were rated moderately contaminated. Evaluation of the BOD_5 resulted in the category II-III for Jordan 2, 3 and 4 and in category III with signs for increased contamination for Jordan 1. Jordan 2 had a BOD_5 of 1.9 mg/ml which is rated class II, however, having a total oxygen content of only 2 mg/ml, the BOD_5 equals nearly all oxygen available for metabolizing bacteria at this sampling site. For all sampling sites at the Lower Jordan River, very high concentrations of chloride ions have been detected with 2.5 g/ml which is a well-known phenomenon at the Jordan River and associated with discharge of the Saline Water carrier and hypersaline springs (Comair et al. 2012, Farber et al. 2004, Shavit et al. 2003, Vengosh 2003). A drop in ammonia levels was evident for Jordan 3, 4 and 5. With the method used, 0.5 mg/ml was the smallest amount to be measured. Thus, the quality for these sites may be better than rated here. Jordan 1 was rated class IV with 2 mg/ml, and the highest concentration was found at Jordan 2 with 10 mg/ml. Similar results were described in studies by Barel-Cohen et al (2006) as well as Holtzman et al. (2005). The values for nitrite significantly improved from 1.0 mg/ml at Jordan 1 over 0.1 mg/ml at sites 2 and 3 to 0.025 mg/ml or even less at Jordan 4 and 5. The concentration of nitrate was 10 mg/ml for all sampling sites except Jordan 1 where it was 25 mg/ml, which was, thus, rated class IV. Fish ponds and agricultural drainage in the northern parts of the Jordan Valley have been identified as reasons for the strong contamination by nitrogen along the upper stretches of the Lower Jordan River (Gat and Dansgaard 1972, Segal-Rozenhaimer et al. 2004). According to phosphate concentrations, Jordan 1 and 2 are classified as category IV and Jordan 3, 4 and 5 as category III-IV with 6.7 and 1 mg/ml, respectively. However, 1 mg/ml was the detection limit for the method used. Thus, the quality for the later sites may be better than rated here. Markel et al. (1994) identified basaltic sources of the Golan Heights and the Crateceous and anthropogenic sources such as fish ponds in the Hula Valley as the major sources for phosphate in the Lake Tiberias and the Upper Jordan River which may also account for the Jordan River.

Tab. 11: Classification of the sampling sites at the Jordan River according to LAWA (1998).

		Physical and chemical parameters [mg/mL]									
		°C[1]	pH	Conduc-tivity[2]	O_2	BOD_5[3]	Cl^-	PO_4^{3-}	NH_4	NO_2^-	NO_3^-
Jordan	1	27.5	8.3	7.4	6.6	5.99	2500	6.7	2	> 1.0	25
	2	25	7.7	6.3	2.0	1.9	2500	6.7	10	0.1	10
	3	25.5	8.2	10.3	7.8	3.1	2500	< 1*	< 0.5*	0.1	10
	4	25.5	8.2	12.4	11	4.77	2500	< 1*	< 0.5*	< 0.025*	10
	5	26	8.3	13.5	8.2	3.5	2500	< 1*	< 0.5*	< 0.025*	10

1: ranking of temperature does not aplly to semi-arid climate; 2: mS/cm; * the method used did not allow a more accurate measurement, thus, a better quality class might as well apply.

3.1.2 King Abdullah Canal

Since the King Abdullah Canal (KAC) is an artificial water conveyor, it did not have a natural riverbed, but was channeled and lined with concrete (Fig. 18Fig. 19). Sedimentation was limited to a thin layer of approximately 10 – 20 cm at the bottom of the channel. The channel had a width of 3 meters and a depths of 1.5 meters. Also the current velocity was the same with 50 m/s and the depths of visibility was 20 cm.

Fig. 18: Sampling site 1 at the King Abdullah Canal (Deir Allah): pipeline on the right is used for pumping drinking water to the Zai treatment station for further use in Amman.


Fig. 19: Sampling site 2 at the King Abdullah Canal with the Zarqa River discharging from the left. Pipelines abstract water for irrigational usage in the Jordan Valley.

Tab. 12 shows the physical parameters as well as the concentrations of nutrients and salts measured for the sampling sites at the KAC. Categorization after LAWA (1998) and Graw and Borchardt (1998) resulted in the overall water quality class II-III indicating considerable eutrophication. The ph values indicated slightly alkaline conditions with values of 8.5 for KAC 1 and 8.2 for KAC 2. Electrical conductivity slightly decreased during the flow direction from 2.3 to 1.2 mS/cm, which was rated class IV. The contents of oxygen were very high for both sampling sites (11.6 and 8.5 mg/ml) and could be assigned category I. However, the BOD_5 with 4.35 mg/ml at sites KAC 1 implied an increased microbiological activity (class III). KAC 2 was classified II with 1.9 mg/ml. For ions and nutrients, the water quality of the KAC generally increases during the river flow: chloride concentrations decreased from 475 to 275 mg/ml and were rated class III-IV and III, respectively. Contamination with phosphate was very high at KAC 1 with 6.7 mg/ml. For the second sampling site, the detection limit of the method used did not allow for a more accurate determination and, thus, 1 mg/ml was set as limit. Although other studies have showed strong eutrophication of the waters from the KAC especially for nitrogen (Alkhoury et al. 2010, Salameh and Harahsheh 2011), results for ammonia and nitrite were rated class II-III and I-II, respectively. Only KAC 2 showed increased levels of nitrate with 10 mg/ml and KAC 1 had 5 mg/ml.

Tab. 12: Classification of sampling sites at the King Abdullah Canal after LAWA (1998).

		Physical and chemical parameters [mg/mL]									
		°C[1]	pH	Conduc-tivity[2]	O_2	BOD_5	Cl^-	PO_4^{3-}	NH_4	NO_2^-	NO_3^-
KAC	1	25	8.5	2.3	11.6	4.35	475	6.7	< 0.5*	< 0.025*	10
	2	27.5	8.2	1.2	8.5	1.9	275	< 1*	< 0.5*	< 0.025*	5

1: ranking of temperature does not aplly to semi-arid climate; 2: mS/cm; * the method used did not allow a more accurate measurement, thus, a better quality class might as well apply.

3.1.3 Wadi Mujib

During the time of sampling, the Mujib reservoir did not have the level of full storage capacity (Fig. 20 a, b). Site 1 was without any findings of odor, but had only a visibility of 5 cm due to strong general turbidity of the stagnant water. Site 2 (Fig. 20 c, d), however, showed strong odor of hydrogen sulfur and the sediment sampled was of nearly black color indicating anaerobic decomposition. Visibility was 50 cm with a current velocity of 60 cm and a width of up to 10 m. Sampling site Mujib 3 (Fig. 20 e, f) had a strong current with 70 cm/s at a depth of 20 – 40 cm with a width of 8 m and a depth of 20 – 40 cm.

Fig. 20: Sampling sites at the Wadi Mujib: a, b) Inlet Mujid Dam (Mujib 1), c, d) Outlet Mujib Dam (Mujib 2), e, f) Mouth to Dead Sea (Mujib 3).

The general water quality class resulting out of the categorization according to LAWA (1998) led to a categorization into class II for Mujib 1 and 3 and class II-III for Mujib 2. A summary of the parameters is given in Tab. 13. The ph value was rated class II for all sampling sites at the Wadi Mujib ranging from slightly acidic (6.4, Mujib 1) to 8.4 (Mujib 2) and 8.1 (Mujib 3). Electrical conductivity as a sign for salinity increased during the course of the wadi from 0.6 to 1.0 and 1.7 mS/cm suggesting that side wadis as the Wadi Wala discharge ions into the conveyor or that ions are dissolved out of the riverbed. The amount of oxygen was rated class 1 except for site Mujib 1 where there were only 7.1 mg/ml to be measured, probably linked to the stagnant water and absence of any major water plants. The BOD_5 suggests a high microbiological activity with 5.36 mg/ml for Mujib 2 which equals class III-IV. Site 1 was rated class II-III (2.73 mg/ml) and site 2 class II with 2.49 mg/ml. The concentration of chloride ions significantly increased (75, 100, 350 mg/ml) during flow direction matching the results from the electric conductivity. The most dominant nutrient was phosphate with 1.5 mg/ml at site Mujib 2 which was classified as category VI. Due to the detection limit of the method used, a more accurate determination than less than 0.5 mg/ml PO_4^{3-} for the other sites could not be detected. The content of ammonia and nitrate could be classified as II-III and the content of nitrate as I-II for all sampling sites at the Wadi Mujib.

Tab. 13: Classification of the sampling sites at the Wadi Mujib according to LAWA (1998).

		Physical and chemical parameters [mg/mL]									
		°C[1]	pH	Conduc-tivity[2]	O_2	BOD_5	Cl^-	PO_4^{3-}	NH_4	NO_2^-	NO_3^-
Wadi Mujib	1	27	6.4	0.6	7.1	2.73	75	< 1*	< 0.5*	< 0.025*	< 5*
	2	27	8.4	1.0	12.3	5.36	100	1.5	0.5	0.05	< 5*
	3	28.8	8.1	1.7	10.1	2.49	350	< 1*	< 0.5*	< 0.025*	< 5*

1: ranking of temperature does not apply to semi-arid climate; 2: mS/cm;* the method used did not allow a more accurate measurement, thus, a better quality class might as well apply.

3.1.4 Yarmouk River

As the river is regulated through the Unity Dam (Fig. 21 a, b) and is diverted to parts into the KAC (Fig. 21 d, e), anthropogenic influence and pressure on the stream is high. Furthermore, the sampling sites 2 and 4 flow through extensively agriculturally used areas with high water abstraction rates and surface runoffs with agricultural drainage (Fig. 21 c, f). There was a tendency of decreasing current velocity (60-10 m/s) as the river gets wider (4 - 10 m) and deeper (30 - 400 cm). Visibilty ranged between 30 – 40 cm at sites 1 and 3, but was only 5 – 10 cm at sites 2 and 4.

Fig. 21: Sampling sites at the Yarmouk River: a, b) Unity Dam (Yarmouk 1), c) Al Hamma (Yarmouk 2), d, e) Diversion to KAC (Yarmouk 3), f) Gesher (Yarmouk4).

The average water quality class for the Yarmouk River was class II-III according to the categorization system of the LAWA (1998) indicating a considerable problem with eutrophication. Parameters are nearly constant during the river flow with slight variations as explained below (Tab. 14). Class I could be allocated to Yarmouk 1 for the ph value of 7.9. The other three sampling sites ranged from 8.1 (Yarmouk 2 and 4) to 8.4 and were rated class II. Electrical conductivity indicated high salinity for all sampling sites (class IV). The oxygen concentration increased during river flow (6.1, 8.6, 9.2 and 9.0 mg/ml) although most water

turbulences were recognized at Yarmouk 1 due to high current velocity and shallow waters. The BOD_5, on the other hand, was best for Yarmouk 1 with 2.6 mg/ml (class II-II). For Yarmouk 2 4.7 mg/ml and for Yarmouk 3 and 4 3.3 mg/ml were detected and rated class III as sign for increased microbiological activity. Chloride concentration was highest at Yarmouk 1 with 250 mg/ml (class III) and then decreased slightly to 175 mg/ml for the other sites (class II-III). The heaviest pollution with nutrients was recorded for phosphate which was categorized as class IV for all sampling sites at the Yarmouk River ranging from 1.3 mg/ml (Yarmouk 2) to 6.7 mg/ml (Yarmouk 4). With the method used, ammonia levels could not be determined precisely resulting in concentrations of less than 0.5 mg/ml which was rated as class II-III. Nitrite concentrations ranged from less that 0.025 to 0.5 mg/ml (class I-II). Class III was allocated to Yarmouk 1.2 and 3 for 10 mg/ml of nitrate. At Yarmouk 4, the concentration decreased to less than 5 mg/ml (class II-III).

Tab. 14: Classification of the sampling sites at the Yarmouk River according to LAWA (1998).

		Physical and chemical parameters [mg/mL]									
		°C[1]	pH	Conduc-tivity[2]	O_2	BOD_5	Cl^-	PO_4^{3-}	NH_4	NO_2^-	NO_3^-
Yarmouk	1	26.6	7.9	1.2	6.1	2.6	250	2.7	< 0.5*	0.05	10
	2	27.6	8.1	1.2	8.6	4.7	175	1.3	< 0.5*	< 0.025*	10
	3	29.5	8.4	1.1	9.2	3.3	175	2.7	< 0.5*	< 0.025*	10
	4	30.4	8.1	1.1	9.0	3.3	175	6.7	< 0.5*	0.025	< 5*

1: ranking of temperature does not aplly to semi-arid climate; 2: mS/cm; * the method used did not allow a more accurate measurement, thus, a better quality class might as well apply.

3.1.5 Zarqa River

Since the Zarqa River basin is the most populated basin in Jordan, anthropogenic influence on the River basin and its water quality is distinct. Morphological alterations were most prominent in the King Talal Dam (Fig. 23 c, d), which was completed in 1977 and stores approximately 86 million m^3. But also the effluent discharge of the water treatment plant Khirbet As-Samra (Fig. 22 a, b) into the basin which later unites with Wadi Dulheil poses an immense change on the natural conditions, especially during summer when there is no natural run off at all. A very strong odor of humic acid and yellowish color of the water was observed at the first three sampling sites, diminishing with increasing distance to the water treatment plant. Being a popular recreational area for picnic and swimming, a lot of litter and solid waste was found alongside the shore and concrete slabs from the bridge were found in the water at Jerash Bridge (Fig. 23 a, b). Apart from Zarqa 5 at the King Talal Dam, the river showed a rather fast flow compared to other Jordanian streams (80 – 120 cm/s). Visibility was good as the bottom was

clearly visible at the sampling sites Zarqa 1 – 4; Zarqa 5, however, only had 20 cm and Zarqa 6 60 cm. The width was between 2 and 9 m with the exception of the King Talal Dam, whose shores were 50 m distant from each other. Depths varried between 40 – 100 cm, agin with the exception of the dam where the exact depths is not known due to permanetn sediment influx and varriying water levels.

Fig. 22: Sampling sites at the Zarqa River: a, b) Khirbet As-Samra (Zarqa 1), c, d) Confluence Zarqa (Zarqa 2), e, f) Seil Jerash (Zarqa 3).

Fig. 23: Sampling sites at the Zarqa River: a, b) Jerash Bridge (Zarqa 4), c, d) Inlet King Talal Dam (Zarqa 5), e, f) Outlet King Talal Dam (Zarqa 6).

Considering all allocable parameters to the same degree and calculating the mean value of the classes rated, the classification after LAWA (1998) resulted in class III-IV for Zarqa 1 and 2, whereas the remaining sites were rated class III (Tab. 15). Thus, the Zarqa River together with the Jordan River (Ch. 3.1.1) is the most polluted and anthropogenically affected River system studied in this thesis. With values from 7.3 to 8.7, the ph values could be rated class I, I-II and II, respectively. Electrical conductivity was very high for all sampling sites ranging from 1.9 mS/cm (Zarqa 1) to 2.4 mS/cm (Zarqa 3) and was, thus, rated class IV for all sites. The

oxygen content was very good for the sampling sites before and right at the King Talal Dam (class I). The upper two sampling sites were categorized as class II with 6.8 and 7.4 mg/ml of oxygen. Only the outlet of the dam was the content of oxygen low with 3.4 mg/ml and was assigned class III-IV. This might be due t the fact that the water that is released from the dam into the Jordan Valley is taken from the lower parts of the reservoir were oxygen contents are generally lower than at the surface (Smith and Smith 2009). At this site also, the BOD_5 was 2.8 mg/ml which is generally rated class II; however, since almost all the oxygen available was consumed after five days, the situation is rather underestimated with this categorization. For the other sampling sites, the BOD_5 ranged between 3.9 mg/ml (Zarqa 3) and 8.1 mg/ml (Zarqa 5) and was allocated class III and III-IV indicating a lot of microbiological activity. Chloride ions were found at concentrations of 375 mg/ml (Zarqa 2 and 6, class III), 400 mg/ml (Zarqa 1, class III), 475 mg/ml (Zarqa 5, class III-IV) and 500 mg/ml (Zarqa 3 and 4, class III-IV). Very high concentrations were found for phosphate and all sampling sites were rated class IV with values from 6.7 mg/ml (Zarqa 5) to 13.4 mg/ml (Zarqa 1 and 2). The concentrations decrease with the river flow direction. High values of ammonia were found for Khirbet As-Samra and the outlet of King Talal Dam with 5 and 2.5 mg/ml, respectively. Class III was allocated to Zarqa 2 with 1 mg/ml and the other sites were rated class II-III as the method used did not allow a more precise measurement. Variations in the nitrite level were observed between Zarqa 1 and 2 (more than 1 mg/ml, class IV) and Zarqa 2, 3 and 6 (0.1 - 0.075 mg/ml, class II). Nitrate concentrations were detected between 5 mg/ml at Zarqa 3, 4 and 5 (class II-III) and 10 mg/ml at Zarqa 1, 2 and 6 (class III).

Tab. 15: Classification of the sampling sites at the Zarqa River according to LAWA (1998).

		Physical and chemical parameters [mg/mL]									
		°C[1]	pH	Conduc-tivity[2]	O_2	BOD_5[3]	Cl^-	PO_4^{3-}	NH_4	NO_2^-	NO_3^-
Zarqa River	1	29.3	7.3	1.9	6.8	6.4	400	13.4	> 5	> 1	10
	2	28.5	8.3	2.1	7.4	7.0	375	13.4	1	> 1	10
	3	24	8.3	2.4	9.6	3.9	500	12	< 0.5*	0.1	5
	4	24	8.2	2.2	10.4	5.0	500	12	< 0.5*	0.075	5
	5	30.1	8.7	2.2	9.2	8.1	475	6.7	< 0.5*	0.9	5
	6	24.7	7.8	2.1	3.4	2.8	375	10	2.5	0.1	10

1: ranking of temperature does not aplly to semi-arid climate; 2: mS/cm; * the method used did not allow a more accurate measurement, thus, a better quality class might as well apply

Tab. 16: Compilation of all physical and chemical data obtained for the sampling sites of Jordanian surface water systems.

Sampling Site		Physical parameters						Chemical parameters [mg/mL]							
		width [m]	depth [cm]	current velocity [cm/s]	depth of visibiliy [cm]	Conductivity [mS/cm]	°C	O_2	BOD_5[1]	NH_4	PO_4^{3-}	Cl^-	NO_2^-	NO_3^-	pH
Jordan	1	3	100	20	5	7.4	27.5	6.6	5.99	2	6.7	2500	1.0	25	8.3
	2	10	100	10	15	6.3	25	2.0	1.9	10	6.7	2500	0.1	10	7.7
	3	4	30	80	10	10.3	25.5	7.8	3.1	> 0.5*	> 1*	2500	0.1	10	8.2
	4	3	10-20	150	10-20	12.4	25.5	11	4.77	> 0.5*	> 1*	2500	> 0.025*	10	8.2
	5	3	20-30	150	20-30	13.5	26	8.2	3.5	> 0.5*	> 1*	2500	> 0.025*	10	8.3
KAC	1	3	150	50	20	2.3	25	11.6	4.35	> 0.5	6.7	475	> 0.025*	10	8.5
	2	3	150	50	20	1.2	27.5	8.5	1.9	> 0.5	> 1*	275	> 0.025*	> 5*	8.2
Wadi Mu	1	200	40	0	5	0.6	27	7.1	2.73	> 0.5*	> 1*	75	> 0.025*	> 5*	6.4
	2	3-10	50-80	60	50	1.0	27	12.3	6.9	0.5	1.5	75	0.05	> 5*	8.4
	3	8	40-50	70	40-50	1.7	28.8	10.1	2.49	> 0.5*	> 1*	350	> 0.025*	> 5*	8.1
Yarmouk Rive	1	4	30-40	60	30-40	1.2	26.6	6.1	2.6	> 0.5*	2.7	250	0.05	10	7.9
	2	5	30-40	50	5	1.2	27.6	8.6	4.7	> 0.5*	1.3	175	> 0.025*	10	8.1
	3	6	400	10	30	1.1	29.5	9.2	3.3	> 0.5*	2.7	175	> 0.025*	10	8.4
	4	10	120	20	10	1.1	30.4	9.0	3.3	> 0.5*	6.7	175	0.025	> 5*	8.1
Zarqa River	1	3	40-50	120	30-40	1.9	29.3	6.8	6.4	5	13.4	400	1	10	7.3
	2	9	50-100	100	50-100	2.1	28.5	7.4	7.0	1	13.4	375	1	10	8.3
	3	7	50-80	120	50-80	2.4	24	9.6	3.9	0.5*	12	500	0.1	> 5*	8.3
	4	8	40-50	100	40-50	2.2	24	10.4	5.0	0.5*	12	500	0.075	> 5*	8.2
	5	30-50	60	5	20	2.2	30.1	9.2	8.1	0.5*	6.7	475	0.9	> 5*	8.7
	6	6	60-100	80	60	2.1	24.7	3.4	2.8	2.5	10	375	0.1	10	7.8

[1]: Classifictaion was done accoording to Graw and Borchardt (1999); * the method used did not allow a more accurate measurement, thus, it is also possible to have a better quality class than depicted; n.d.: not detectable

3.2 Acute cytotoxicity of sediment extracts in the neutral red assay with RTL-W1 cells

3.2.1 Jordan River

All sediment extracts from the Jordan River showed cytotoxic effects in three independent replicates of the neutral red assay with RTL-W1 cells. All replicas were valid according to the criteria in Ch.2.4.2 .The concentrations at which 50 % of the neutral red were retained by the cells (NR_{50}) are displayed in Fig. 24 for all sampling sites. NR_{50} values were determined graphically as shown exemplarily in Fig. 25. A decrease in cytotoxicity was detectable with the river flow direction. Jordan 1 was moderately toxic with an NR_{50} value of 34.1 mg SEQ/ml. Jordan 2 showed slightly cytotoxic effects with 85.3 mg SEQ/ml. At Jordan 3, 4 and 5, the effects decreased further to little to no toxicity with NR_{50} values of 93.5, 97.1 and 137.8 mg SEQ/ml, respectively. NR_{80} values for usage as initial concentrations in the micronucleus and comet assay of Jordan 1 - 5 were also determined graphically and rounded for easier handling. Thus, 20 mg SEQ/ml were assigned to Jordan 1, 60 mg SEQ/ml to Jordan 2, 70 mg SEQ/ml to Jordan 3 and 4 and 110 mg SEQ/ml to Jordan 5.

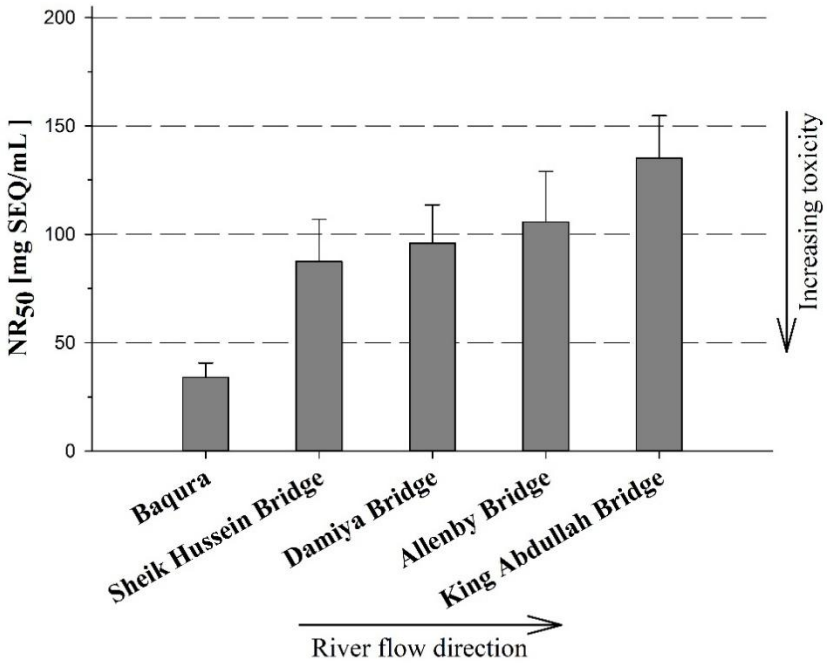

Fig. 24: Cytotoxicity of the sediment extracts of the Jordan River in the neutral red assay with RTW-W1 cells (n = 3). NR_{50} values are displayed in sediment equivalent/ml.

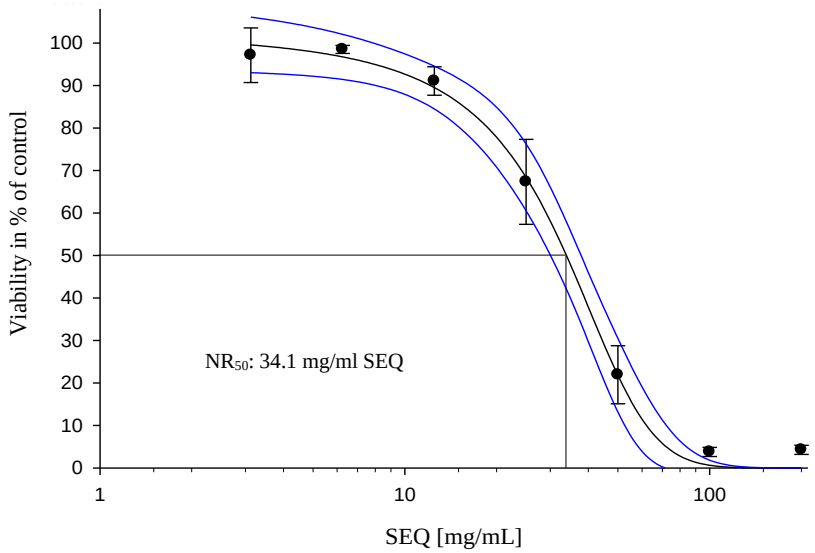

Fig. 25: Cytotoxicity of extracts from Jordan 1 at Gesher Bridge in viability [%] of the control cells as medians, ± standard deviation. A sigmoid curve fitting with three parameters was applied with 95-%-confidence interval (blue lines).

3.2.2 King Abdullah Canal

Only the first sampling site at the KAC taken showed cytotoxic effects in the neutral red assay. All replicas were valid according to the criteria in Ch.2.4.2 With an NR_{50} value of 45.4 mg SEQ/ml the sediment extract was considered moderately toxic (Fig. 26). The concentrations of the NR_{80} values needed for the micronucleus and comet assay were also determined. Thus, 30 mg SEQ/ml were detected for KAC 1 and 200 mg SEQ/ml for KAC 2. No effec ts were recorded for testing of the water eluates as described in Ch.2.3.3.

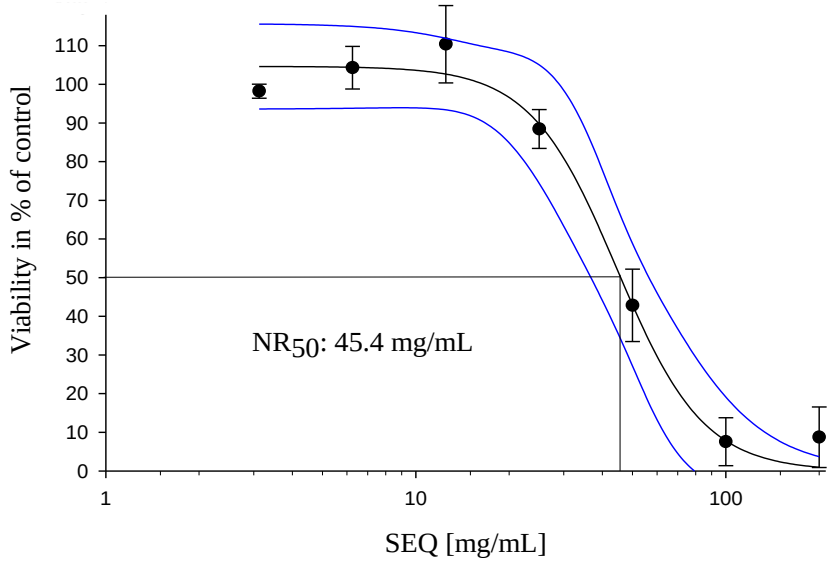

Fig. 26: Cytotoxicity of the 2010 extracts from KAC 1 (Deir Allah) in viability [%] of the vitality of control cells as medians with standard deviation. A sigmoid curve fitting with three parameters with 95-%-confidence interval (blue lines) was applied.

3.2.3 Wadi Mujib

The effects of the acetonic extracts from Wadi Mujib observed for cytotoxicity in the neutral red assay with RTL-W1 cells were heterogeneous (Fig. 27). Hence, the highest cytotoxicity found for all Jordanian samples was determined at Mujib 2 at the outlet of Mujib reservoir with an NR_{50} value of only 16.4 mg SEQ/ml. On the other hand, Mujib 1 at the reservoir inlet showed only slight to no cytotoxicity with a value of 144.7 mg SEQ/ml and at Mujib 3 no effects could be observed at the concentrations range tested. NR_{80} values for usage as initial concentrations in the micronucleus and comet assay were derived and rounded for easier handling from the graphs as follows: 120 mg SEQ/ml for Mujib 1 and 10 mg/ml for Mujib 2. As no effect could be observed for Mujib 3, 200 mg SEQ/ml were used. All replicas were valid according to the criteria in Ch. 2.4.2

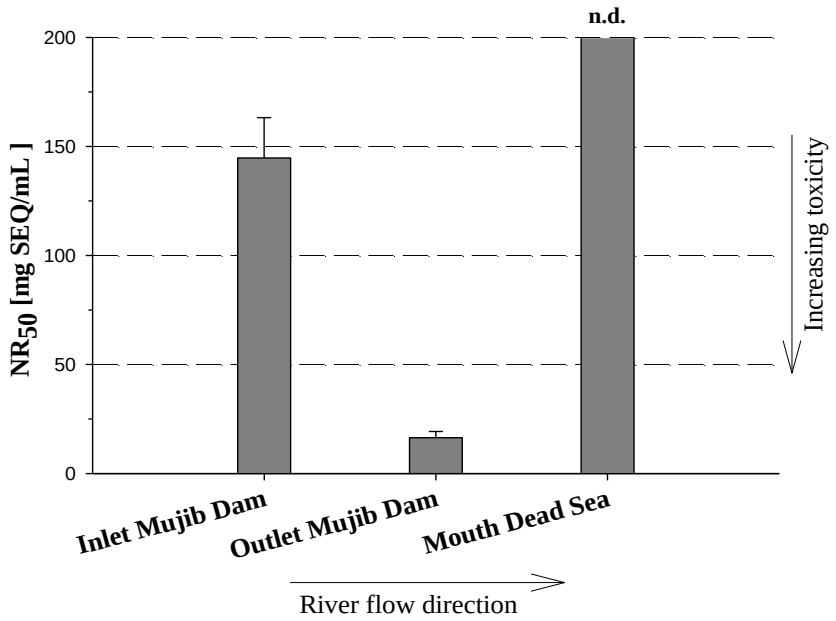

Fig. 27. Cytotoxicity of the sediment extracts of the Wadi Mujib in the neutral red assay with RTW-W1 cells (n = 3). NR_{50}-values are displayed in sediment equivalent/ml. n.d.: NR_{50} not detectable.

3.2.4 Yarmouk River

The NR_{50} values for the sediment extracts of the Yarmouk River in the neutral red assay with RTL-W1 cells are displayed in Fig. 28. All replicas were valid according to the criteria in Ch.2.4.2 All sampling sites showed cytotoxic effects. Moderate toxicity was recorded for the sampling sites Yarmouk 1 at the Unity Dam, Yarmouk 2 at Al Hamma and Yarmouk 4 at Gesher Bridge with NR_{50} values of 80.0 mg SEQ/ml, 46.5 mg SEQ/ml and 51.5 mg SEQ/ml, respectively. Yarmouk 3 at the Diversion to King Abdullah Canal showed little to no cytotoxic

potential with 126.9 mg SEQ/ml. The following NR_{80} values were obtained graphically for the sampling sites listed in river flow direction after rounding for easier handling in the genotoxicity tests as follows: 60 mg SEQ/ml, 25 mg SEQ/ml, 75 mg SEQ/ml and 35 mg SEQ/ml.

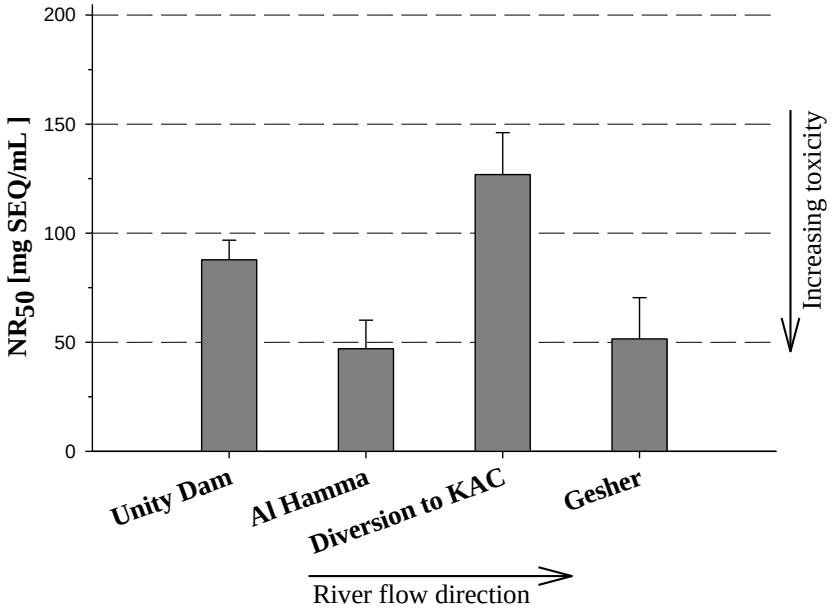

Fig. 28: Cytotoxicity of the sediment extracts of the Yarmouk River in the neutral red assay with RTW-W1 cells (n = 3). NR_{50}-values are displayed in mg sediment equivalent/ml.

3.2.5 Zarqa River

All extracts from the Zarqa River were tested in the neutral red assay with RTL-W1 cells fulfilling the criteria in Ch. 2.4.2 and exhibited cytotoxic effects. The corresponding NR_{50} values are shown in Fig. 29. Apart from the sampling sites at the King Talal Dam, the Zarqa River showed increasing toxicity upstream. The closer the sampling sites were to the water treatment plant Khirbet As-Samra, the higher was the cytotoxic potential of the extracts. The second most toxic sample of all Jordanian samples following Mujib 1 was found to be Zarqa 1 directly at the effluent of Khirbet As-Samra. There, 16.5 mg SEQ/ml induced 50 % effects on the cells. At the moderately toxic sampling sites Zarqa 2, Zarqa 3 and Zarqa 4, the NR_{50} values were 38.1 mg SEQ/ml, 39.3 mg SEQ/ml, and 79.8 mg SEQ /ml, respectively. At the inlet of the dam at Zarqa 5, the NR_{50} value was determined at 160.7 mg SEQ/ml and, thus, it was assigned little to no toxicity. At the outlet of the dam, however, toxicity increased to moderate toxicity with an NR_{50} value of 75.8 mg SEQ/ml. The NR_{80} values that were needed to determine the initial concentrations used in the genotoxicity assays were also determined graphically and rounded for easier pipetting of the volumes as follows: Zarqa 1. 10 mg SEQ/ml, Zarqa 2 and a: 30 mg SEQ/ml, Zarqa 4: 70 mg SEQ/ml, Zarqa 5: 120 mg SEQ/ml and Zarqa 6: 50 mg SEQ/ml. No effects wer observed for the water elutes as described in Ch. 2.3.3.

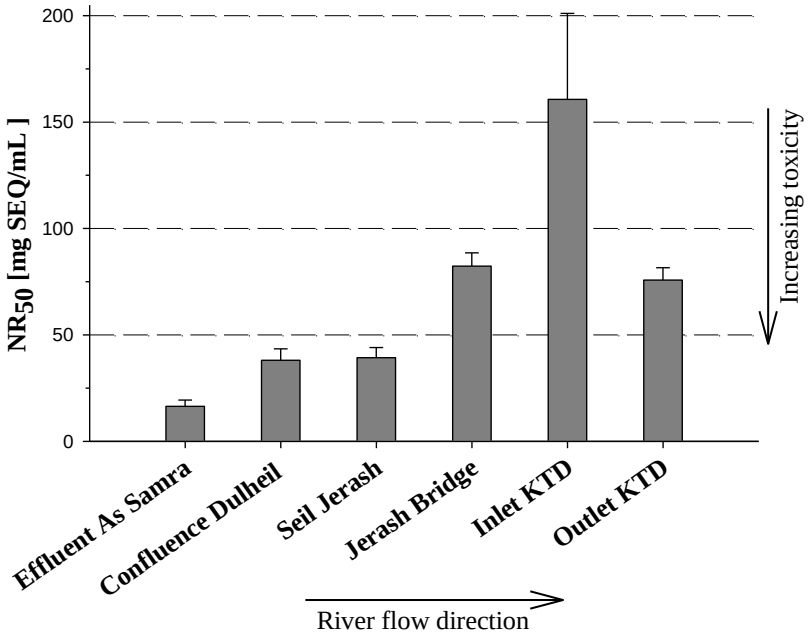

Fig. 29 Cytotoxicity of the 2010 sediment extracts of the Zarqa River in the neutral red assay with RTW-W1 cells (n = 3). NR_{50}-values are displayed in sediment equivalent/ml.

3.2.6 Comprehensive presentation of effects in the neutral red assay

On the basis of the threshold values for cytotoxicity in the neutral red assay developed by Keiter et al. (Keiter et al. 2009b) (Ch. 2.6), all samples with an NR_{50} value of less than 31 mg SEQ/ml were rated strongly toxic. NR_{50} values between 31 and 80 mg SEQ/ml were considered moderately toxic and those higher than 80 mg SEQ/ml non-toxic. A ranking of the cytotoxicity of all Jordanian sampling sites is shown in Fig. 30.

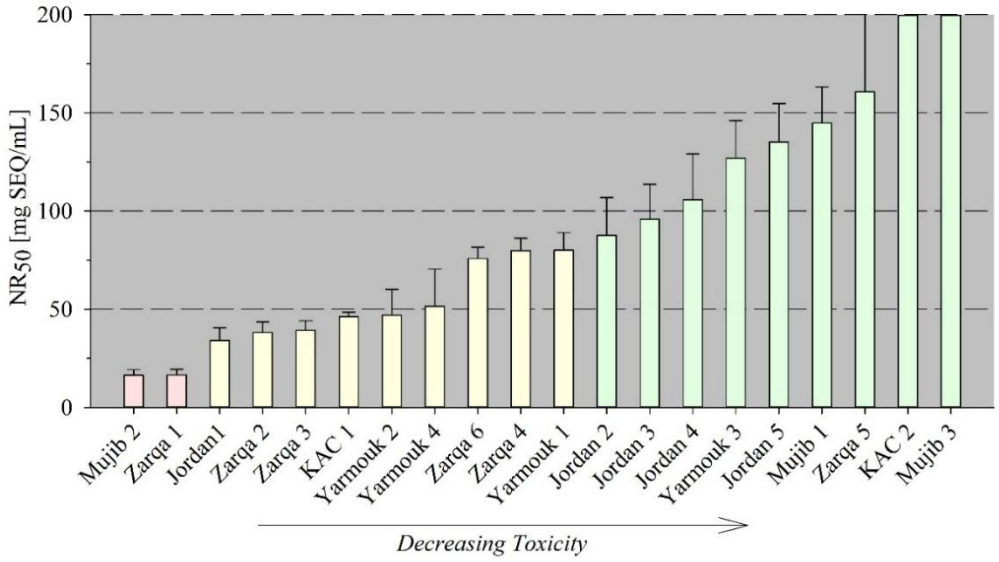

Fig. 30: Ranking of Jordanian rivers in terms of toxicity of sediment extracts in the neutral red assay with RTL-W1 cells given as NR_{50} values [mg SEQ/ml]. Colors indicate toxicity (Keiter et al. 2009); red: strongly toxic, yellow: moderately toxic, green: non-toxic.

3.3 Acute cytotoxicity in the neutral red assay with V79 cells

All acetonic sediment extracts were tested for cytotoxicity with the endpoint neutral red retention with V79 cells under exogenous metabolic activation with S-9 mix. In two independent replicates, neither of the sampling sites showed significant effects that allowed a determination of NR_{50} or NR_{80} values (Fig. 31) with the exception of Zarqa 1 and Mujib 2. For Zarqa 1 an NR_{50} value of 96.3 mg SEQ/ml and for Mujib 2 an NR_{50} of 112.1 mg SEQ/ml was detected. The NR_{80} was determined graphically and rounded to 50 mg/ml for both locations. For the other sampling sites, 200 mg SEQ/ml were used as maximum concentration for testing of genotoxic potential in the micronucleus assay. Nearly all sampling sites showed vitality stimulation at almost all concentrations.

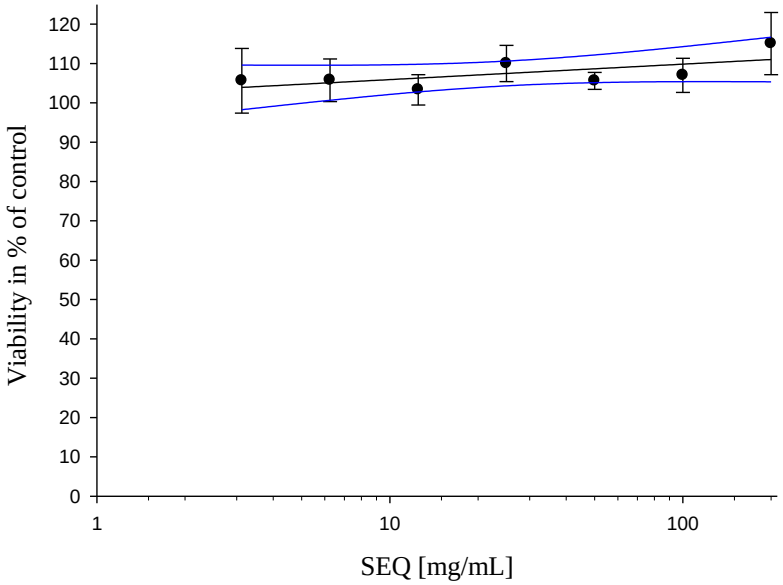

Fig. 31: Cytotoxicity of the acetonic extract from Yarmouk 2 (Al Hamma) with V79 cells in viability [%] of the vitality of control cells as medians with standard deviation. A logarithmic curve fitting with three parameters with 95-%-confidence interval (blue lines) was applied.

3.4 Comet assay with RTL-W1 cells

3.4.1 Jordan River

In two independent replicates each, the sediment extracts from all five sampling sites at the Jordan River showed significant genotoxic effects in terms of DNA fragmentation (Olive tail moment) when compared to the negative controls. Generally, a dose-response relationship could be observed as illustrated in Fig. 32Fig. 33. Concentrations of 20 mg SEQ/ml (Jordan 1), 60 mg SEQ/ml (Jordan 2 and 4), 70 mg SEQ/ml (Jordan 3) to 110 mg SEQ/ml (Jordan 5) were used as maximum concentrations as obtained from rounded NR_{80} values of the neutral red assay (Ch. 3.2.1). For all locations, the maximum induction factors (IF_{max}), LOEC values and concentration dependent induction factors (CDI) were determined and are listed in Tab. 16.

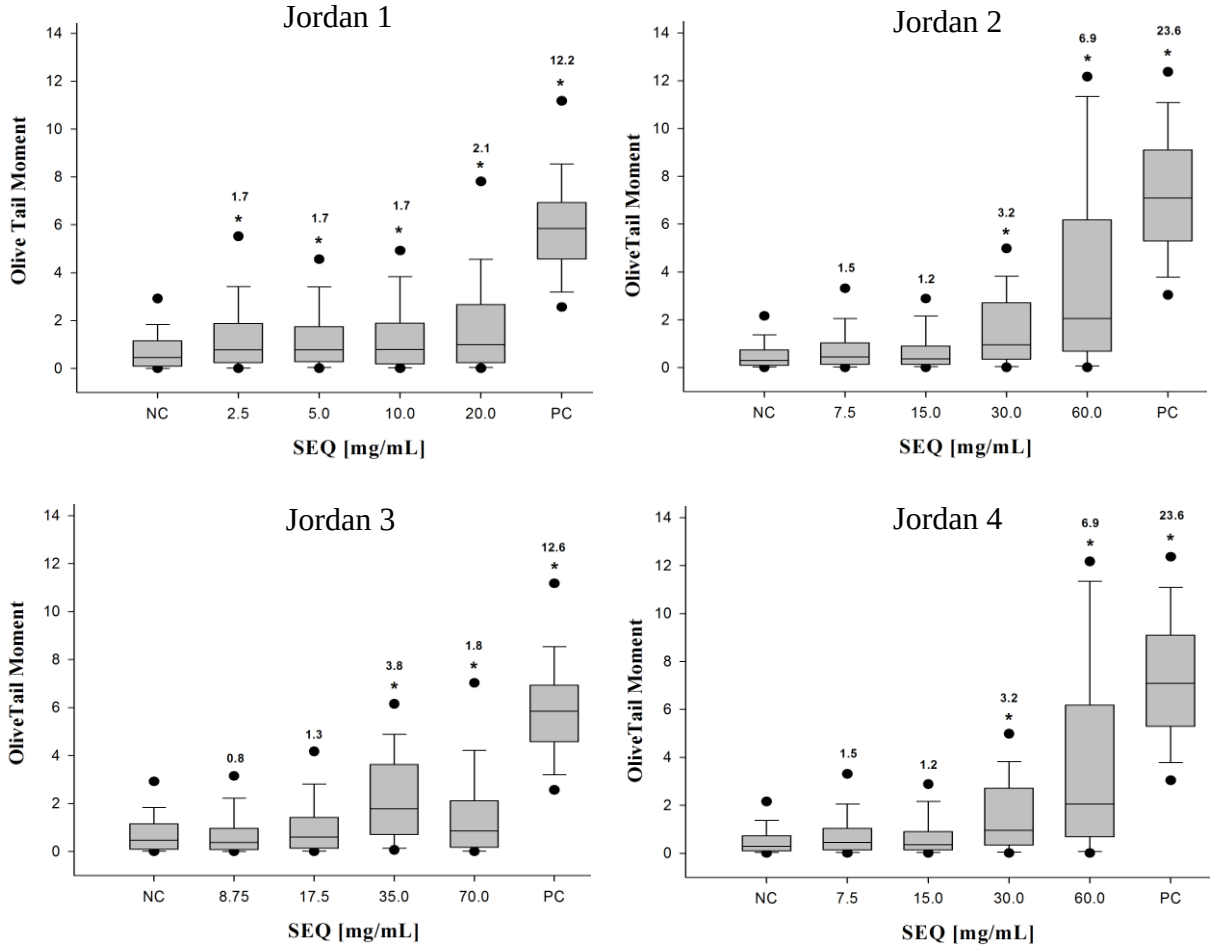

Fig. 32: Genotoxic effects of the acetonic sediment extract of Jordan 1 - , negative (NC) and positive controls (PC) displayed in Olive Tail Moment with median. The boxes indicate 25 and 75 percentiles, dots 5 and 95 percentiles, whiskers minimum and maximum values and numbers induction factors. An ANOVA-on-ranks test was conducted with Dunn's test with $p < 0.05$. * = significantly different from negative control.

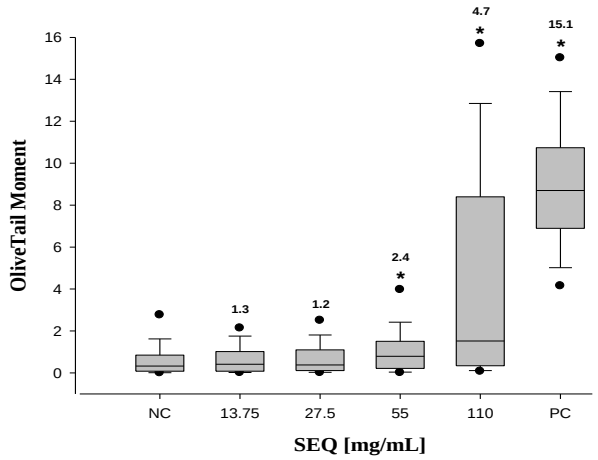

Fig. 33: Genotoxic effects of the acetonic sediment extract of Jordan 5 as well as negative (NC) and positive control (PC) displayed in Olive Tail Moment with median. The boxes indicate 25 and 75 percentiles, dots 5 and 95 percentiles, whiskers minimum and maximum values and numbers induction factors. An ANOVA-on-ranks test was conducted with Dunn's test with $p < 0.05$. * = significantly different from negative control.

Tab. 16: Genotoxicity of sediment extracts from sampling sites at the Jordan River given as LOEC, IF_{max} and CDI values.

	LOEC [mg SEQ/mL]	IF_{max}	CDI
Jordan 1, Baqura	2.5	2.1	1.3
Jordan 2, Sheik Hussein Bridge	30	6.9	0.5
Jordan 3, Damiya Bridge	35	3.8	0.3
Jordan 4, Allenby/King Hussein Bridge	17.5	2.4	0.3
Jordan 5, King Abdullah Bridge	55	4.7	0.2

With respect to IF_{max} values, Jordan 2 with 6.9 and Jordan 3 with 3.8 were the most toxic sites at the Jordan River. However, the IF_{max} does not consider the concentration at which the effect occurs. Thus, consideration of the LOEC values is also necessary for the understanding of toxicity. Jordan 2 would then be only the third toxic site with an LOEC of 30 mg SEQ/ml. Jordan 1 with only 2.5 mg SEQ/ml was by far the most toxic site followed by Jordan 4 with 17.5 mg SEQ/ml. Jordan 5 was the least toxic location with an LOEC of 55 mg/ml. A concept that allows the combination of the IF_{max} and the LOEC is the CDI according to Seitz etal. (2008). Based on the CDI, the ranking of toxicity paralleled the river flow direction with Jordan 1 being the site with a strongly genotoxic potential as it induced comets significantly at concentrations as low as 2.5 mg SEQ/ml. Jordan 2 was also rated strongly genotoxic, whereas

Jordan 3 and 4 were moderately and Jordan 5 slightly to non-toxic. For better comparison of the data, a three-step analysis was conducted (Fig. 34).

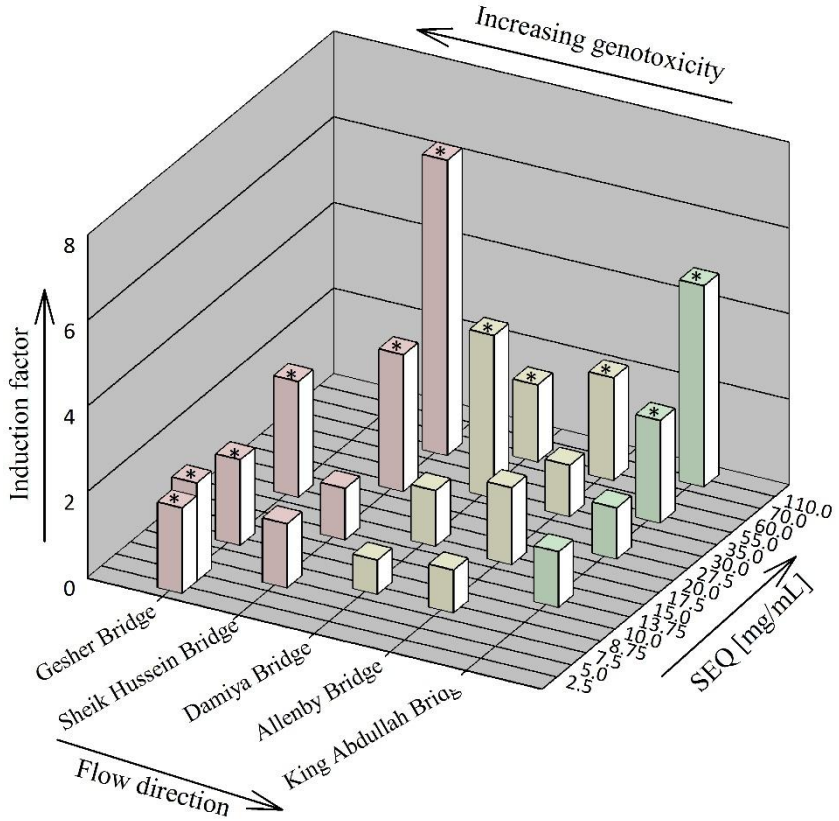

Fig. 34: Induction factors and LOECs of the extracts from the Jordan River sediments in the comet assay with RTL-W1 cells. * = significant difference to negative control. Colors indicate toxicity degree according to Keiter et al. (2009); red: strongly toxic, yellow: moderately toxic, green: non-toxic.

3.4.2 King Abdullah Canal

Both sampling sites at the King Abdullah Canal showed significant effects in the comet assay with RTL-W1 cells in terms of Olive Tail Moment (Fig. 35). As there was no cytotoxic potential detected for KAC 2 that allowed a determination of a NR_{80} value, the highest concentration tested was 200 mg SEQ/ml. For KAC 1, however, a maximum of 30 mg SEQ/ml was applied. A dose-dependency with the exception of one concentration each was observed. At KAC 1, the IF was only 2 at 15 mg SEQ/ml whereas it was 2.3 at only 7.5 mg SEQ/ml. At KAC 2, the IF was 1.2 and not significantly different from the negative control, whereas it was 2.6 at only 25 mg SEQ/ml. LOEC, IF_{max} and CDI values are shown in Tab. 17.

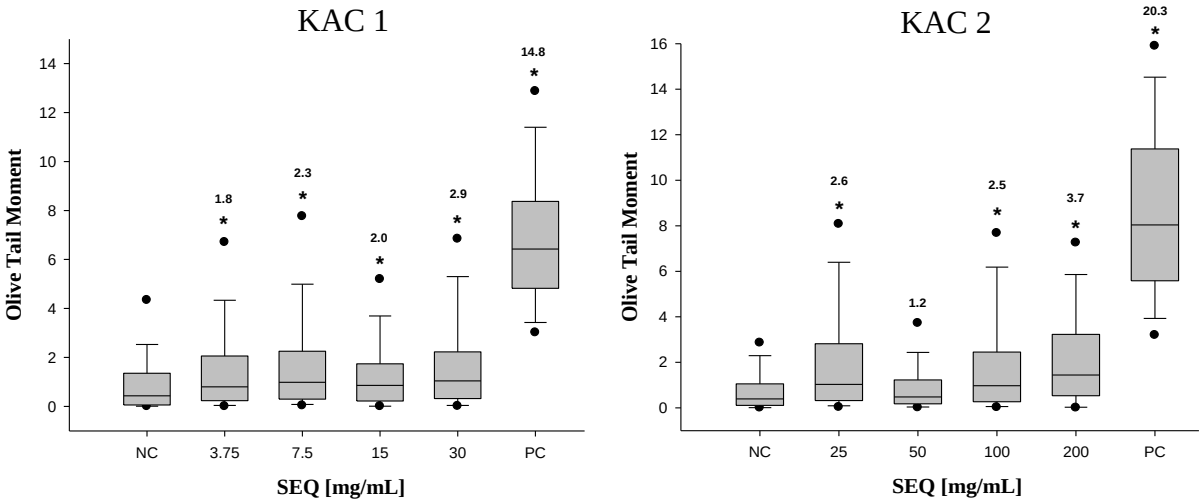

Fig. 35: Genotoxic effects of the acetonic sediment extract of KAC 1 at Deir Allah and KAC 2 at the Confluence with the Zarqa River (right) and negative (NC) and positive controls (PC) displayed in Olive Tail Moment with median. The boxes indicate 25 and 75 percentiles, dots 5 and 95 percentiles, whiskers minimum and maximum values and numbers induction factors. An ANOVA-on-ranks test was conducted with Dunn's test with $p < 0.05$. * = significantly different from negative control.

Tab. 17: Genotoxicity of the sediment extracts sampling sites at the King Abdullah Canal given as LOEC, IF_{max} and CDI values.

	LOEC [mg SEQ/mL]	IF_{max}	CDI
King Abdullah Canal 1, Deir Allah	3.75	2.9	1.0
King Abdullah Canal 2, Confluence with Zarqa River	25	3.7	0.2

Considering the LOEC value of 3.75 mg SEQ/ml compared to 25 mg SEQ/ml, KAC 1 would be the most toxic site at the KAC. The IF_{max} of KAC 2, however, with 3.7 is higher than that of KAC 1 with 2.9. The CDI as a value combining both qualities, identifies KAC 1 as exhibiting a strong genotoxic potential with 1.0, whereas extracts from KAC 2 showed little to no toxicity with a CDI of 0.2. No effects wer observed for the water elutes as described in Ch. 2.3.3.

3.4.3 Wadi Mujib

A genotoxic potential as detected in the comet assay with RTL-W1 cells was only observed for the outlet of Mujib Dam and a dose-dependency was visible as shown in Fig. 36. According to the NR_{80} values from the cytotoxicity test (Ch. 3.1.3) 10 mg SEQ/ml was the highest

concentration tested. As even the lowest concentration of 1.25 mg SEQ/ml induced high effects with an IF_{max} of 8.2, this sampling site was strongly toxic one in terms of the endpoint Olive Tail Moment compared to the negative control. All concentrations induced significant effects. As the LOEC of 1.25 mg SEQ/ml was the lowest concentrations tested, the actual LOEC may be even smaller. A CDI of 6.1 assigned a very high genotoxic potential to the sediments. At Mujib 1, only the two highest concentrations of 120 and 60 mg SEQ/ml induced significant effects with induction factors of 2.6 and 2.5, respectively (Fig. 36). The CDI was found to be 0.2 and was thus rates slightly to non-genotoxic. The complete data are listed in Tab. 18 and the three step analysis is shown in Fig. 37

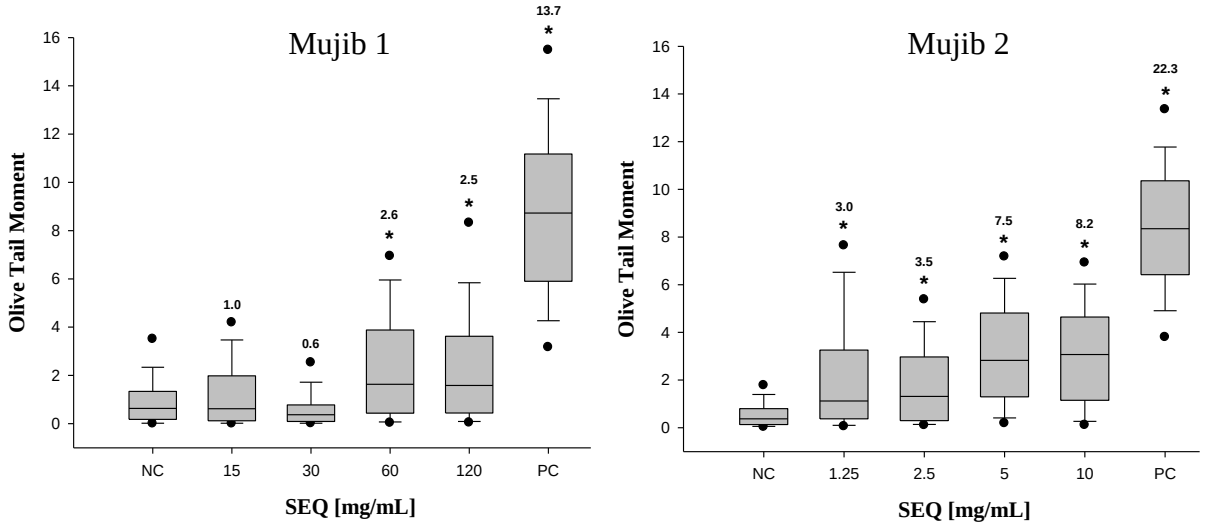

Fig. 36: Genotoxic effects of the acetonic sediment extract of Mujib 1 and Mujib 2, negative (NC) and positive controls (PC) displayed in Olive Tail Moment with medians. Boxes indicate 25 and 75 percentiles, dots 5 and 95 percentiles, whiskers minimum and maximum values and numbers induction factors. An ANOVA-on-ranks test was conducted with Dunn's test with $p < 0.05$. * = significantly different from negative control.

Tab. 18: Genotoxicity of sediment extracts from sampling sites at the Wadi Mujib given as LOEC, IF_{max} and CDI values.

	LOEC [mg SEQ/mL]	IF_{max}	CDI
Mujib 1, Inlet Mujib Dam	60	2.6	0.2
Mujib 2, Outlet Mujib Dam	1.25	8.2	6.1
Mujib 3, Mouth Dead Sea	n.d.	1.3	0.1

n.d.: not detectable with the concentrations tested

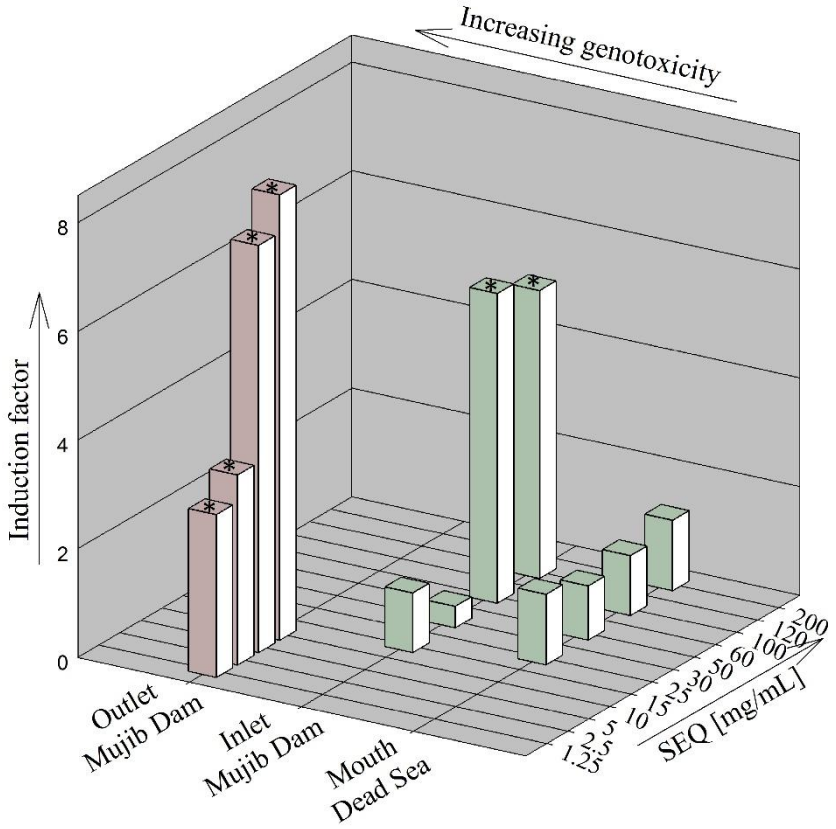

Fig. 37: Induction factors and LOECs of the extracts from the Wadi Mujib in the comet assay with RTL-W1 cells. * = significant difference to negative control. Colors indicate toxicity degree according to Keiter et al. (2009); red: strongly toxic, green: non-toxic.

3.4.4 Yarmouk River

For all sediment extracts from the Yarmouk River with the exception of Yarmouk 3 at the diversion to the KAC, a genotoxic potential was detected in the comet assay with RTL-W1 cells. A compilation of LOEC, IF_{max} and CDI values is shown in Tab. 19. The NR_{80} values as

determined in the neutralred assay (Ch. 3.2.4) set the limit for the highest concentration to be tested: for Yarmouk 1 to 60 mg, for Yarmouk 2 to 25 mg, for Yarmouk 3 to 75 mg, and for Yarmouk 4 to 35 mg SEQ/ml. As can be seen in Fig. 38, effects on the cells were very heterogeneous between the different concentrations of the various sampling sites, and a dose-dependency could only be detected at Yarmouk 4. At Yarmouk 1, on the other hand, the induction factor decreased from 2.4 at 7.5 mg SEQ/ml to 1.9 at 60 mg SEQ/ml (Fig. 38). Yarmouk 2 showed the highest significant effect of 6.1 at the second-lowest concentration of 6.25 mg SEQ/ml, whereas the induction factor ranged from 2.6 to 1.8 at the other concentrations (Fig. 38).

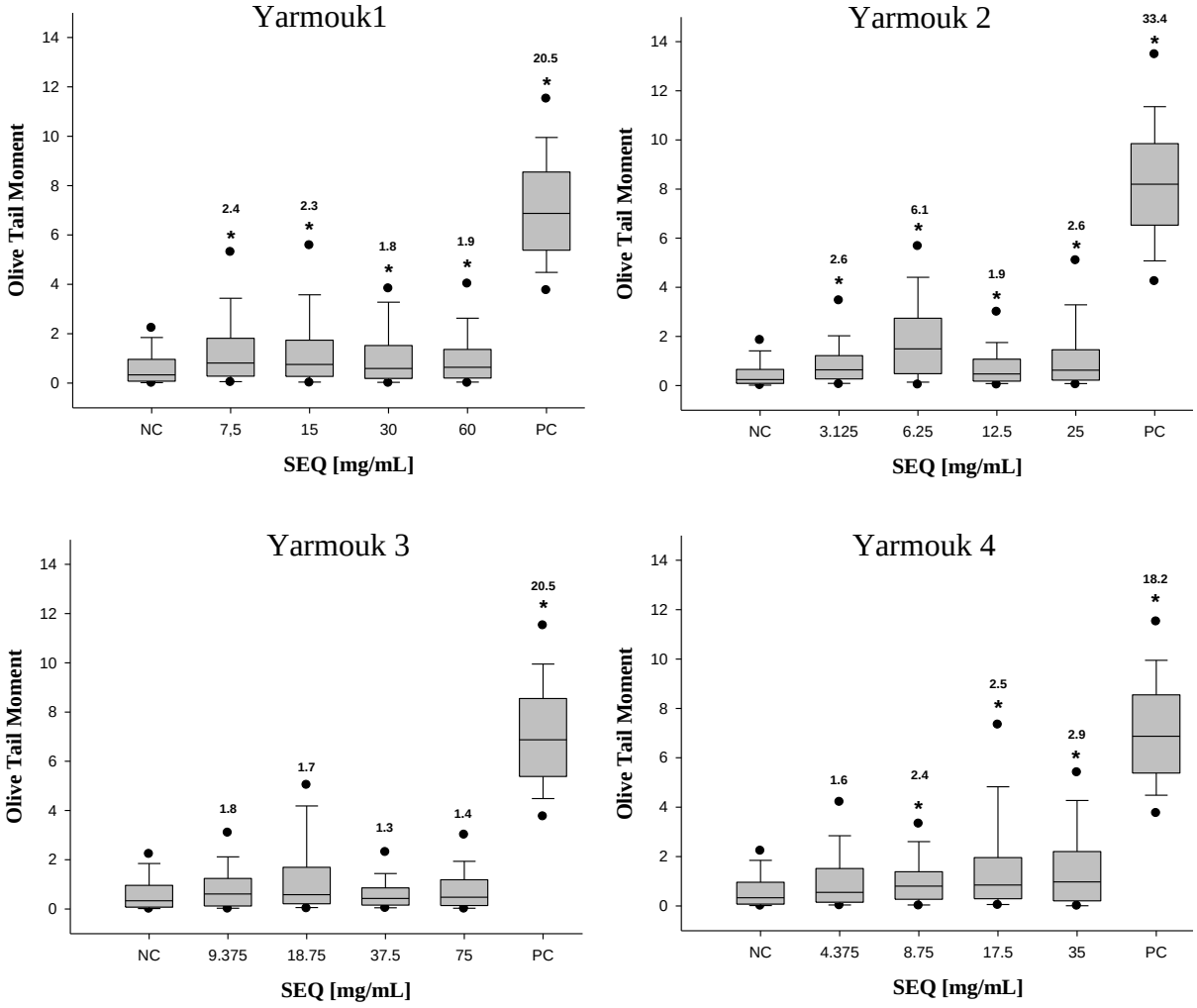

Fig. 38: Genotoxic effects of the acetonic sediment extract of Yarmouk 1 - 4, negative (NC) and positive controls (PC) displayed in Olive Tail Moment with median. Boxes indicate 25 and 75 percentiles, dots 5 and 95 percentiles, whiskers minimum and maximum values and numbers induction factors. An ANOVA-on-ranks test was conducted with Dunn's test with $p < 0.05$. * = significantly different from negative control.

Tab. 19: Genotoxicity of the sediment extracts from sampling sites at the Yarmouk River given as LOEC, IF_{max} and CDI values.

	LOEC [mg SEQ/mL]	IF_{max}	CDI
Yarmouk 1, Unity Dam	7.5	2.4	0.6
Yarmouk 2, Wadi Raqab	3.125	6.1	2.1
Yarmouk 3, Diversion to KAC	18.75	1.8	0.4
Yarmouk 4, Gesher	8.75	2.9	1.1

n.d.: not detectable within the concentration range tested.

Due to the LOEC of 3.1 mg SEQ/ml, the IF_{max} of 6.1 as well as the CDI of 2.1, the second sampling site at the Yarmouk showed very strong genotoxic potential. Second-most toxic was Yarmouk 4 at Gesher as the LOEC of 8.75 mg SEQ/ml still induced 2.4 times more DNA fragmentation then the negative control. The IF_{max} of 2.9 was found at the highest test concentration of 35 mg SEQ/ml. A similar LOEC (7.5 mg SEQ/ml) with also 2.4 times induction was determined for Yarmouk 1 at the Unity Dam. Both sites exhibited a strongly genotoxic potential. The LOEC values of Yarmouk 1 and 2 were the lowest concentration used in the test. Thus, the actual values might have been even less. Yarmouk 3 was classified as moderately toxic with a CDI of 0.4. The three step analysis is shown in Fig. 39.

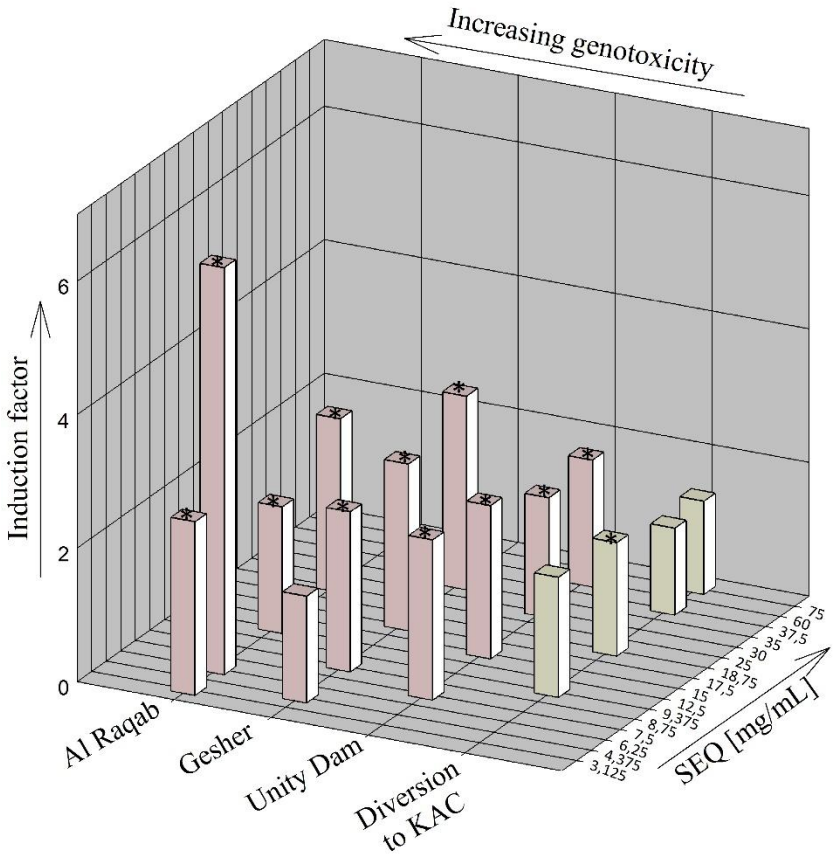

Fig. 39: Induction factors and LOECs of extracts from Yarmouk River in the comet assay with RTL-W1 cells. * = significant difference to negative control. Colors indicate toxicity degree according to Keiter et al. (2009); red: strongly toxic, yellow: moderately toxic.

3.4.5 Zarqa River

Effects on the DNA fragmentation as detected by the Olive tail Moment in two independent replicates in the comet assay with RTL-W1 cells were visible for all sampling sites at the Zarqa River (Tab. 20, Fig. 40Fig. 41). A dose dependency was identified with the exception of the sampling sites Zarqa 2 and 3. For Zarqa 2, a low-dose effect with an induction of 1.8 occurred at 3.75 mg SEQ/ml, which was also the LOEC. Zarqa 3 showed a significant rise of induction from 3.0 fold at 3.75 mg SEQ/ml to 7.9 fold at 7.5 mg SEQ/ml and then a decrease to a 2.4 fold induction at 15 and 30 mg SEQ/ml. In general, substantial differences in the effective range (3.75 to 120 mg SEQ/ml) between the locations were observed. Furthermore, significant induction factors ranged from 7.9 at Zarqa 3 to 1.8 at Zarqa 2.

Tab. 20: Genotoxicity of the sediment extracts from Zarqa River given as LOEC, IF_{max} and CDI values.

	LOEC [mg SEQ/mL]	IF_{max}	CDI
Zarqa River 1, Khirbet As Samra	10	2.2	1.7
Zarqa River 2, Confluence Zarqa	3.75	1.8	0.8
Zarqa River 3, Seil Jerash	3.75	7.9	2.1
Zarqa River 4, Jerash Bridge	8.75	3.3	1.2
Zarqa River 5, Inlet King Talal Dam	15	5.3	0.3
Zarqa River 6, Outlet King Talal Dam	6.25	3.2	0.6

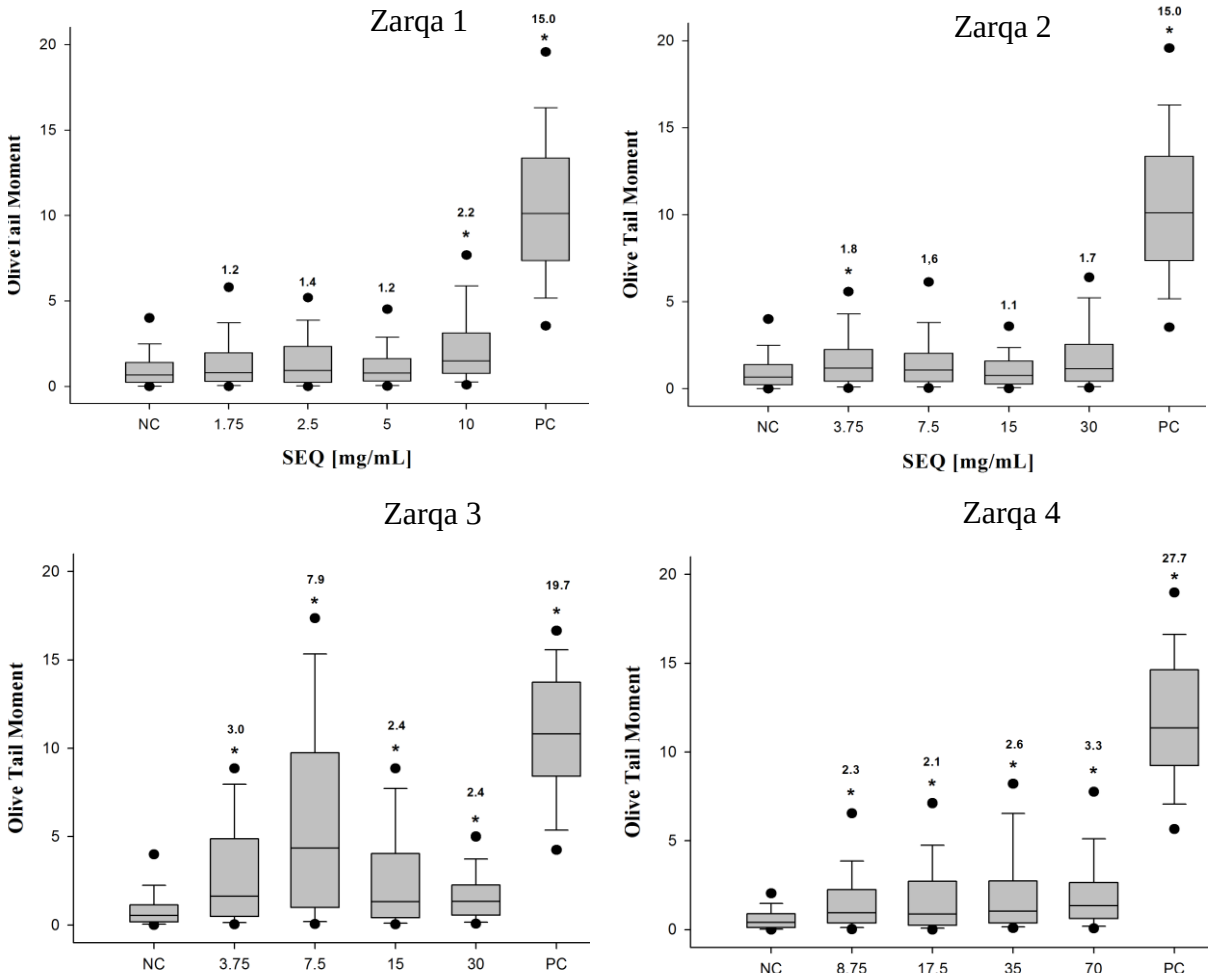

Fig. 40: Genotoxic effects of the acetonic sediment extract of Zarqa 1 – 4, negative (NC) and positive controls (PC) displayed in Olive Tail Moment with median. Boxes indicate 25 and 75 percentiles, dots 5 and 95 percentiles, whiskers minimum and maximum values and numbers induction factors. An ANOVA-on-ranks test was conducted with Dunn's test with $p < 0.05$. * = significantly different from negative control.

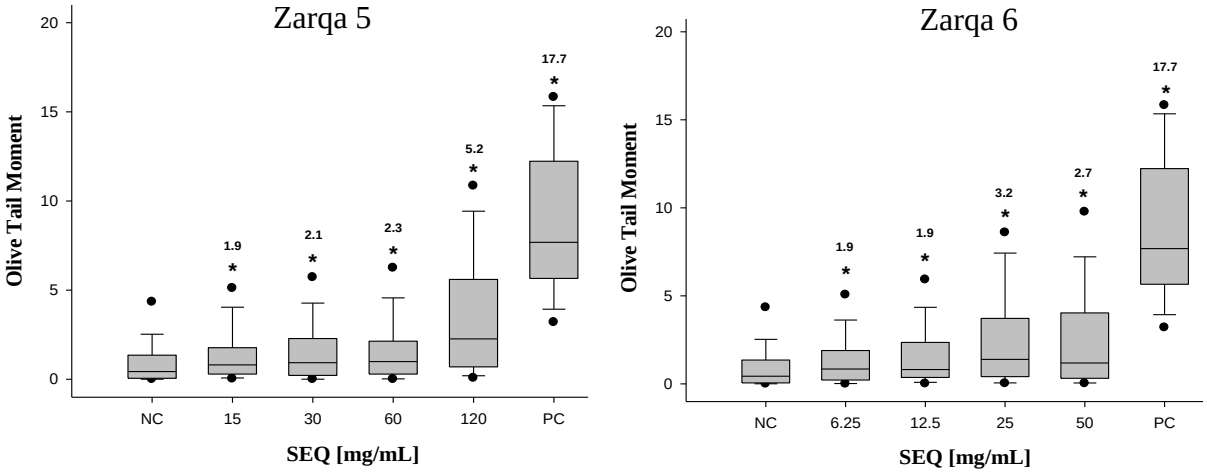

Fig. 41: Genotoxic effects of the acetonic sediment extract of Zarqa 5 and 6, negative (NC) and positive controls (PC) displayed in Olive Tail Moment with median. Boxes indicate 25 and 75 percentiles, dots 5 and 95 percentiles, whiskers minimum and maximum values and numbers induction factors. An ANOVA-on-ranks test was conducted with Dunn's test with $p < 0.05$. * = significantly different from negative control.

Considering only the LOEC values, Zarqa 2 and 3 would both be rated as the most genotoxic sampling sites within this river. However, considering the IF_{max} and CDI, it became obvious that Zarqa 3 had higher toxicities with significant effects at all other tested concentrations, whereas for Zarqa 2 the LOEC was also the only significant concentration tested. A high induction of DNA fragmentation was also found at Zarqa 5 with an IF_{max} of 5.3. As this occurred at a rather high concentration of 120 mg SEQ/ml, however, the CDI of 0.3 was rather small and rated moderately toxic. Strong toxicity could be ascribed to all sites with the exception of Zarqa 5 which was moderately genotoxic. For a better comparison of the sampling sites, a three-step analysis was conducted listing the sites according to their genotoxic potentials (Fig. 42). No effects wer observed for the water elutes as described in Ch. 2.3.3.

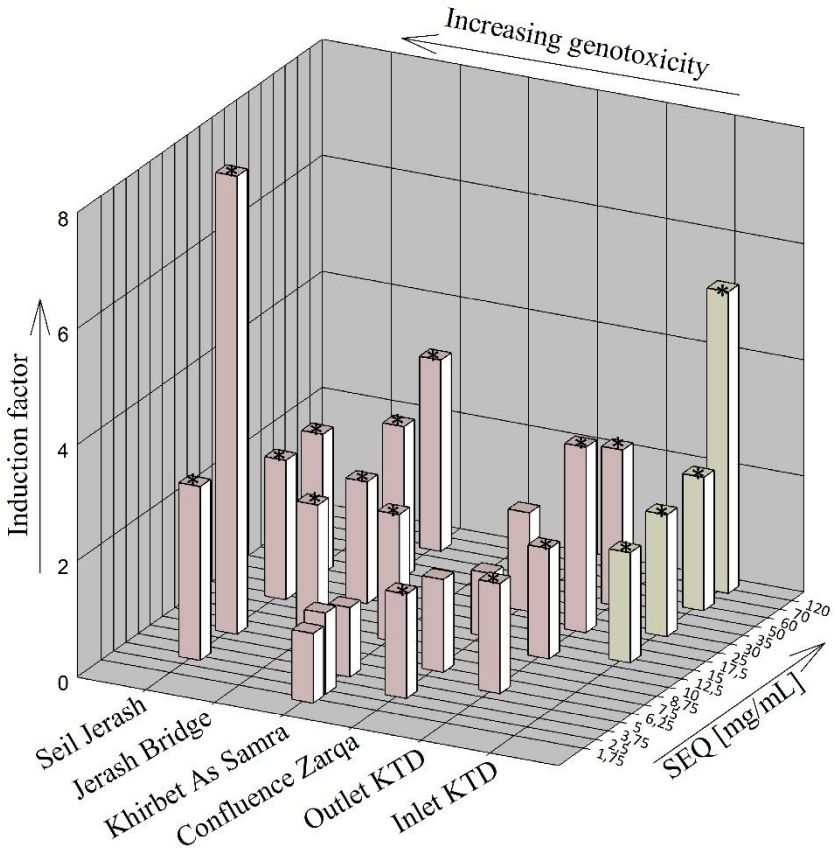

Fig. 42: Induction factors and LOECs of the extracts from the Zarqa River in the comet assay with RTL-W1 cells. * = significant difference to negative control. Colors indicate toxicity degree according to Keiter et al. (2009); red: strongly toxic, yellow: moderately toxic.

3.5 Micronucleus assay with RTL-W1 cells

In order to complete and supplement the results of the comet assay and to reveal another mode of genotoxic action, the miconucleus assay was conducted. Micronuclei may result from acentric chromosome fragments detaching from a chromosome after breakage during mitosis (clastogenic effects) or from whole chromosomes which do not integrate into the daughter nuclei due to damage of the spindle apparatus (aneugenic effects; (Fenech 2000).

In this study, RTL-W1 cells did not prove suitable for the assessment of genotoxicity in the micronucleus assay. As is shown for the example of the sediments from KAC 1 in Fig. 43, the negative controls constantly exceeded the maximum allowed micronuclei rate of 3% (ISO 2004, OECD 2010). The number of micronucleated and abnormal cells did in many cases even exceed the number of affected cells in the exposure series (Fig. 44).

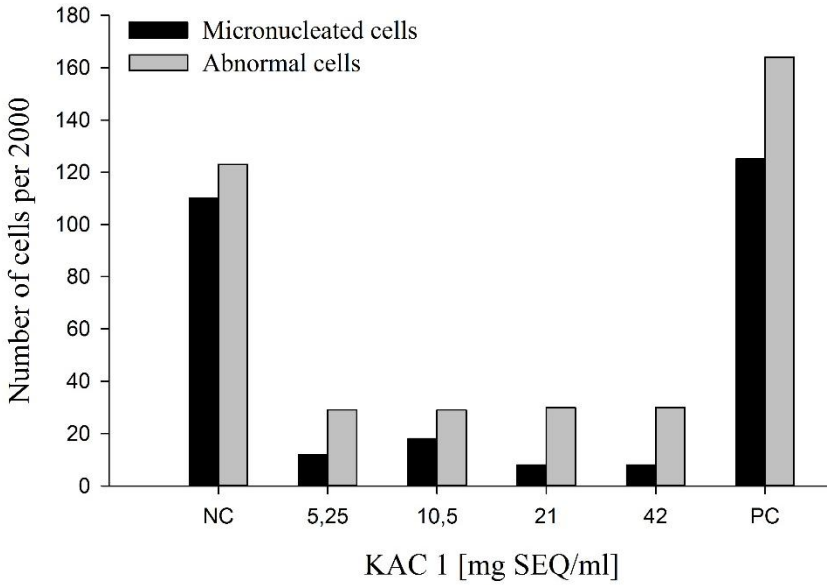

Fig. 43: Micronuclei and abnormality rate in RTL-W1 cells after exposure to sediment extracts of KAC 1 from 2009 and negative (NC) and positive control (PC) with 100 mg/ml NQO.

A contamination with mycoplasms was identified as a possible reason for this phenomenon during the time of the experiment. Paton and coworkers (1965) showed that mycoplasms may induce chromosome anomalies in cell cultures. However, tests with freshly thawed and uncontaminated cell stocks showed similar results. At least, the results for the negative controls were not reproducible. Various kinds of anomalies and micronucleated or even polynucleated cells occurred more often than allowed by the validity criteria according to ISO/DIS 21427-2

(2004) and the OECD Guideline 487 (2010). The micronucleus test series with RTL-W1 cells was therefore not continued, and V79 cells were used instead.

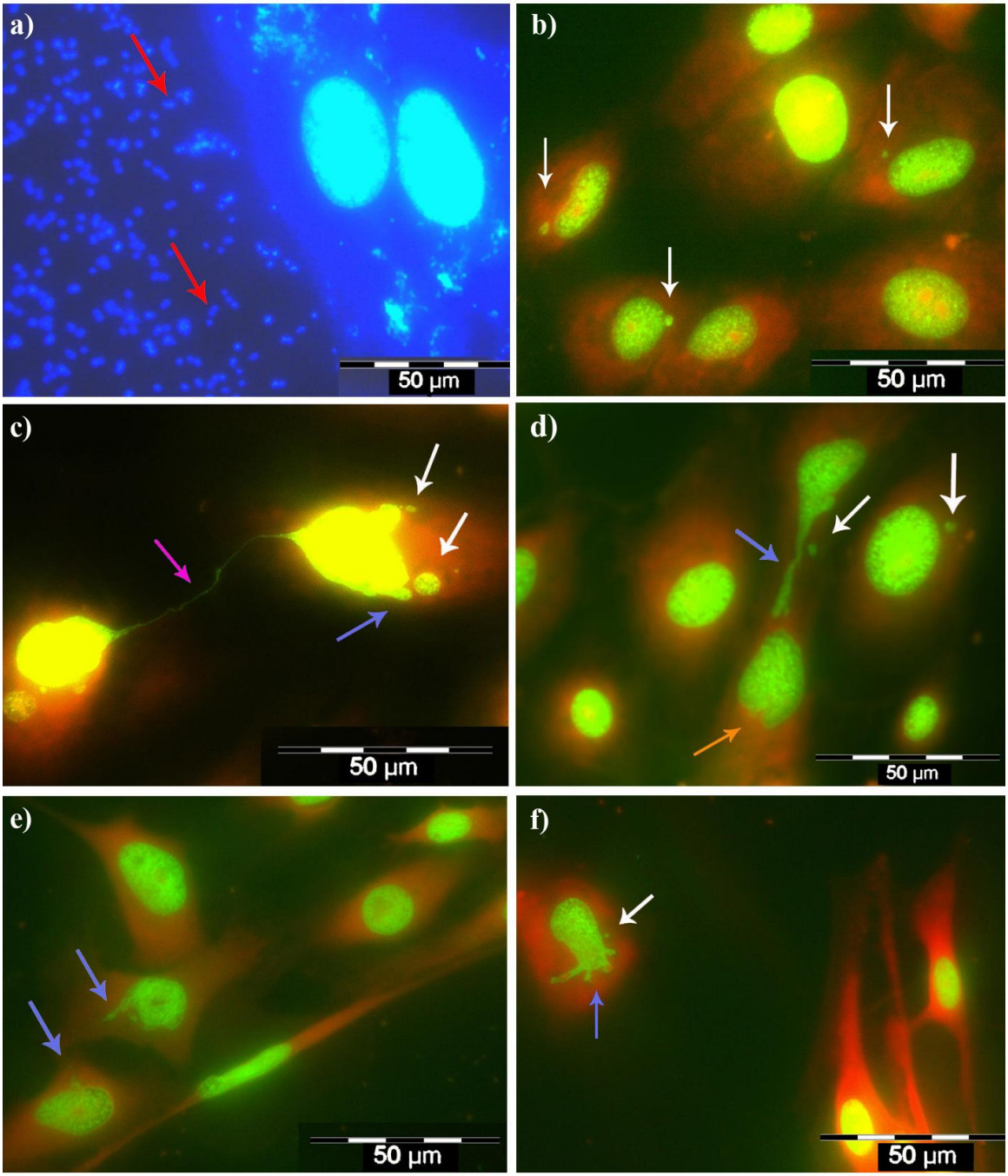

Fig. 44: a) Hoechst 3258 staining of RTL-W1 cells with *Mycoplasma* contamination, red arrows indicate single mycoplasms; b-f) RTL-W1 cells as negative controls in the micronucleus assay stained with acridine orange with micronuclei and various anomalies, white arrows indicate micronuclei, pink arrows indicates a plasma bridge between two nuclei, blue arrows indicate leaking nuclei and noses, orange arrow indicates nucleus malformation.

3.6 Micronucleus assay with V79 cells

Since RTL-W1 cells did not proved suitable as test system for the micronucleus assay (Ch. 3.5), the micronucleus assay was conducted with V79 cells instead. Metabolic activation through S9-mix was also used in this test approach to detect clastogenic and aneugenic genotoxic effects of the sediment extracts (Fenech 2000, ISO 2004). Besides micronuclei, various anomalies were also recorded. The micronucleus assay with V79 cells was only conducted with extracts from the 2010 sampling period. Each sampling sites was tested in three independent replicates. Results are given as (1) the percentage of micronucleated and abnormal cells out of 2000 cells counted for each concentration, (2) the induction factors (IFs) as compared to the negative control and (3) the lowest observed effect concentrations (LOECs) for the induction of micronuclei. Examples for anomalies detected in exposed V79 cells are given in fig. 45 and 47.

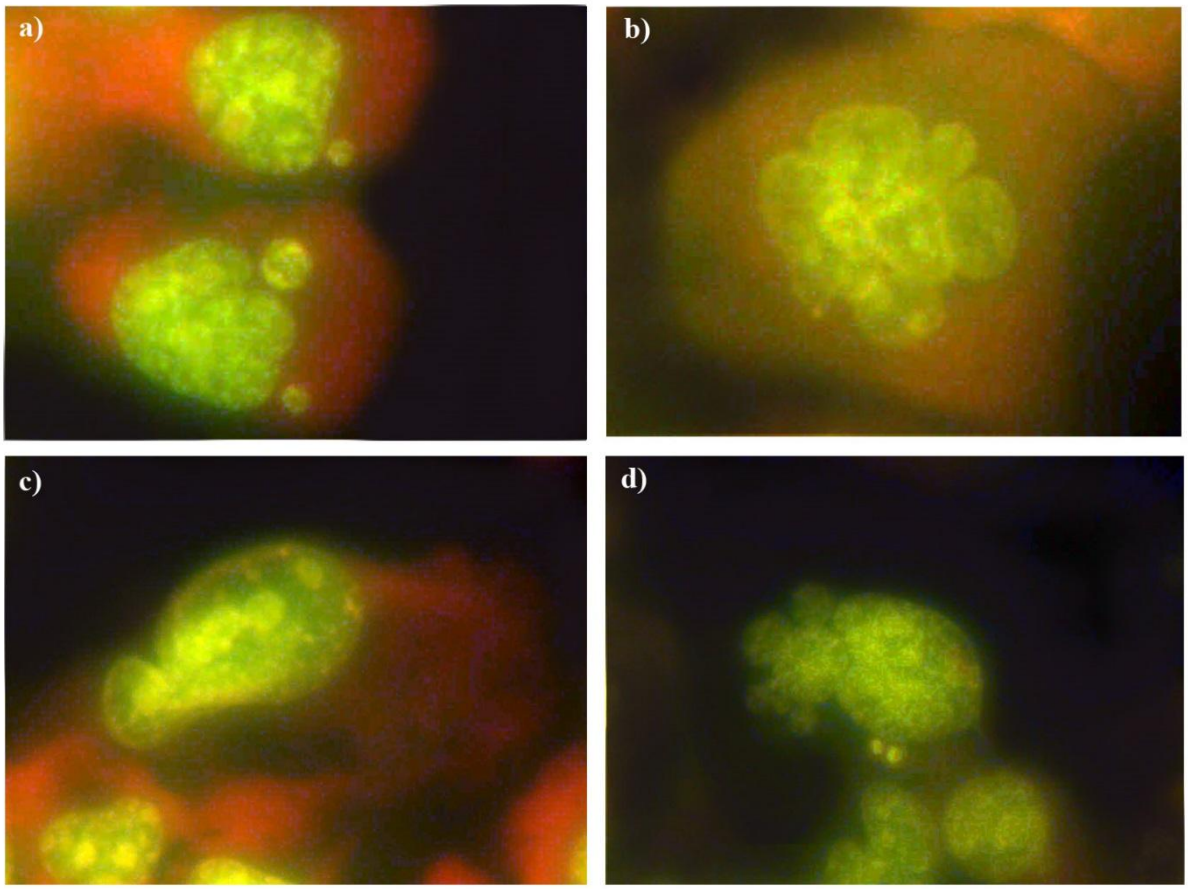

Fig. 45: V79 cells stained with acridine orange in the micronucleus assay after exposure to sediment extracts; a) two cells with micronuclei, the lower one with two and further nucleus deformation after exposure to 25 mg SEQ/ml of Jordan 1, b) polynucleated cell after exposure to 100 mg SEQ/ml of KAC 1, c) nucleus with indention after exposure to 200 mg SEQ/ml of KAC 2, d) blebbing after exposure to 200 mg SEQ/ml of Yarmouk 4.

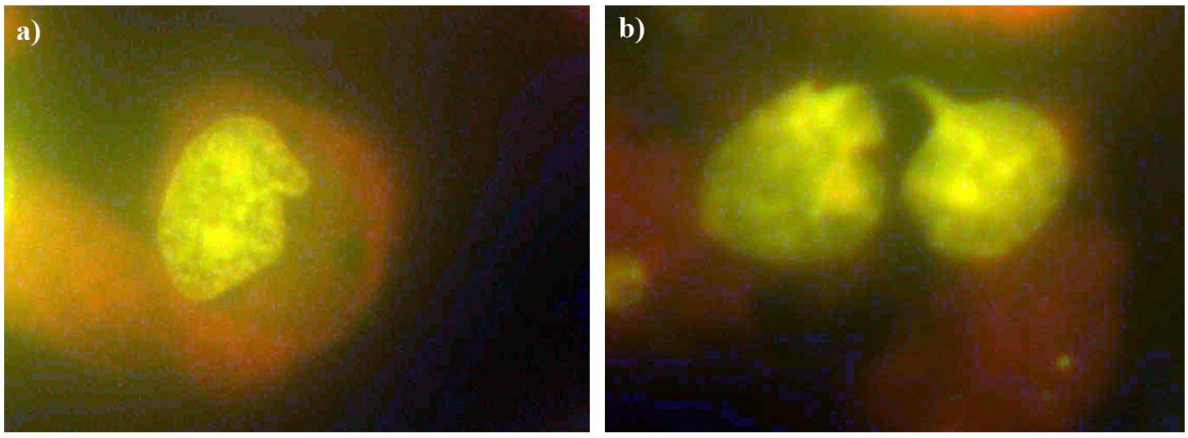

Fig. 46: V79 cells stained with acridine orange in the micronucleus assay after exposure to sediment extracts; a) nucleus deformation (nose) after exposure to 100 mg SEQ/ml of Zarqa 3, b) plasma-bridge between two nuclei after exposure to 50 mg SEQ/ml of Zarqa 4.

3.6.1 Jordan River

Since the cytotoxicity assay did not show any significant effects (Ch. 3.3), the highest concentration tested was defined by the maximum allowed concentration of 1 % DMSO, which corresponded to 200 mg SEQ/ml. Significant genotoxic effects of the sediment extracts from the Jordan River as detected by formation of micronuclei were only observed at the sampling sites Jordan 1 and Jordan 4 (Tab. 21). The other sampling sites did not show any significant increase in the rate of micronucleated or abnormal cells and thus did also not allow the determination of LOEC values. Anomalies generally occurred at rates less than 2.5 % and were statistically not significant. At Jordan 1, the highest and the lowest concentrations tested induced a significant number of micronuclei compared to the negative control with IFs of 3.3 and 2.2, respectively (Fig. 47). At 50 and 100 mg SEQ/ml, no increase in the frequency of micronucleated cells was observed. 100 and 200 mg SEQ/ml of the extract from Jordan 2 induced 3.4 and 2 times more micronuclei than in the negative controls (Fig. 47). The other concentrations tested did not induce significantly. A compilation of the complete data is shown in Tab. 21.

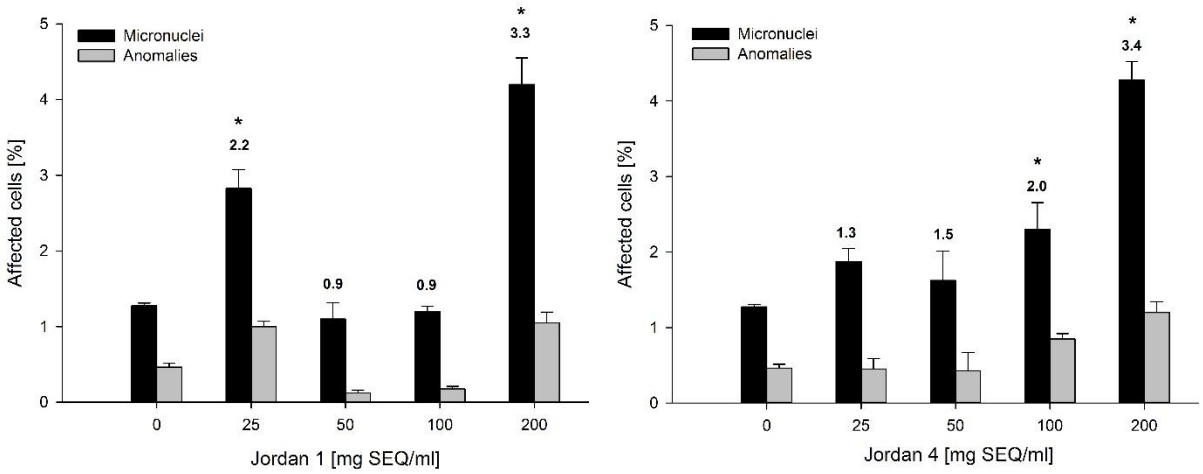

Fig. 47: Induction of micronuclei and anomalies in the micronucleus assay with V79 cells after exposure to sediment extracts from Jordan 1 at Baqura and Jordan 4 at the Allenby Bridge in three replicates. An ANOVA-on-ranks test was conducted followed by Dunnett's method with $p < 0.05$; * = significantly different from negative controls. Numbers indicate induction factors. n = 3.

Tab. 21: Induction factors in the micronucleus assay with V79 cells for the sediment extracts from Jordan River. Grey boxes indicate LOEC values, * = significantly different from negative controls.

	mg SEQ/mL			
	25	50	100	200
Jordan 1, Baqura	2.2*	0.9	0.9	3.3*
Jordan 2, Sheik Hussein Bridge	1.3	1.4	1.1	0.5
Jordan 3, Damiya Bridge	0.8	1.0	1.6	1.4
Jordan 4, Allenby/King Hussein Bridge	1.3	1.5	2.0*	3.4*
Jordan 5, King Abdullah Bridge	0.8	1.2	1.2	1.3

3.6.2 King Abdullah Canal

Both sampling locations at the King Abdullah Canal induced the micronucleus rate significantly in three independent replicates. The highest concentration tested (200 mg SEQ/ml) was provided by the maximum allowed concentration of the solvent DMSO (0.5 %). In both cases, the two highest concentrations of 200 and 100 mg SEQ/ml yielded in significant results with induction factors of 2.3 and 2.5 for KAC 1 and in 3.2 and 2.8 for KAC 2 (Tab. 22). As the negative controls showed a slightly higher micronucleus rate than in the replicates for the Jordan River, an induction factor of 2.4 as observed for 25 mg SEQ/ml of KAC 2 was not found to differ significantly. The rate of anomalies did not show a significant increase when compared

to negative controls. A graphic illustration is given in fig. 48. No effects wer observed for the water eluates as described in Ch. 2.3.3.

Tab. 22: Induction factors in the micronucleus assay with V79 cells for the sediment extracts from King Abdullah Canal. Grey boxes indicate LOEC values,* = significantly different from controls.

	mg SEQ/mL			
	25	50	100	200
KAC 1, Deir Allah	2.0	1.9	2.5*	2.3*
KAC 2, Confluence with Zarqa River	2.4	2.0	2.8*	3.2*

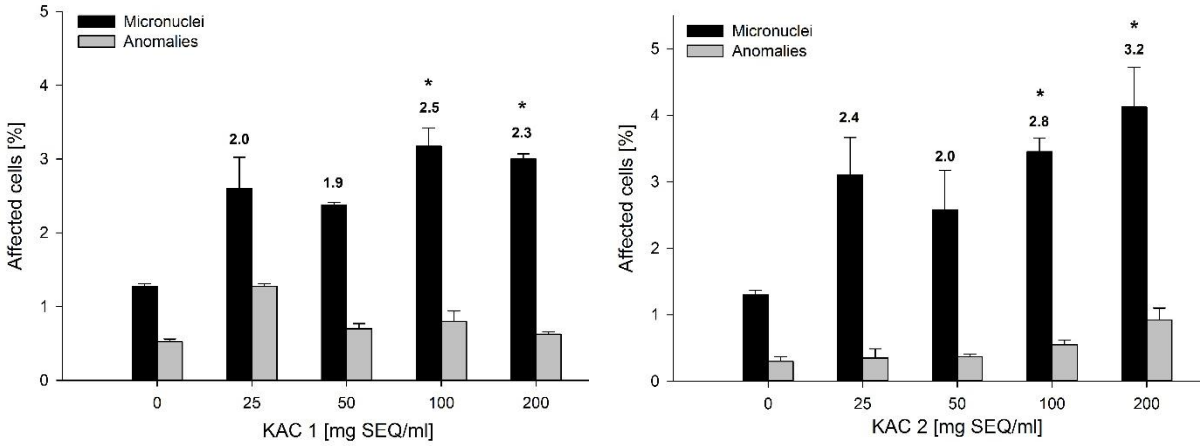

Fig. 48: Induction of micronuclei and anomalies in the micronucleus assay with V79 cells after exposure to sediment extracts of KAC 1 and KAC 2. An *ANOVA on the ranks* was conducted with *Dunnett's* method with $p < 0.05$. * = significantly different from negative controls. Numbers indicate induction factors. n = 3.

3.6.3 Wadi Mujib

Given that the cytotoxicity test with V79 cells did not show any effects for the sampling sites Mujib 1 and Mujib 3 (Ch. 59), 200 mg SEQ/ml of the extracts were used as highest concentration in the micronucleus assay. The NR_{80} for Mujib 2 was determined at 50 mg SEQ/ml which set the limit for testing. 200 and 100 mg SEQ/ml of Mujib 1 induced a 1.9 fold rate of nuclei and the concentrations of 12.5, 25, 50 mg SEQ/ml of Mujib 2 resulted IFs of 3.2, 3.5 and 3.3, respectively (Fig. 49). The LOEC of 12 mg SEQ/ml was the lowest recorded during the whole experiment (Tab. 23). Mujib 3, the LOEC was determined at 200 mg SEQ/ml which induced micronuclei 2.4 fold.

Tab. 23: Induction factors in the micronucleus assay with V79 cells for the sediment extracts from Jordan River. Grey boxes indicate LOEC values, * = significantly different from controls.

	mg SEQ/mL			
	25	50	100	200
Mujib 1, Inlet Mujib Dam	1.1	1.4	1.9*	1.9*
Mujib 2, Outlet Mujib Dam	2.0[1]	3.2*[1]	3.5*[1]	3.3*[1]
Mujib 3, Mouth Dead Sea	0.8	2.3	1.9	2.4*

[1]: the equivalent concentrations for Mujib 2 were 50, 25, 12.5 and 6.25 mgSEQ/ml

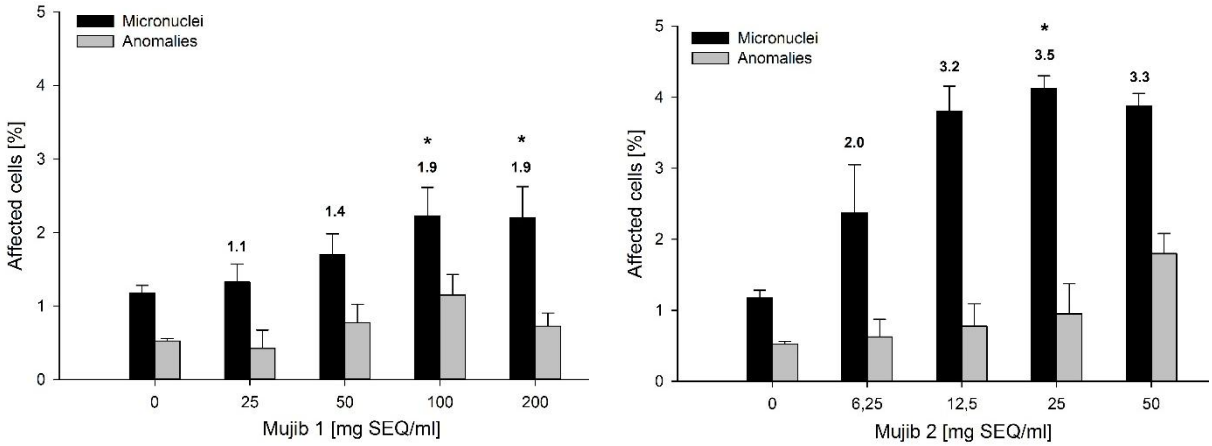

Fig. 49: Induction of micronuclei and anomalies in the micronucleus assay with V79 cells after exposure to sediment extracts of Mujib 1 and 2. An *ANOVA on the ranks* was conducted with *Dunnett's* method with $p < 0.05$. * = significantly different from negative controls. Numbers indicate induction factors. n = 3.

3.6.4 Yarmouk River

As is illustrated in fig. 50, the sediment extracts of the sampling sites Yarmouk 1 and Yarmouk 4 showed significant effects in the micronucleus assay with V79 cells. For Yarmouk 1, only the highest concentration of 200 mg SEQ/ml induced a significant number of micronuclei with an IF of 4.1 which was the highest to be measured in the whole experiment. The extracts of Yarmouk 4 resulted in a low dose effect where 25 mg SEQ/ml induced significantly 2.4 fold but the other concentrations did not. The LOEC were, thus, 25 mg SEQ/ml and 200 for Yarmouk 1 (Tab. 24).

Tab. 24: Induction factors in the micronucleus assay with V79 cells for the sediment extracts from Yarmouk River. Grey boxes indicate LOEC values, * = significantly different from controls.

	mg SEQ/mL			
	25	50	100	200
Yarmouk 1, Unity Dam	0.7	1.8	2.3	4.1*
Yarmouk 2, Wadi Raqab	0.7	0.8	1.3	1.6
Yarmouk 3, Diversion to KAC	1.2	1.3	1.5	1.3
Yarmouk 4, Gesher	2.4*	2.0*	1.5	1.6

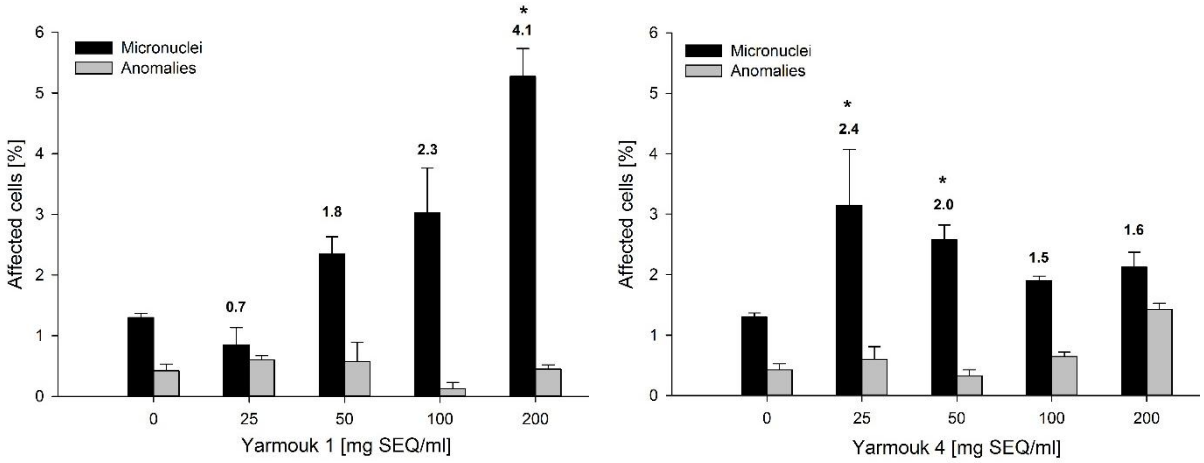

Fig. 50: Induction of micronuclei and anomalies in the micronucleus assay with V79 cells after exposure to sediment extracts of Yarmouk 1 and Yarmouk 4. An *ANOVA on the ranks* was conducted with *Dunnett's* method with $p < 0.05$. * = significantly different from negative controls. Numbers indicate induction factors. n = 3.

3.6.5 Zarqa River

While the cytotoxicity test with V79 cells limited the highest concentration to be tested to a NR_{80} of 50 mg SEQ/ml for the sampling site Zarqa 1, 200 mg SEQ/ml as set by the maximum allowed DMSO concentration of 1 % was used for the other extracts. No mutagenic effects in the micronucleus assay was found for Zarqa 2, whereas the other five sampling sites showed significant mutagenic effects (Fig. 51). The highest IFs for sediments from the Zarqa River were found at Zarqa 1 with a 3.9 fold induction of micronucleus rate at 25 mg SEQ/ml, which was also the LOEC value (Tab. 25). The same LOEC with an IF of 3.5 was found for extracts from Zarqa 5. Zarqa 4 and 6 had a maximum IF of 3.1 and Zarqa 3 of 2.4 at 200 mg SEQ/ml. No effects wer observed for the water elutes as described in Ch. 2.3.3.

Tab. 25: Induction factors in the micronucleus assay with V79 cells for the sediment extracts from Zarqa River. Grey boxes indicate LOEC values, * = significantly different from controls.

	mg SEQ/mL			
	25	50	100	200
Zarqa 1, Khirbet as Samra	2.1[1]	2.8[2]	3.9[3]*	3.6[4]*
Zarqa 2, Confluence Zarqa	1.1	1.2	1.4	1.8
Zarqa 3, Seil Jerash	0.7	1.0	1.4	2.4*
Zarqa 4, Jerash Bridge	0.9	1.6	1.8	3.1*
Zarqa 5, Inlet King Talal Dam	3.5*	2.2	1.9	2.4
Zarqa 6, Outlet King Talal Dam	1.1	1.7	1.9	3.1*

1: 6.25 mg SEQ/mL, 2: 12.5 mg SEQ/mL, 3: 25 mg SEQ/mL, 4: 50 mg SEQ/mL

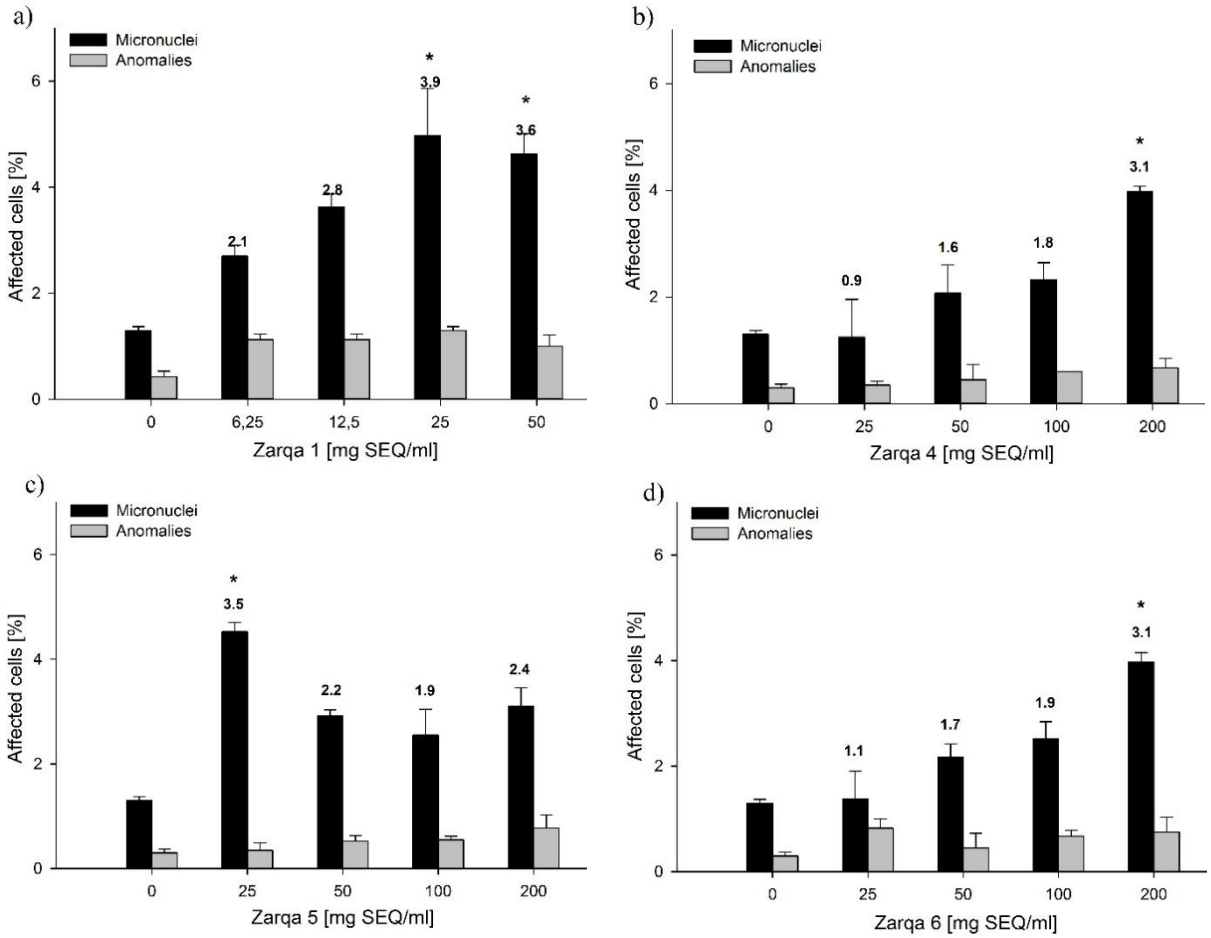

Fig. 51: Induction of micronuclei and anomalies in the micronucleus assay with V79 cells after exposure to sediment extracts of a) Zarqa 1, b) Zarqa 4, c) Zarqa 5 and d) Zarqa 6. An *ANOVA on the ranks* was conducted with *Dunnett's* method with $p < 0.05$. * = significantly different from negative controls. Numbers indicate induction factors. n = 3.

3.7 EROD assay

Since 1 % DMSO was the maximum concentration allowed in *in vitro* tests with the RTL-W1 cell line, 200 mg SEQ/ml was the highest concentration of sediment extracts used in the EROD and MTT assays. Seven 1:2 dilutions were prepared from this concentration, resulting in 1.56 mg SEQ/ml as the lowest concentration. Three independent replicates were conducted for each sampling site. Results are depicted in the concentration of sediment extracts that induced the highest EROD activity, which was then compared to the BNF concentration in pmol evoking an equal induction. Thus, the higher the equivalent concentration of BNF and the lower the concentration of sediment extract, the higher was the dioxin-like activity of a sampling site. To simplify the comparison of the toxicity of the sampling sites, the quotient of the induction equivalent of BNF in pmol, and the mg SEQ/ml was calculated. The higher the quotient, the higher the dioxin-like toxicity in the EROD assay.

3.7.1 Jordan River

All sites along the Jordan River showed dioxin-like induction of the EROD activity in RTL-W1 cells (Tab. 26). The results from the replicates correlated well; however, some minor deviations remained probably due to differences in the moment of exposure during cell cycle. The highest dioxin-like toxicity was found at sampling site Jordan 1, as 12.5 mg SEQ/ml induced EROD activity as 3.4 pmol BNF resulting in a mean quotient of 0.21 (Fig. 52). The second highest induction was found at Jordan 4 because 25 mg SEQ/ml induced in the same way 1.79 – 2.83 pmol BNF with a mean quotient of 0.09 (Fig. 55). Jordan 2, 3 and 5 slightly induced EROD activity with quotients of 0.03 to 0.05 (Figs. 54, Fig. 54). As was detected by the MTT assay, at all sampling sites and replicates the concentration with the highest induction of EROD activity was always linked to a cell viability of about 100 %. At concentrations beyond this point, the cell viability rose in the cases of Jordan 1, 2, and 3, but then declined to a mortality of up to 100 % at 200 mg SEQ/ml as for Jordan 1. Subsequent to the peak of induction, a decrease in the EROD activity of the RTL-W1 cells was detectable in all replicates. This was not only to be observed in cases where cell viability declined as at Jordan 1 at 200 mg SEQ/ml (Fig. 52), but also during increased cell viability as at 100 mg SEQ/ml of the extracts from Jordan 2 (Fig. 54).

Tab. 26: Induction of EROD activity of sediment extracts from the Jordan River given as concentration with the respective highest induction, its BNF equivalent and the quotient of BNF induction and concentration for simplification of the ranking of sampling sites.

	Replicate 1 maximum induction at		Replicate 2 maximum induction at		Replicate 3 maximum induction at		Mean value pmol BNF/ mg SEQ/ml
	mg SEQ/ml	pmol BNF	mg SEQ/ml	pmol BNF	mg SEQ/ml	pmol BNF	
Jordan 1	12.5	1.46	6.25	1.64	12.5	3.4	0.21
Jordan 2	50	1.06	50	2.64	50	0.97	0.03
Jordan 3	50	1.24	25	0.83	25	2.34	0.05
Jordan 4	25	1.79	25	2.16	25	2.83	0.09
Jordan 5	50	1.98	50	1.31	50	2.15	0.04

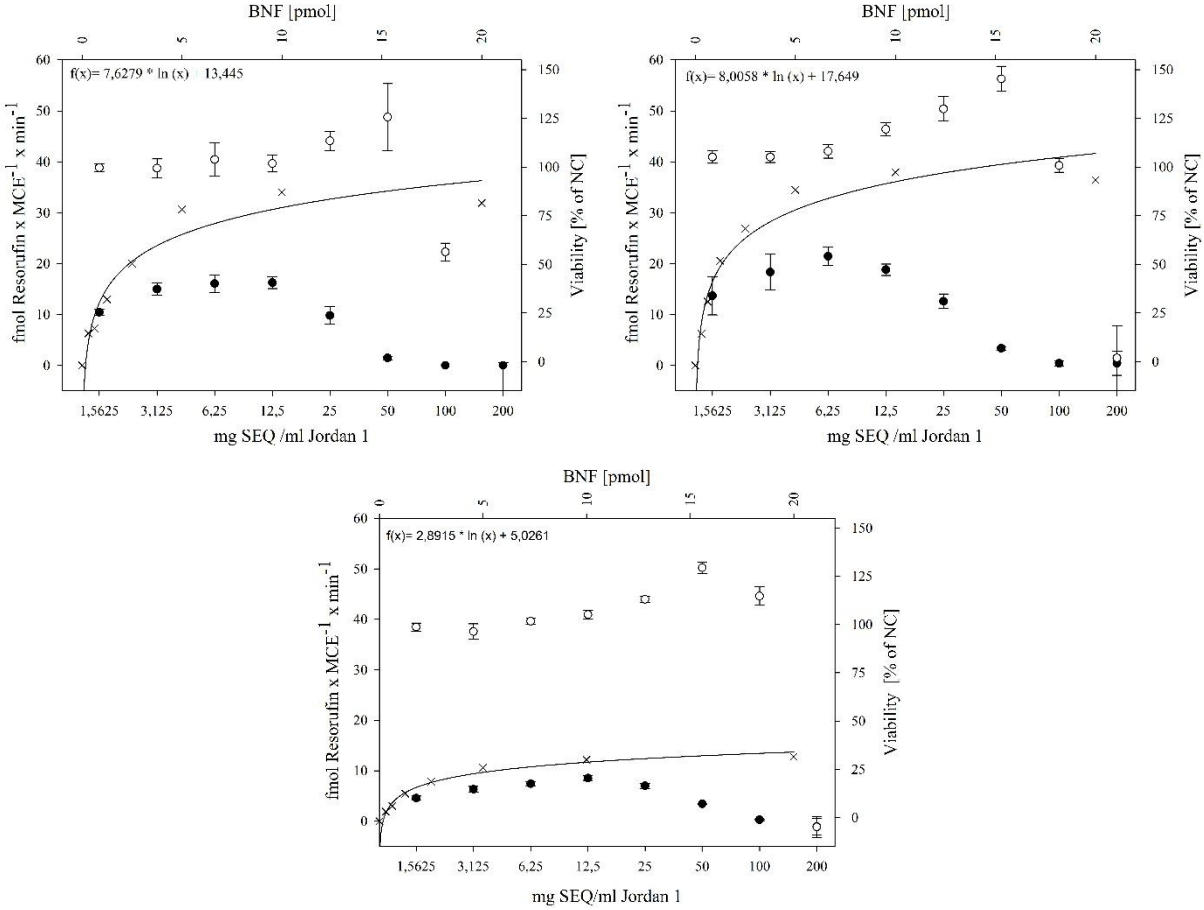

Fig. 52: Dioxin-like toxicity of sediment extracts from Jordan 1 in the EROD assay with RTL-W1 cells. Induction and inhibition of EROD activity is plotted against the viability of cells in the MTT assay. A regression for the induction of BNF was conducted and the equivalent concentration inducing as much as the respective concentration of the sediment extract was determined mathematically. ●: EROD activity of sample, ○: viability in % of negative control, x: EROD activity of BNF as reference.

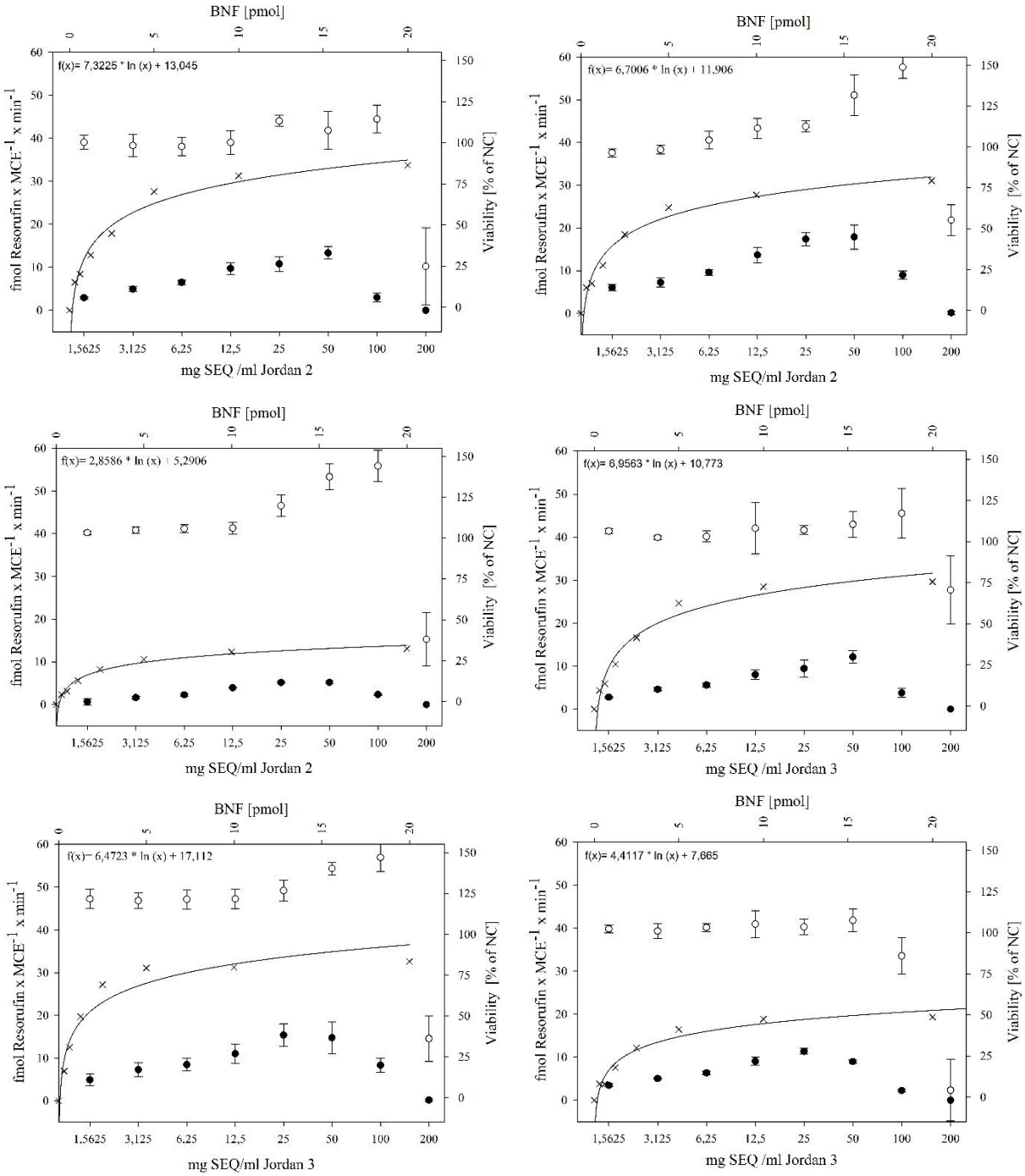

Fig. 53: Dioxin-like toxicity of sediment extracts from Jordan 2 and 3 in the EROD assay with RTL-W1 cells. Induction and inhibition of EROD activity is plotted against the viability of cells in the MTT assay. A regression for the induction of BNF was conducted and the equivalent concentration inducing as much as the respective concentration of the sediment extract was determined mathematically. ●: EROD activity of sample, ○: viability in % of negative control, x: EROD activity of BNF as reference.

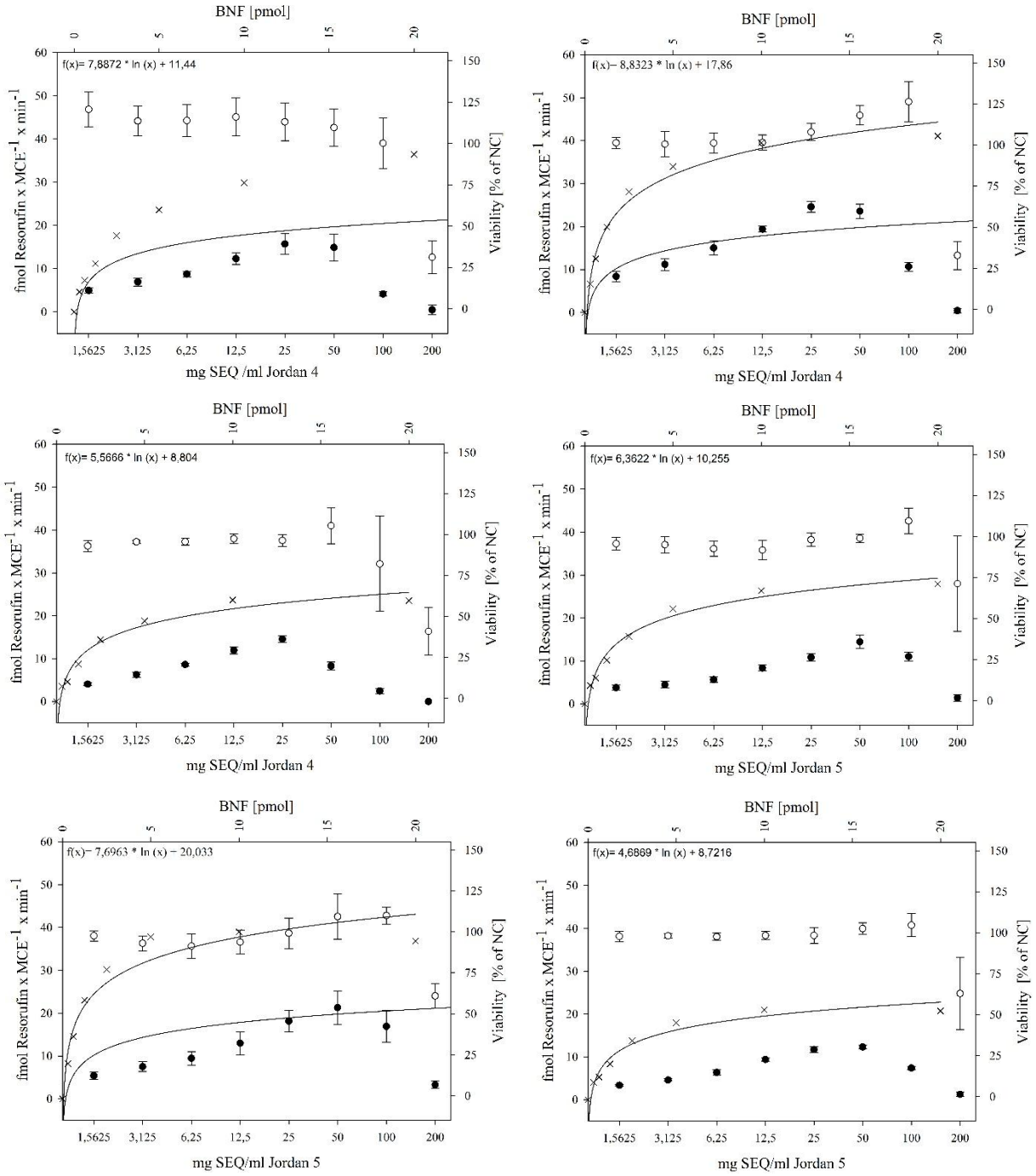

Fig. 54: Dioxin-like toxicity of sediment extracts from Jordan 4 and 5 in the EROD assay with RTL-W1 cells. Induction and inhibition of EROD activity is plotted against the viability of cells in the MTT assay. A regression for the induction of BNF was conducted and the equivalent concentration inducing as much as the respective concentration of the highest observed induction caused by the sediment extract was determined mathematically. ●: EROD activity of sample, ○: viability in % of negative control, x: EROD activity of BNF as reference.

3.7.2 King Abdullah Canal

Little dioxin-like toxicity as determined by the EROD assay with RTL-W1 cells was found for KAC 1 as well as for KAC 2. In all three replicates with sediment extracts from KAC 1, a slight induction of the ERDOD activity was noted up to a concentration of 12.5 mg SEQ/ml followed by a decrease of activity down to no activity at 100 and 200 mg SEQ/ml (Fig. 55). Cell viability as measured in the MTT assay was roughly 100 % up to 25 mg SEQ/ml and then decreased until complete mortality at 100 and 200 mg SEQ/ml, correlating with the decline of EROD activity. The extracts from KAC 2 did not show such a decline in cell viability and EROD activity Cell viability rose at 200 mg SEQ/ml and ERDOD activity was increased until 100 mg SEQ/ml followed by a slight decrease. Comparing the quotient of the equivalent BNF concentration and the SEQ, KAC 1 showed a higher toxicity than KAC 2 as 12.5 mg SEQ/ml exhibited the same induction as 0.58 to 1.18 pmol of BNF. 100 mg SEQ/ml of KAC 2 induced EROD activity as much as 0.22 to 1.63 pmol BNF (Tab. 27).

Tab. 27: Induction of EROD activity of sediment extracts from King Abdullah sediment extracts expressed by the concentration with the respective highest induction, its BNF equivalent and the quotient of BNF induction and concentration for simplification of the

	Replicate 1 maximum induction at		Replicate 2 maximum induction at		Replicate 3 maximum induction at		Mean value pmol BNF/ mg SEQ/ml
	mg SEQ/ml	pmol BNF	mg SEQ/ml	pmol BNF	mg SEQ/ml	pmol BNF	
KAC 1	12.5	0.58	12.5	1.18	12.5	0.6	0.06
KAC 2	100	1.43	100	1.62	100	0.22	0.01

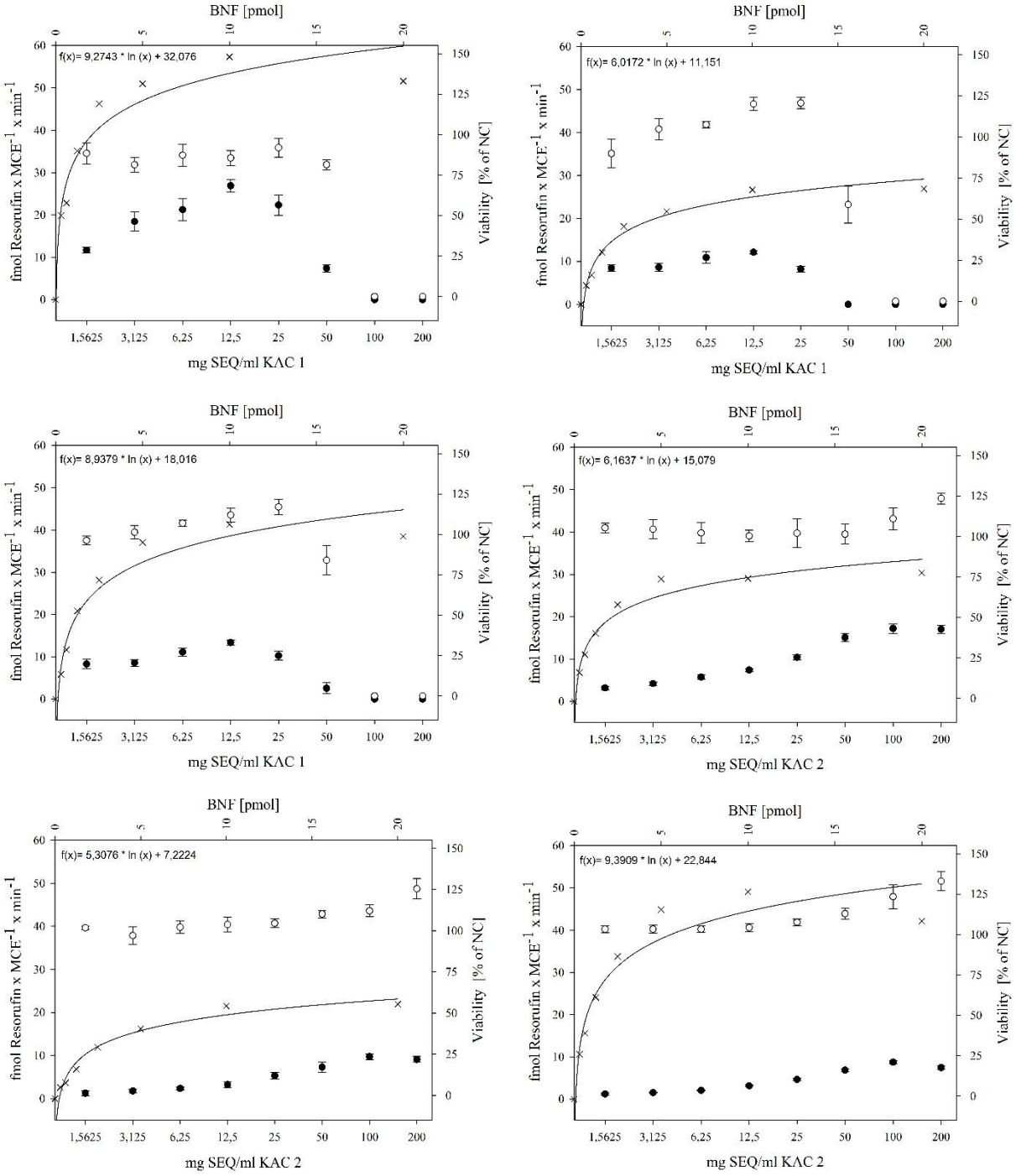

Fig. 55: Dioxin-like toxicity shown for KAC 1 and 2 in the EROD assay with RTL-W1 cells. Induction and inhibition of EROD activity is plotted against the viability of cells measured in the MTT assay. A regression for the induction of BNF was conducted and the equivalent concentration inducing as much as the respective concentration of the highest observed induction caused by the sediment extract was determined mathematically. ●: EROD activity of sample, ○: viability in % of negative control, x: EROD activity of BNF as reference.

3.7.3 Wadi Mujib

The sediment extracts of the three sampling sites of Wadi Mujib were all slightly positive for the induction of EROD activity in RTL-W1 cells. Mujib 1 and Mujib 2 showed gradual induction up to a concentration of 25 and 12.5 mg SEQ/ml, respectively, followed by a strong decrease of the activity at 200 and 100 mg SEQ/ml (Fig. 56). In the case of Mujib 2, this decrease correlated with a complete decline of the cell viability measured with the MTT assay (Fig. 56). Only a slight decline of cell viability was found at 200 mg SEQ/ml of the extracts from Mujib 1. At Mujib 3, cell viability rose slightly at 200 mg SEQ/ml, and a slight decrease of EROD activity was observed after the maximum induction at 100 mg SEQ/ml (Fig. 57). The highest induction, when related to the BNF equivalent, was found in the second replicate of Mujib 2, where 25 mg SEQ/ml induced as much EROD activity as 2.64 pmol BNF (Tab. 28). However, the BNF induction in this replicate was smaller than in the other tests. The same applied to Mujib 3 where the second highest induction of an equivalent of 1.62 pmol BNF was found at 100 mg SEQ/ml. The remaining induction equivalents of the other replicates of Mujib 3 were 1.43 and 0.53 pmol BNF, and 0.29 and 0.12 for Mujib 2. At 25 mg SEQ/ml, Mujib 1 induced as much EROD activity as 0.52 to 0.71 pmol BNF.

Tab. 28: Induction of EROD activity of the Wadi Mujib sediment extracts expressed by the concentration with the respective highest induction, its BNF equivalent and the quotient of BNF induction and concentration for simplification of the ranking of sampling sites.

	Replicate 1 maximum induction at		Replicate 2 maximum induction at		Replicate 3 maximum induction at		Mean value pmol BNF/ mg SEQ/ml
	mg SEQ/ml	pmol BNF	mg SEQ/ml	pmol BNF	mg SEQ/ml	pmol BNF	
Wadi Mujib 1	25	0.59	25	0.52	25	0.71	0.02
Wadi Mujib 2	12.5	0.29	25	2.64	12.5	0.12	0.05
Wadi Mujib 3	100	1.43	100	1.52	100	0.53	0.01

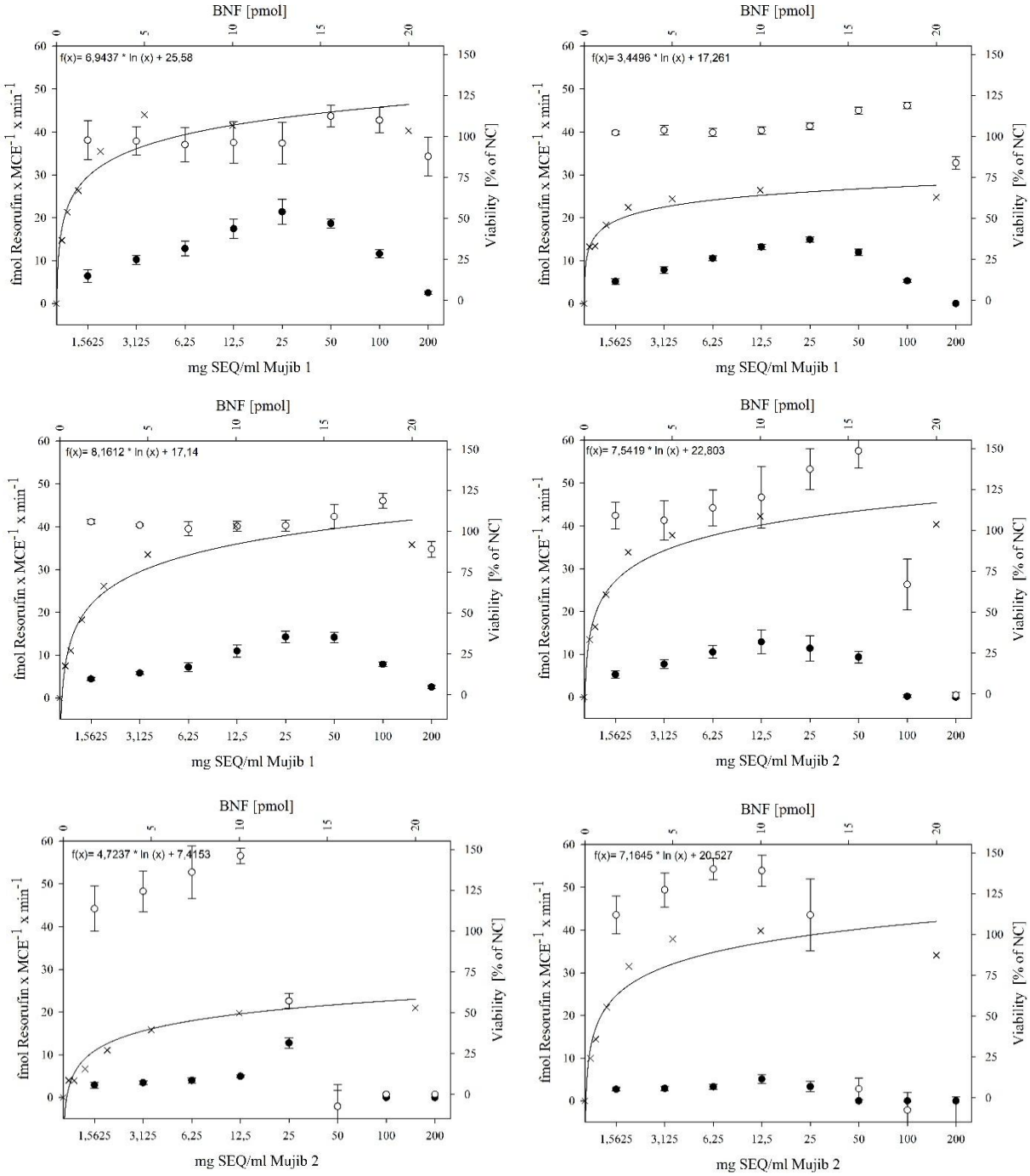

Fig. 56: Dioxin-like toxicity of sediment extracts from Mujib 1 and 2 in the EROD assay with RTL-W1 cells. Induction and inhibition of EROD activity is plotted against the viability of cells measured in the MTT assay. A regression for the induction of BNF was conducted and the equivalent concentration inducing as much as the respective concentration of the highest observed induction caused by the sediment extract was determined mathematically. ●: EROD activity of sample, ○: viability in % of negative control, x: EROD activity of BNF as reference.

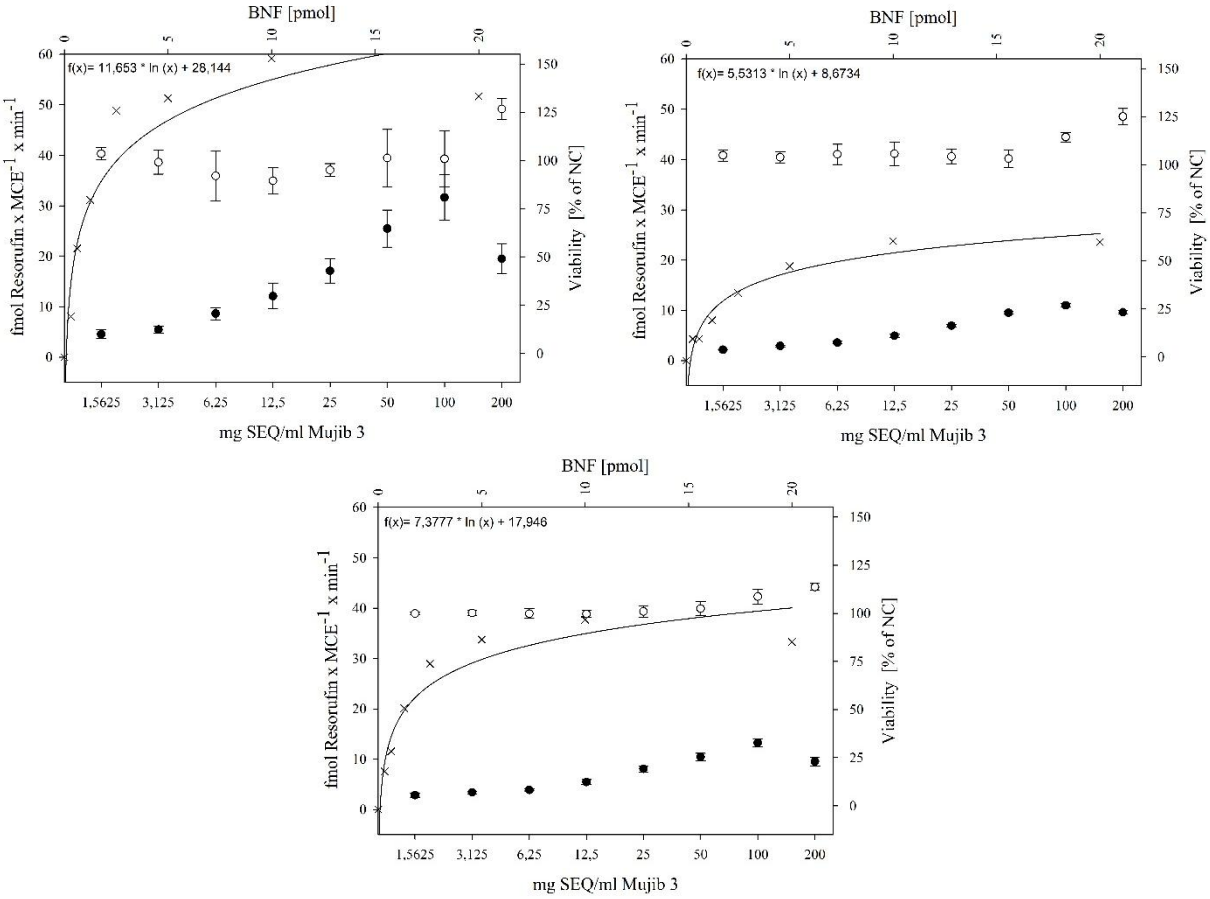

Fig. 57: Dioxin-like toxicity assed in the EROD assay with RTL-W1 cells for sediment extract of Mujib 3. Induction and inhibition of EROD activity is plotted against the viability of cells measured in the MTT assay. A regression for the induction of BNF was conducted and the equivalent concentration inducing as much as the respective concentration of the highest observed induction caused by the sediment extract was determined mathematically. ●: EROD activity of sample, ○: viability in % of negative control, x: EROD activity of BNF as reference.

3.7.4 Yarmouk River

All sediment extracts of the Yarmouk River induced EROD activity at rather low concentrations of 6.25 mg SEQ/ml (Yarmouk 1 and 2) and 12.5 mg SEQ/ml (Yarmouk 3 and 4). Subsequently, for all sites, the activity decreased totally at 100 and at 200 mg SEQ/ml at Yarmouk 3. With the exception of Yarmouk 3, this decrease was accompanied by a decline in the cell viability as was measured by the MTT assay. This decline was preceded by an increase in cell viability where the decrease of EROD activity, however, had already started. The highest EROD induction by means of BNF equivalents was found at the sampling site Yarmouk 1 (Fig. 58), where 12.5 mg SEQ/ml induced as much EROD activity as 2.22 pmol BNF (Tab. 29). The second highest induction equaled 1.98 pmol BNF at 12.5 mg SEQ/ml of Yarmouk 3, followed

by 1.41 pmol BNF at 6.25 mg SEQ/ml of the extracts of Yarmouk 2 (Fig. 59), and 0.93 pmol BNF at 12.5 mg SEQ/ml of Yarmouk 4 (Fig. 60).

Tab. 29: Induction of EROD activity of the Yarmouk River sediment extracts expressed by the concentration with the respective highest induction, its BNF equivalent and the quotient of BNF induction and concentration for simplification of the ranking of sampling sites.

	Replicate 1 maximum induction at		Replicate 2 maximum induction at		Replicate 3 maximum induction at		Mean value pmol BNF/ mg SEQ/ml
	mg SEQ/ml	pmol BNF	mg SEQ/ml	pmol BNF	mg SEQ/ml	pmol BNF	
Yarmouk 1	6.25	1.03	6.25	0.94	12.5	2.22	0.16
Yarmouk 2	6.25	0.86	6.26	1.19	6.25	1.41	0.19
Yarmouk 3	12.5	0.69	12.5	0.53	12.5	1.98	0.09
Yarmouk 4	12.5	0.93	12.5	0.86	25	0.25	0.05

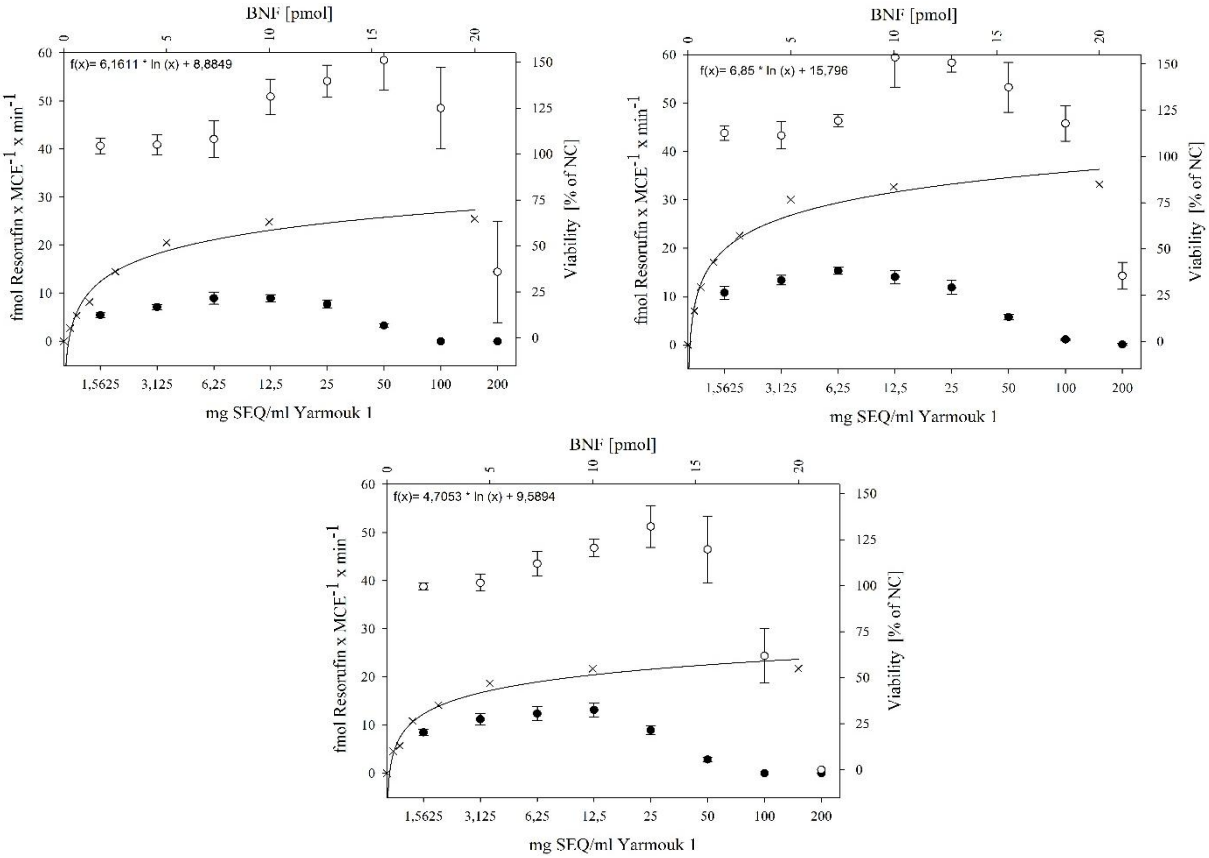

Fig. 58: Dioxin-like toxicity of sediment extracts from Yarmouk 1 in the EROD assay with RTL-W1 cells. Induction and inhibition of EROD activity is plotted against the viability of cells measured in the MTT assay. A regression for the induction of BNF was conducted and the equivalent concentration inducing as much as the respective concentration of the highest observed induction caused by the sediment extract was determined mathematically. ●: EROD activity of sample, ○: viability in % of negative control, x: EROD activity of BNF as reference.

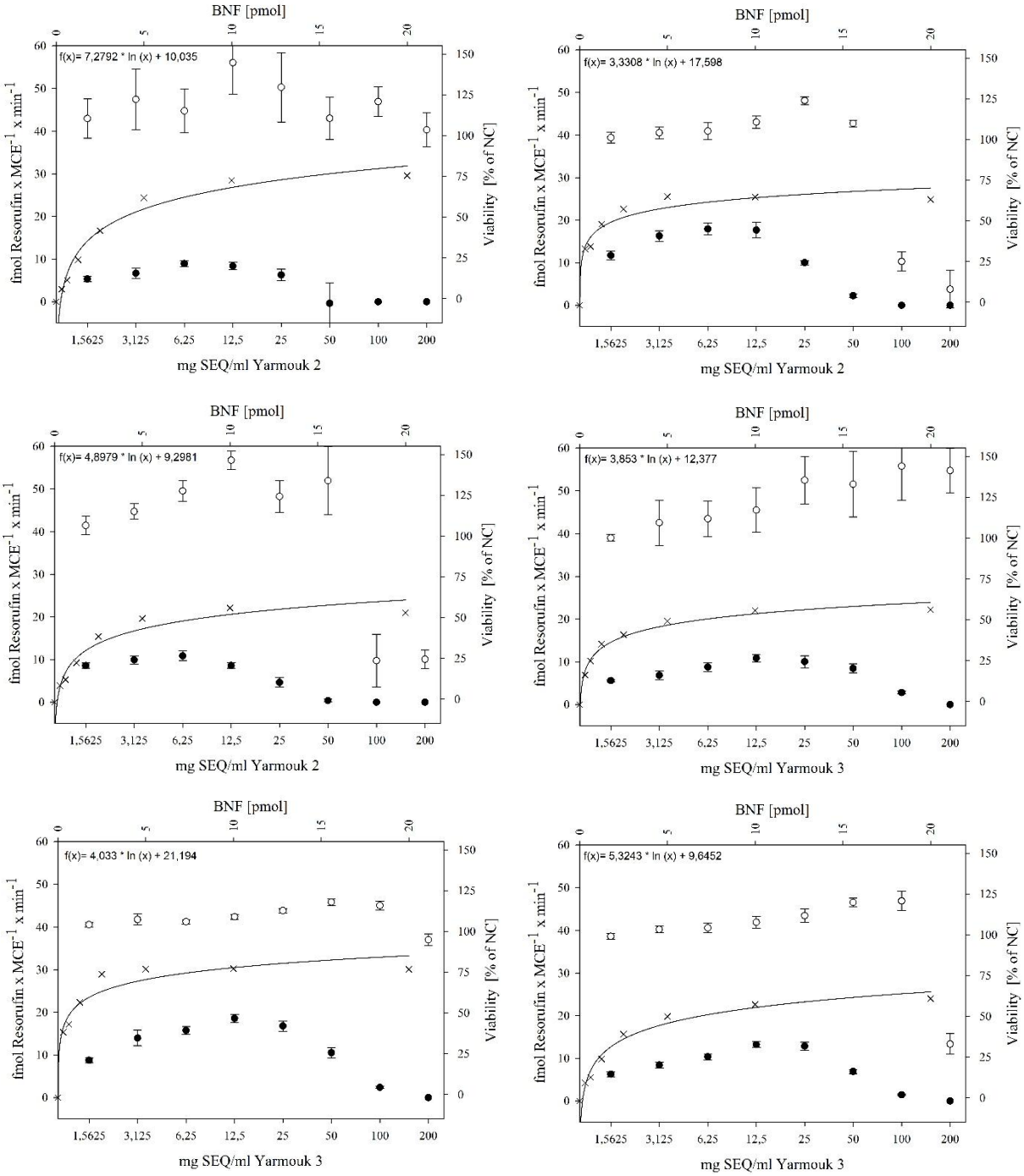

Fig. 59: Dioxin-like toxicity as assed in the EROD assay with RTL-W1 cells for the sampling sites Yarmouk 2 and 3. Induction and inhibition of EROD activity is plotted against the viability of cells measured in the MTT assay. A regression for the induction of BNF was conducted and the equivalent concentration inducing as much as the respective concentration of the highest observed induction caused by the sediment extract was determined mathematically. ●: EROD activity of sample, ○: viability in % of negative control, x: EROD activity of BNF as reference.

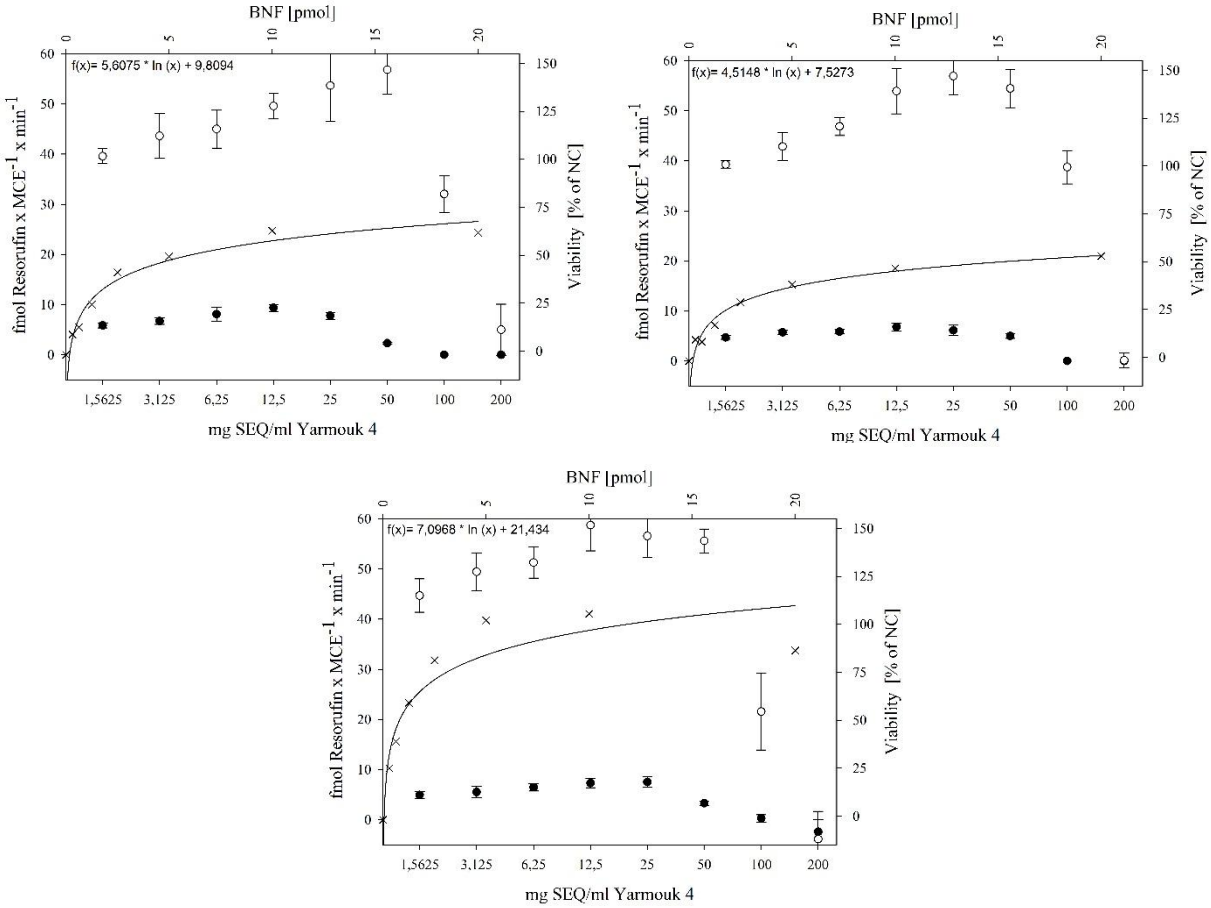

Fig. 60: Dioxin-like toxicity as assed in three independent replicates in the EROD assay with RTL-W1 cells for the sampling site Yarmouk 4. Induction and inhibition of EROD activity is plotted against the viability of cells measured in the MTT assay. A regression for the induction of BNF was conducted and the equivalent concentration inducing as much as the respective concentration of the highest observed induction caused by the sediment extract was determined mathematically. ●: EROD activity of sample, ○: viability in % of negative control, x: EROD activity of BNF as reference.

3.7.5 Zarqa River

All sites along the Zarqa River showed induction of dioxin-like EROD activity in RTL-W1 cells (Tab. 30). Minor deviations between the replicates occured probably due to differences in the moment of exposure during cell cycle, although the overall correlation was good. The highest EROD induction in terms of BNF equivalent was found for the sediment extracts of sampling site Zarqa 2 as 25 mg SEQ/ml induced in the same way as 3.89 pmol BNF (Fig. 61). This was followed by the sites Zarqa 5 and Zarqa 3 as here 50 mg SEQ/ml and 12.5 mg SEQ/ml induced EROD activity as much as 3.75 and 3.15 pmol BNF, respectively (Fig. 62, Fig. 63). The highest BNF equivalents found for the sites Zarqa 1, 6 and 4 were 2.91, 2.56 and 2.43, respectively. In terms of the concentration of the sediment extracts inducing the maximum

EROD activity, Zarqa 1 proved to be most potent in terms of dioxin-like activity as only 3.13 mg SEQ/ml were needed. To simplify the comparison of the toxicity of the sampling sites, the quotient of the induction equivalent of BNF in pmol and the mg SEQ/ml was calculated. Accordingly, the ranking in dioxin-like toxicity for the Zarqa River was as follows: Zarqa 3 (0.17), Zarqa 4 (0.15), Zarqa 6 (0.14), Zarqa 1 (0.12), Zarqa 2 (0.11) and Zarqa 5 (0.06). Induction of EROD activity was always followed by a decrease in activity. With the exception of the sampling site Zarqa 5 (Fig. 63), this decrease correlated with a decline in cell viability as was simultaneously measured by the MTT assay. Preceding the decreasing cell viability, an increase in viability was observed in the cases of Zarqa 1, 2, 4 and 6.

Tab. 30: Induction of EROD activity of the Zarqa River sediment extracts expressed by the concentration with the respective highest induction, its BNF equivalent and the quotient of BNF induction and concentration for simplification of the ranking of sampling sites.

	Replicate 1 maximum induction at		Replicate 2 maximum induction at		Replicate 3 maximum induction at		Mean value pmol BNF/ mg SEQ/ml
	mg SEQ/ml	pmol BNF	mg SEQ/ml	pmol BNF	mg SEQ/ml	pmol BNF	
Zarqa 1	3.13	0.34	3.13	0.31	3.13	2.91	0.12
Zarqa 2	12.5	0.68	12.5	1.43	25	3.89	0.11
Zarqa 3	12.5	1.49	12.5	1.58	12.5	3.15	0.17
Zarqa 4	12.5	2.19	25	1.50	12.5	2.45	0.15
Zarqa 5	50	2.57	50	3.75	50	2.43	0.06
Zarqa 6	12.5	2.56	12.5	1.31	12.5	1.41	0.14

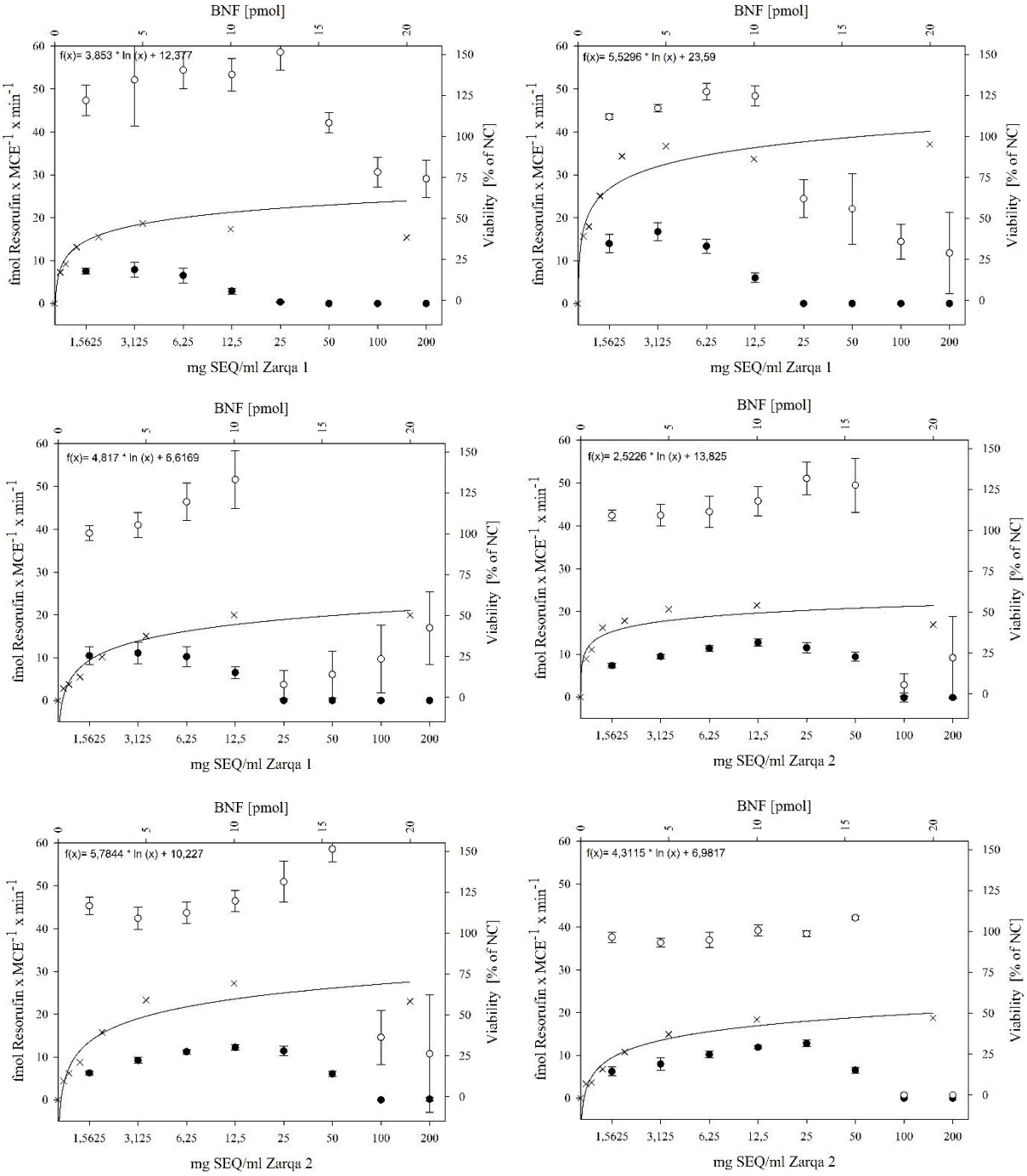

Fig. 61: Dioxin-like toxicity for sediment extracts of Zarqa 1 and 2 in the EROD assay with RTL-W1 cells. Induction and inhibition of EROD activity is plotted against the viability of cells measured in the MTT assay. A regression for the induction of BNF was conducted and the equivalent concentration inducing as much as the respective concentration of the highest observed induction caused by the sediment extract was determined mathematically. ●: EROD activity of sample, ○: viability in % of negative control, x: EROD activity of BNF as reference..

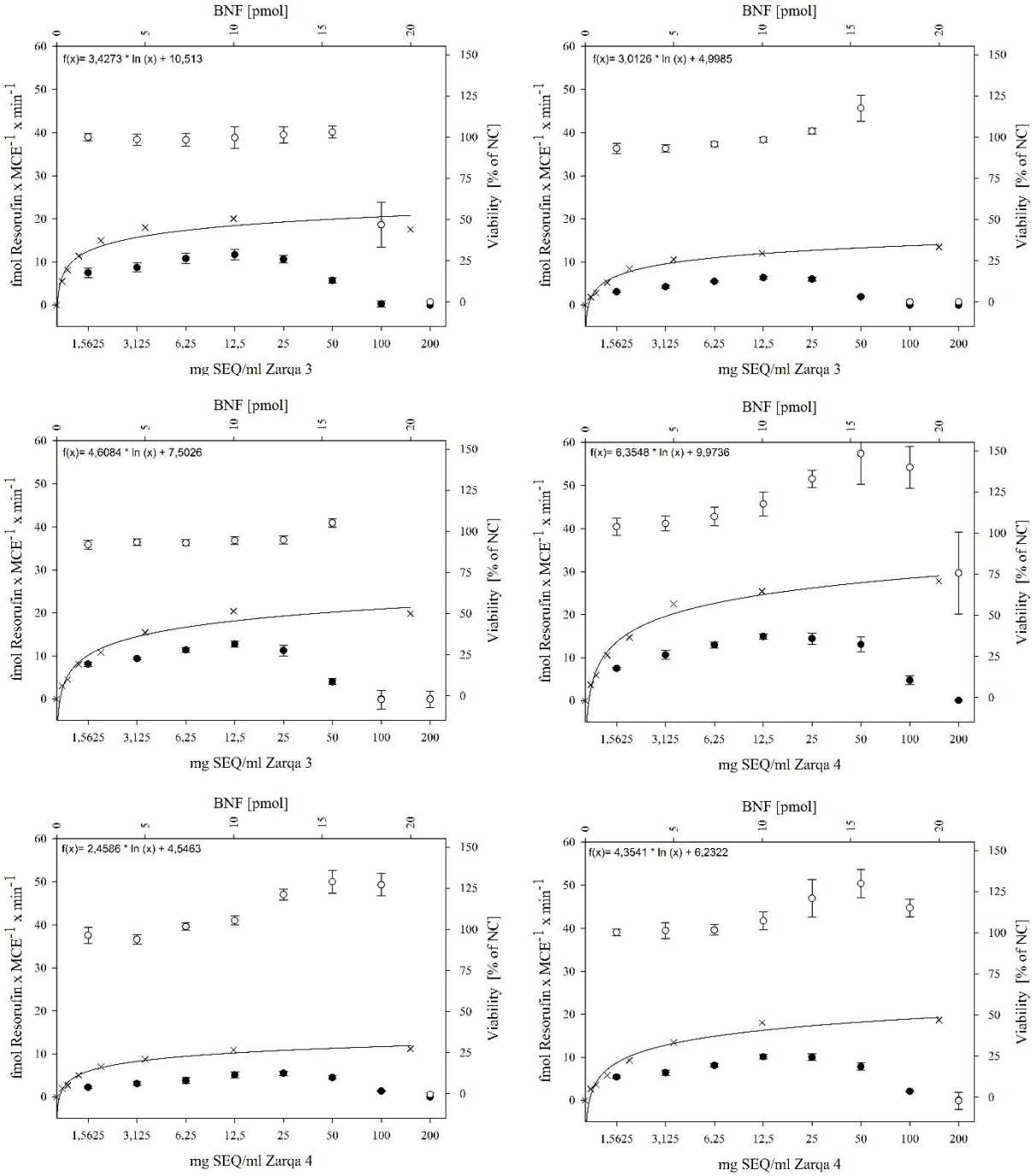

Fig. 62: Dioxin-like toxicity as assed in the EROD assay with RTL-W1 cells for the sampling sites Zarqa 3 and 4. Induction and inhibition of EROD activity is plotted against the viability of cells measured in the MTT assay. A regression for the induction of BNF was conducted and the equivalent concentration inducing as much as the respective concentration of the highest observed induction caused by the sediment extract was determined mathematically. ●: EROD activity of sample, ○: viability in % of negative control, x: EROD activity of BNF as reference.

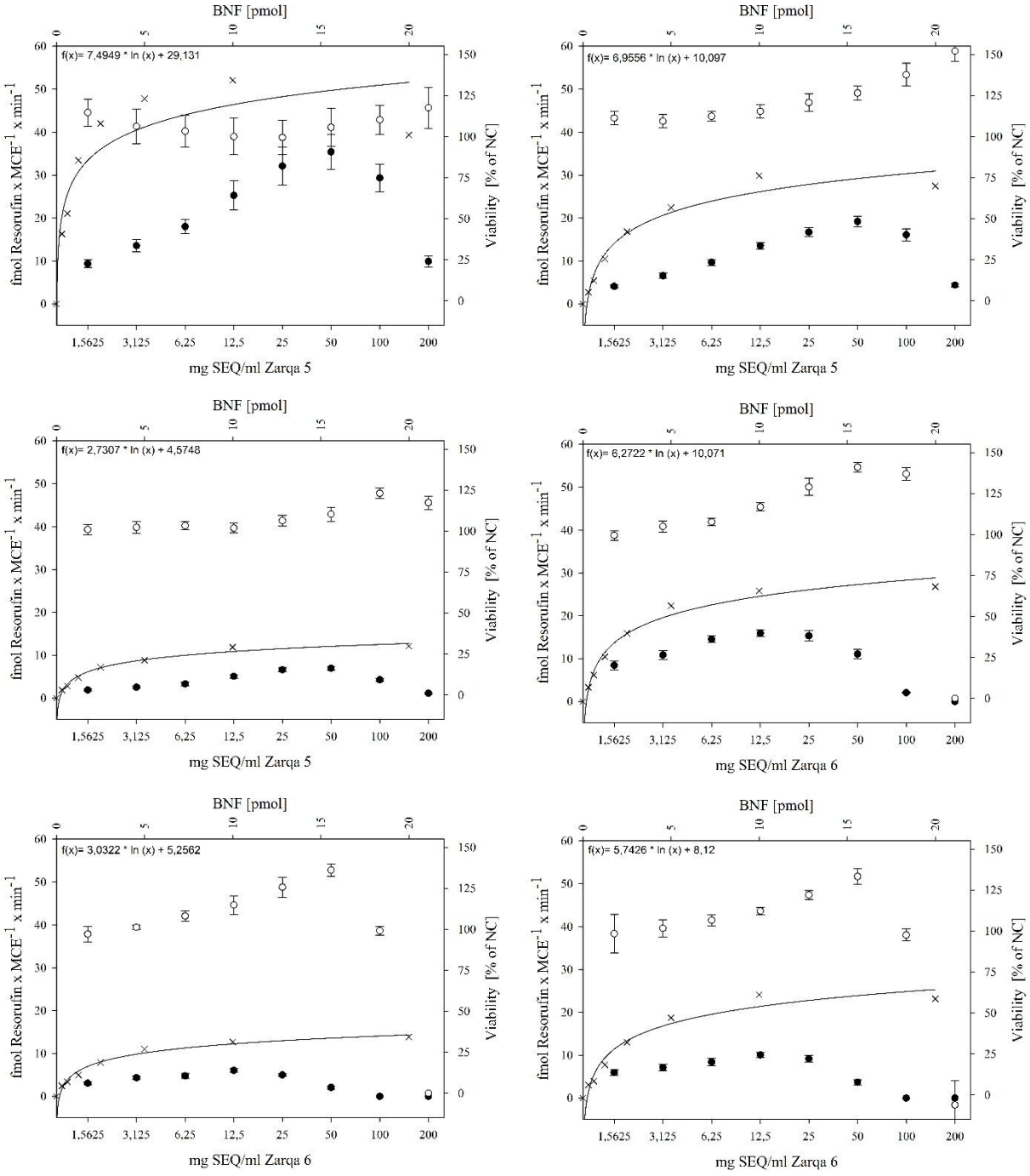

Fig. 63: Dioxin-like toxicity as assed in the EROD assay with RTL-W1 cells for the sampling sites Zarqa 5 and 6. Induction and inhibition of EROD activity is plotted against the viability of cells measured in the MTT assay. A regression for the induction of BNF was conducted and the equivalent concentration inducing as much as the respective concentration of the highest observed induction caused by the sediment extract was determined mathematically. ●: EROD activity of sample, ○: viability in % of negative control, x: EROD activity of BNF as reference.

3.8 Acute toxicity of sediment extracts in the fish embryo test with zebrafish

3.8.1 Jordan River

The effects of sediment extracts as determined by the fish embryo toxicity test with *Danio rerio* were very heterogeneous for the Jordan River, since only sediments from three out of the five sampling sites showed any lethal effects at all. However, following Mujib 2, Jordan 1 was found to be the second most toxic site within this thesis with an LC_{50} value of 13.8 mg SEQ/ml after 96 hpf (Fig. 64 and Tab. 31). After 24 hpf, 100 % of the embryos at 50 and 37.5 mg SEQ/ml and 75 and 7 % of the embryos at 25 and 12.5 mg SEQ/ml were coagulated, respectively. At 25 and 12.5 mg SEQ/ml, other lethal effects such as non-detachment of the tails and retarded somite stages were recorded three times, all of which eventually resulted in coagulation or lack of heartbeat at 48 hpf. Acute toxicity rose to 90 % at 25 mg SEQ/ml and to 13 % at 12.5 mg SEQ/ml. Furthermore, sublethal effects in terms of edemata, reduction of blood circulation and heartbeat rate were recorded for 42 % of the remaining living embryos at 12.5 mg SEQ/ml and for 100 % at 25 mg SEQ/ml at 48 and 72 hpf. Hence, the EC_{50} value after 48 hpf differed considerably from the equivalent LC_{50} value (Fig. 64). This was even more evident after 96 hpf, as 100 % of the remaining living embryos at 25 mg SEQ/ml, 87 % at 12.5 mg SEQ/ml and 93 % at 1.0 mg SEQ/ml showed no hatching success (Fig. 66 g). At 12.5 mg SEQ/ml, embryos showed severe edemata and reduction of heartbeat rate and blood circulation in 39 %. Thus, the EC_{50} value could not be determined with ToxRat and had to be less than 1.0 mg SEQ/ml (Fig. 64).

Tab. 31: Toxicity data of the fish embryo test with *Danio rerio* for the Jordan River in mg SEQ/ml.

	LC_{50}		EC_{50}		LOEC	NOEC
	96 hpf	48 hpf	96 hpf	48 hpf	96 hpf	96 hpf
Jordan 1, Baqura	13.8	14.8	< 1*	5.2	1.0	< 1*
Jordan 2, Sheik Hussein Bridge	33.9	35.4	6.6	24.8	1.0	< 1*
Jordan 3, Damiya Bridge	n.d.	n.d.	28.7	n.d.	12.5	1.0
Jordan 4, Allenby/King Hussein Bridge	n.d.	n.d.	n.d.	n.d.	37.5	25
Jordan 5, King Abdullah Bridge	n.d.	n.d.	n.d.	n.d.	50	37.5

n.d.: not detectable within the concentration range tested since the amount of effects observed did not allow for a detection of the corresponding values. *: Since all embryos showed sublethal effects even at the lowest concentration of 1 mg SEQ/mL, the EC50 value must be less than this.

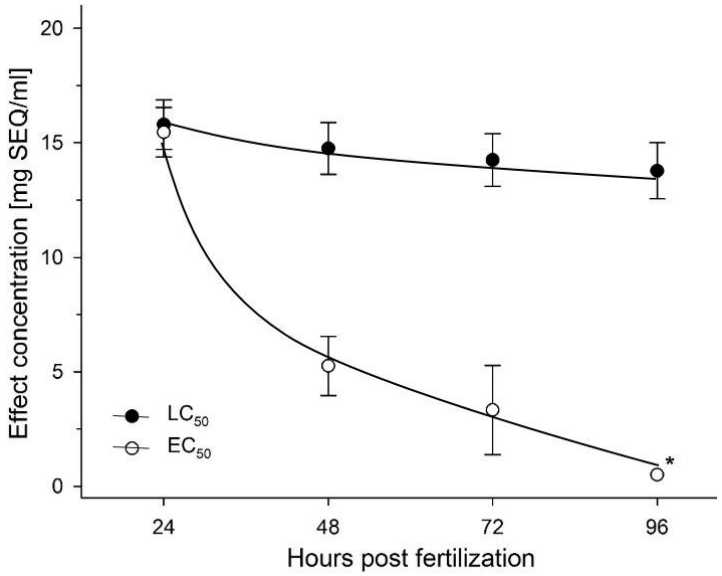

Fig. 64: Correlation and development of LC_{50} and EC_{50} values for the sediment extracts of Jordan 1. *: Since embryos at all concentrations tested showed no hatching success at 96 hpf, the EC_{50} value must be less than 1.0 mg SEQ/ml.

Jordan 2 at the Sheik Hussein Bridge with an LC_{50} at 96 hpf of 33.9 mg SEQ/ml produced acute embryo toxicity as well and was the fourth most toxic sampling site after Jordan 1, Mujib 2 and Zarqa 6 in terms of embryo toxicity with *Danio rerio* (Tab. 31). Besides 66 % coagulation, two embryos showed the effect of no heartbeat at 50 mg SEQ/ml after 48hpf and seven after 96 hpf. By then, 90 % of the embryos at 50 mg SEQ/ml, 57 % at 37.5 mg SEQ/ml 13 % at 25 mg SEQ/ml were mortally affected. 40 and 30 % of the not lethally affected embryos showed effects on the cardiovascular system after 48 hpf at 37.5 and 25 mg SEQ/ml, respectively. At 25 mg SEQ/ml, a recovery of these effects could be observed in three out of nine individuals after 96 hpf. As did also account for Jordan 1, the hatching success of embryos was affected after 96 hpf at all concentrations. Of the remaining living embryos, 100 % failed to hatch at 50 mg SEQ/ml, 93 % at 37.5 mg SEQ/ml, 96 % at 25 mg SEQ/ml, 38 % at 12.5 mg SEQ/ml (Fig. 66 f) and 7 % at 1.0 mg SEQ/ml. Furthermore, teratogenic effects in terms of severe malformations of notochord/spine and tail were observed in 60 %, 30 %, 27 % and 7 % at 50, 37.5, 25 and 12.5 mg SEQ/ml, respectively (Figs. 66,Fig. 66 a - f). This led to a significant difference between the EC_{50} and LC_{50} values. Correlation of the LC_{50} values after 48 and 96 hpf was good for Jordan 1 as well as for Jordan 2.

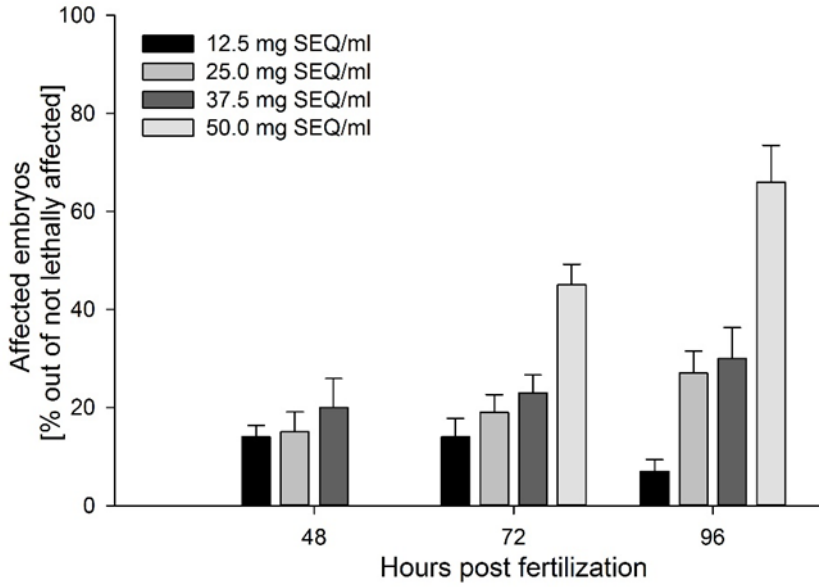

Fig. 65: Effects of sediment extracts from Jordan 2 on spine and tail malformations.

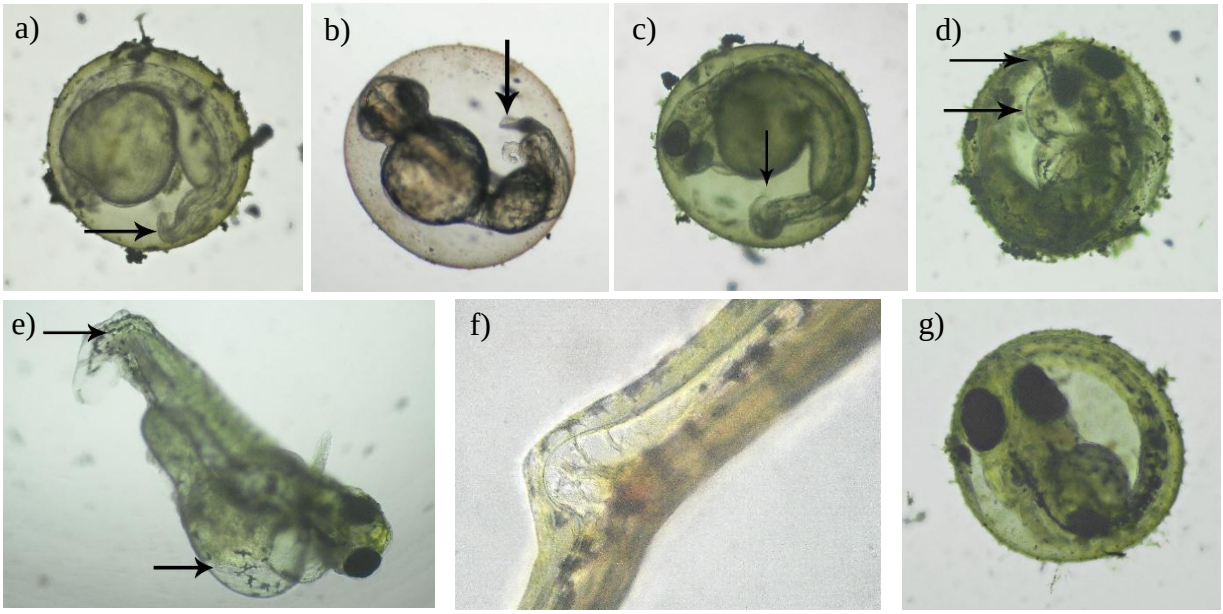

Fig. 66: Embryos of *Danio rerio* exposed to sediment extracts from Jordan 1 and 2 (J 1, 2). Flocculated extract was accumulated at the chorion at higher concentrations. **a)** 48 hpf at 50 mg SEQ/ml J 2, tail malformation, **b)** 72 hpf at 50 mg SEQ/ml J 2, tail malformation, **c)** 48 hpf at 25 mg SEQ/ml J 2, tail malformation, **d)** 96 hpf at 12.5 mg SEQ/ml J 2, lack of hatching, edema and tail malformation, **e)** 96 hpf at 25 mg SEQ/ml J 2, tail malformation, edema and reduced heartbeat, **f)** 96 hpf at 25 mg SEQ/ml J 2, severe malformation of the notochord/spine **g)** 96 hpf at 12.5 mg SEQ/ml J 1, no hatching success.

Although extracts of the sampling site Jordan 3 did not result in a sufficient number of lethal effects that allowed a determination of LC_{50} values, an EC_{50} of 28.7 mg SEQ/ml after 96 hpf (Tab. 31) could be determined, since reduced hatching success and spine and tail malformations

were observed in some embryos at all concentrations except for 1.0 mg SEQ/ml. Teratogenicity could therefore be assigned to this sampling site. Only very few embryos showed any sublethal effect after exposure to extracts from the sampling sites Jordan 4 and 5, which are not to be mentioned further as they did allow the determination of LC_{50} or EC_{50} values.

3.8.2 King Abdullah Canal

Neither of the sediment extracts from the King Abdullah Canal did show significant lethal effects in the Embryo Toxicity Test with *Danio reri*o that would allow for the determination of LC_{50} values. However, extracts from KAC 1 led to a reduced hatching rate of at concentrations of 12.5, 25, 37.5 and 50 mg SEQ/ml. At 50 mg SEQ/ml all embryos in the three replicates except for one showed this effect. A dose-dependency was evident (Fig. 67). Furthermore, few embryos showed reduced blood circulation and reduced heartbeat rate. An EC_{50} of 19.1 mg SEQ/ml could thus be determined after 96 hpf.

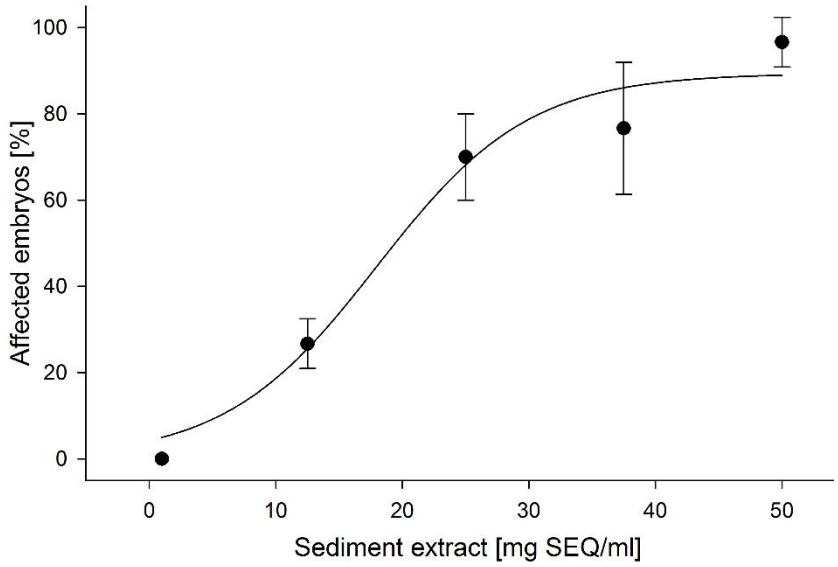

Fig. 67: Effects of sediment extracts from KAC 1 on hatching success of zebrafish embryos.

3.8.3 Wadi Mujib

Acute toxicity as determined in the fish embryo test with *Danio rerio* could only be observed for the sampling site Mujib 2 at the outlet of Mujib Dam. It was, however, with an LC_{50} of 21 mg SEQ/ml after 48 hpf and 9.9 mg SEQ/ml after 96 hpf (Tab. 32) the most toxic sampling site of this thesis and was rated strongly acute toxic according to Keiter et al. (2009). 24 hpf 100 % and 100 % of the embryos were lethally affected at 50 and 37.5 mg SEQ/ml, respectively (Fig. 68). Besides 36 % of coagulation, 30 % of the embryos showed retarded somite stage and

non-detachment of tails at 25 mg SEQ/ml, which led to coagulation or no heartbeat at 48 hpf. One embryo lacked tail detachment at 12.5 mg SEQ/ml and showed no heartbeat at 48 hpf. No lethal or sublethal effects were recorded for 1.0 mg SEQ/ml at any time during the experiment. Mortality rate rose to 80 and 100 % at 25 mg SEQ/ml after 48 and 72 hpf (Fig. 68), of which 50 % were coagulated (Fig. 69 a) and 50 % ´had no heartbeat (Fig. 69 c). At 12.5 mg SEQ/ml 17 % were coagulated and 50 % lacked heartbeat at 96hpf. Of the not lethally affected embryos at 25 and 12.5 mg SEQ/ml, 100 % showed effects on the cardiovascular system as reduced heartbeat rate and blood circulation after 48, 72 and 96 hpf. Furthermore, pericardial or yolk sack edemata or a combination of both were recorded for all individuals (Fig. 69 b - d). 40% of the remaining embryos at 12.5 mg SEQ/ml did not hatch after 96 hpf. Moreover, teratogenic effects in terms of spine/notochord and tail malformation were observed in 40 and 100 % (Fig. 69 c - e).

Tab. 32: Toxicity data of the fish embryo test with *Danio rerio* for sediment extracts of Wadi Mujib in mg SEQ/ml.

	LC_{50}		EC_{50}		LOEC	NOEC 96
	96 hpf	48 hpf	96 hpf	48 hpf	96 hpf	hpf
Mujib 1, Intlet Mujib Dam	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Mujib 2, Outlet Mujib Dam	9.9	21.0	1.4	2.6	12.5	1.0
Mujib 3, Mouth Dead Sea	n.d.	n.d.	34.1	n.d.	25	12.5

n.d.: not detetctable within the concentration range tested since the amount of effects observed did not allow for a detection of the corresponding values.

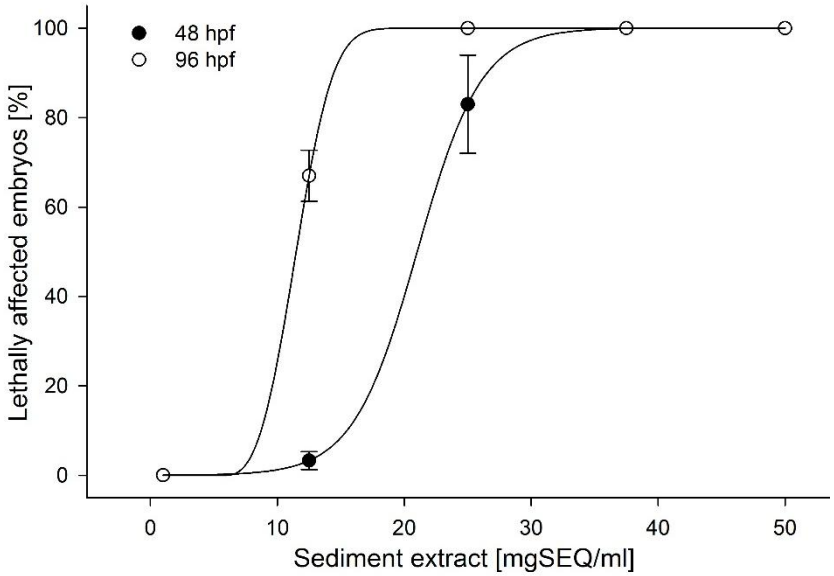

Fig. 68: Acute toxicity of sediment extracts from Mujib 2 to zebrafish embryos at 48 and 96 hpf.

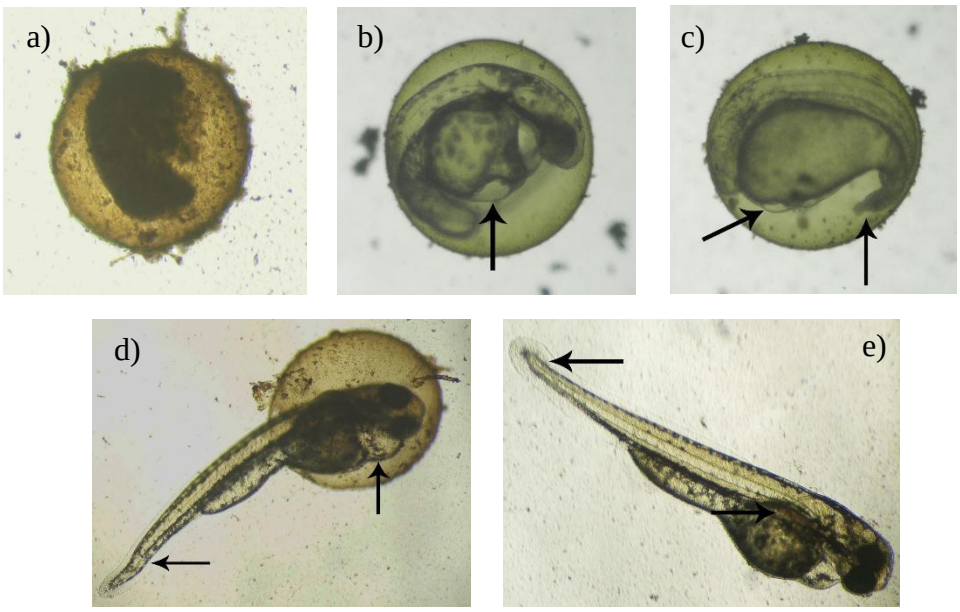

Fig. 69: Effects on embryos of *Danio rerio* exposed to sediment extracts of Mujib 2. **a)** 72 hpf at 25 mg SEQ/ml, coagulation, **b)** 48 hpf at 12.5 mg SEQ/ml, yolk sack edema, **c)** 48 hpf at 25 mg SEQ/ml, tail malformation, yolk sack edema and lack of heartbeat, **d)** 72 hpf at 12.5 mg SEQ/ml, tail malformation, edema and reduced heartbeat, **e)** 72 hpf at 12.5 mg SEQ/ml, tail malformation.

As many embryos did not hatch at 96 hpf after treatment with 50, 37.5 and 25 mg SEQ/ml from Mujib 3, and EC_{50} of 34.1 mg SEQ/ml could be determined for this site (Tab. 32). Apart from that, no other effect was to be observed.

3.8.4 Yarmouk River

In the embryo toxicity test with *Danio rerio*, no lethal effects could be found for the sediment extracts within the concentration range of 50 to 1 mg SEQ/ml. Embryonic development proceeded normally, except for the sampling site Yarmouk 2 at Wadi Raqab. In all replicates, hatching of embryos could not be observed at all concentration except for 1.0 mg SEQ/ml for between 70 and 100 % of individuals. Therefore, an EC_{50} value after 96 hrs of exposure could be determined at 6.0 mg SEQ/ml, assigning a still highly toxic potential to this site. In rare cases, effects on the cardiovascular system and edemata were recorded.

3.8.5 Zarqa River

Sediment extracts of the sampling sites Zarqa 2, 4 and 5 did not lead to lethally affected embryos. Only few sublethal effects as edemata were recorded for Zarqa 2 and 4. The sampling sites Zarqa 1 and Zarqa 6 showed acute toxicity as LC_{50}s were determined at 43.1 and 18.5 mg SEQ/ml at 48 hpf and at 48.5 and 19.8 mg SEQ/ml at 96 hpf, respectively (Tab. 33).

Thus, the correlation of LC_{50} values after these two evaluation intervals was good (Fig. 70). 24 hpf, 55 and 23 % of the embryos were lethally affected either with coagulation (Fig. 72 a) or non-detachment of tail (Fig. 72 b) without spontaneous movement at 50 and 37.5 mg SEQ/ml of Zarqa 1. The cardiovascular system of all remaining individuals was affected at the later evaluation intervals at 50 mg SEQ/ml and of 75 % at 37.5 mg SEQ/ml (Fig. 72 d). At 25 mg SEQ/ml, coagulation was only recorded once. Severe edemata were recorded for this concentration in 25 to 35 % of the embryos at 48 and 96 hpf, respectively (Fig. 72 c - g). In 30 % of the cases, this was combined with a reduced heartbeat rate and blood circulation. Some embryos at 50 and 37.5 mg SEQ/ml also lacked pigmentation (Fig. 72 c). Teratogenic effects (Fig. 72 e - g) could be observed at the three highest concentrations and is displayed in Fig. 71. 96 hpf, no success of hatching was detected 67, 65, 35 and 10 % of embryos at 50, 37.5, 25 and 12.5 mg SEQ/ml, respectively. Apart from that, no other sublethal effects were recorded for 12.5 mg SEQ/ml of Zarqa 1, and no effect at all was recorded at 1.0 mg SEQ/ml. The EC_{50} values were determined at 23.8 mg SEQ/ml at 48 hpf and 18.2 mg SEQ/ml at 96 hpf (Tab. 33)

Tab. 33: Toxicity data of the fish embryo test with *Danio rerio* for Zarqa River in mg SEQ/ml.

	LC_{50}		EC_{50}		LOEC	NOEC
	96 hpf	48 hpf	96 hpf	48 hpf	96 hpf	96 hpf
Zarqa River 1, Khirbet As Samra	43.1	48.5	18.2	23.8	12.5	1.0
Zarqa River 2, Confluence Zarqa	n.d.	n.d.	n.d.	n.d.	50	37.5
Zarqa River 3, Seil Jerash	n.d.	n.d.	22.1	34.4	12.5	1.0
Zarqa River 4, Jerash Bridge	n.d.	n.d.	n.d.	n.d.	50	37.5
Zarqa River 5, Inlet King Talal Dam	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Zarqa River 6, Outlet King Talal Dam	18.5	19.8	16.2	17.4	12.5	1.0

n.d.: Not detectable within the concentration range tested since the amount of effects observed did not allow for a detection of the corresponding values.

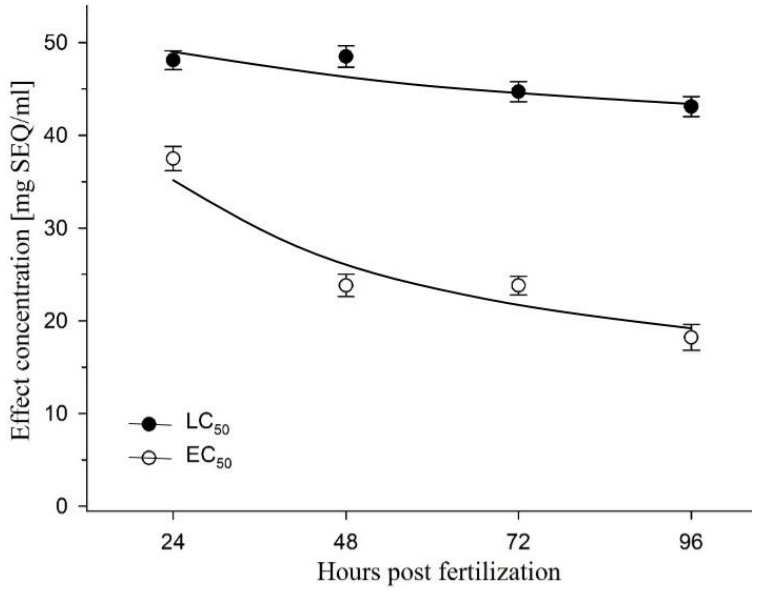

Fig. 70: Correlation and development of LC_{50} and EC_{50} values for the sediment extracts from Zarqa 1.

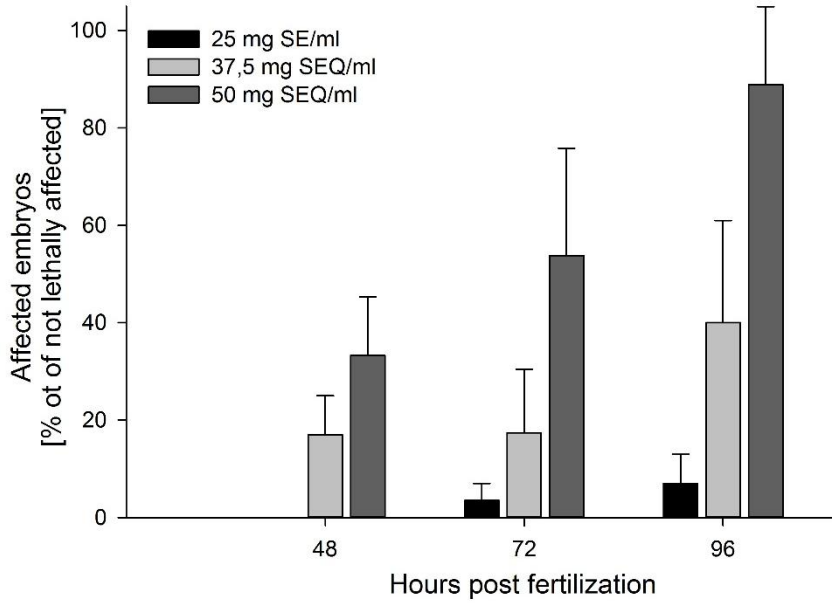

Fig. 71: Sublethal effects of sediment extract from Zarqa 1 on spine/notochord and tail malformations.

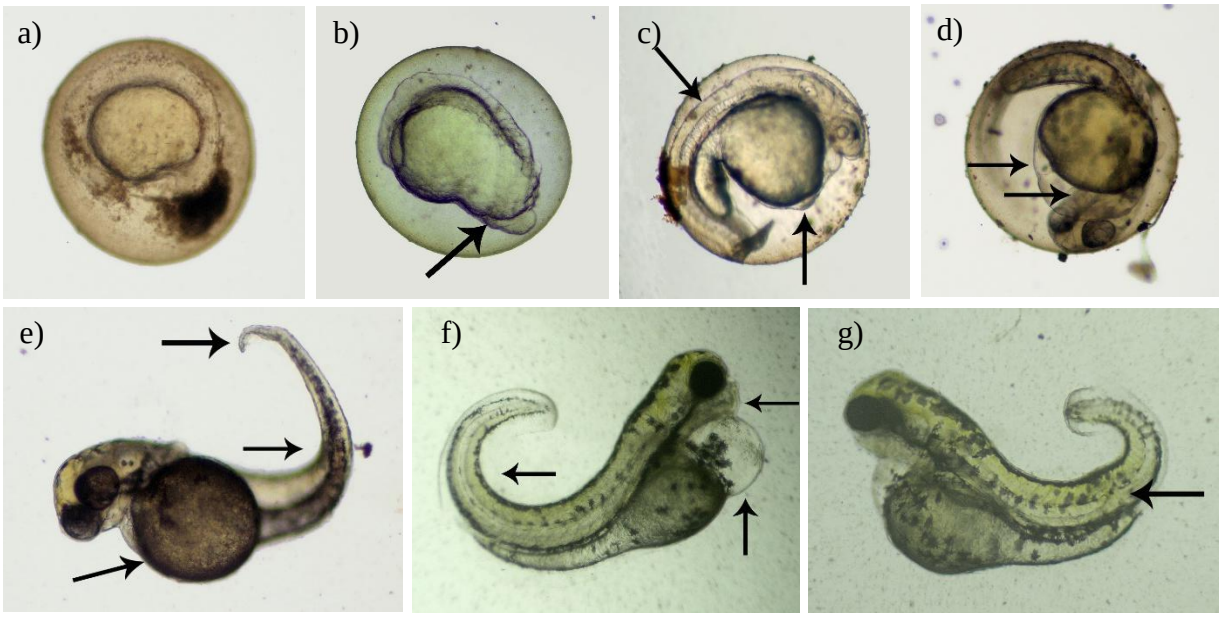

Fig. 72: Lethal and sublethal effects of sediment extracts from Zarqa 1 on zebrafish embryos. **a)** 37 mg SEQ/ml at 24 hpf, beginning coagulation, **b)** 50 mg SEQ/ml at 24 hpf, non-detachment of tail, **c)** 50 mg SEQ/ml at 48 hpf, yolk sack edema and lack of pigmentation, **d)** 37.5 mg SEQ/ml at 48 hpf, pericardial edema and reduced heartbeat rate, **e)** 50 mg SEQ/ml at 72 hpf, spine/notochord, tail and yolk malformation, **f)** 37.5 mg SEQ/ml at 96 hpf, malformation of spine and mouth and pericardial edema, **g)** 50 mg SEQ/ml at 96 hpf, malformation of spine/notochord.

Out of the embryos exposed to sediment extracts from Zarqa 6, 100 % were coagulated at 24 hpf at the two highest concentrations and 60 % at 25 mg SEQ/ml. Here, an additional 10 % showed no detachment of the tail. Therefore, the LC_{50} values resulted in 19.8 mg SEQ/ml at 48 hpf and 18.5 mg SEQ/ml at 96 hpfb (Tab. 33). Sublethal effects on the cardiovascular system in combination with edemata were found in 66 % of the not lethally affected embryos at 25 mg SEQ/ml at 48 hpf (Fig. 73). Two embryos managed a full recovery at 96 hpf. Apart from one coagulated and two unhatched embryos, no effects were recorded at 12.5 mg SEQ/ml, and none at all for 1.0 mg SEQ/ml. Thus; EC_{50} values were determined at 17.4 mg SEQ/ml at 48 hpf and at 16.2 mg SEQ/ml at 96 hpf.

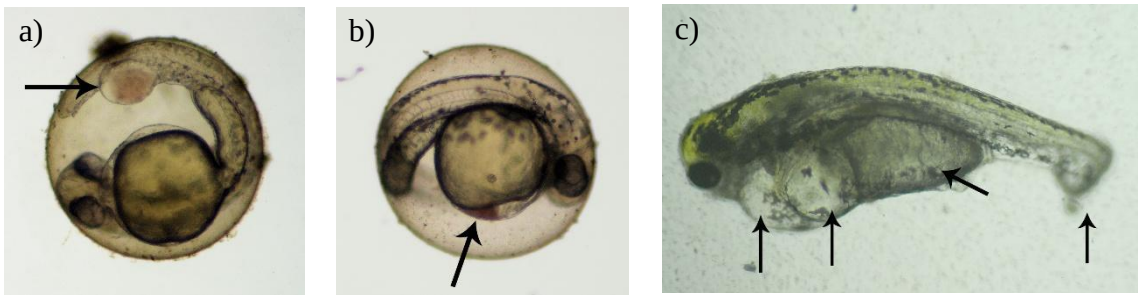

Fig. 73: Sublethal effects of 25 mg SEQ/ml from Zarqa 6 on zebrafish embryos. **a)** at 48 hpf, tail edema, **b)** at 48 hpf, yolk sack edema, **c)** at 96 hpf, yolk sack and pericardial edema, yolk and tail malformation.

As the lethality did not exceed 30 %, a determination of LC_{50} values was not possible within the concentration range tested for the sampling site Zarqa 3. However, EC_{50} values of 34.4 and 22.1 mg SEQ/ml were recorded at 96 and 48 hpf since there were major effects on the cardiovascular system and edemata formation. For 63, 31 and 14 % of the not lethally affected individuals reduce heartbeat rate and blood circulation was identified at 50, 37.5 and 25 mg SEQ/ml at 48 hpf. Three embryos managed to recover from this cardiovascular deficiency after 72 hrs and another one after 96 hrs. Development of this effect is shown in Fig. 74. Furthermore, some embryos had general malformations or malformations of the spine/notochord or tail or a combination of this. Since between 7 and 60 % of the embryos did not hatch at 96 hpf at all concentration except 1.0 mg SEQ/ml, the EC_{50} increases at this evaluation point.

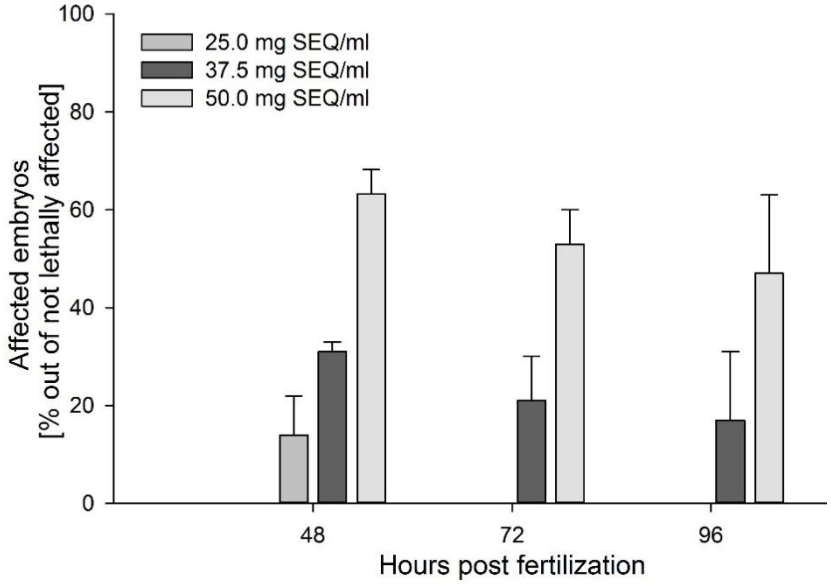

Fig. 74: Sublethal effects on the cardiovascular system of zebrafish embryos after exposure to sediment extracts from Zarqa 3

3.8.6 Comprehensive presentation of effects in the fish embryo test

On the basis of the threshold values for fish embryos toxicity developed by Keiter et al. (Keiter et al. 2009b), all samples that allowed a detection of LC_{50} values between a concentrations of 10 to 28 mg SEQ/ml were classified as moderately toxic in terms of embryo toxicity on the zebrafish. Mujib 2, however, was rated strongly toxic. Extracts that led to values higher than 28 mg SEQ/ml were considered not embryo toxic. The ranking of the relevant sampling sites in terms of LC_{50} values at 48 and 96 hpf is shown in Fig. 75. An equivalent ranking for EC_{50} values is displayed in Fig. 76, which accounts for more sampling sites as the occurrence of

sublethal effects did often allow for a determination of EC_{50} values although no LC_{50} values could be determined.

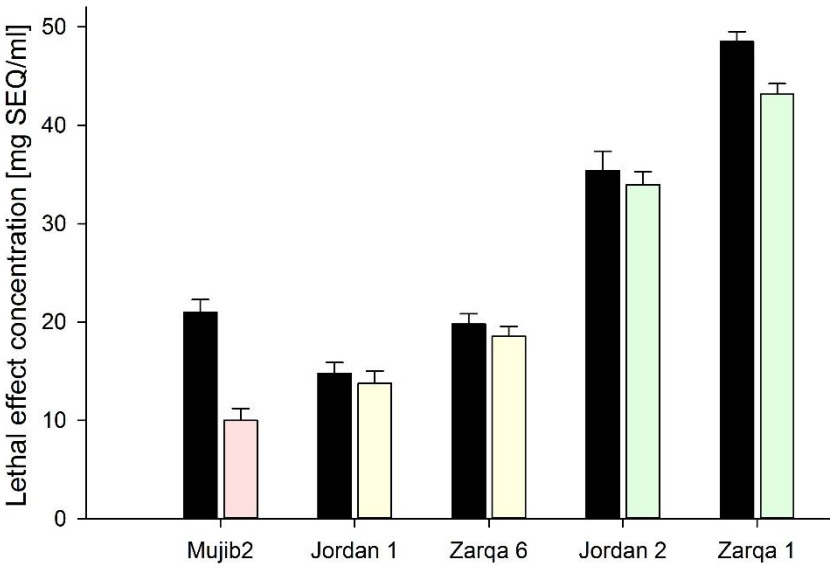

Fig. 75: Ranking of the five Jordanian sampling sites that showed acute toxicity in the zebrafish embryo test with *Danio rerio* according to LC_{50} values at 96 hpf. LC_{50} values at 48 hpf are included for better traceability. Colors indicate toxicity degree according to Keiter et al. (2009); red: strongly toxic, yellow: moderately toxic, green: non toxic.

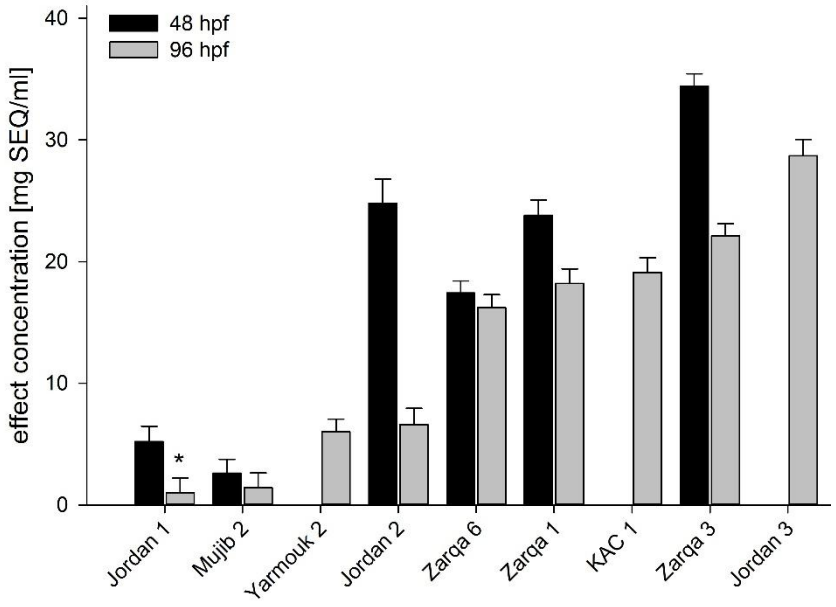

Fig. 76: Ranking of Jordanian sampling sites that showed teratogenic effects in the zebrafish embryo test with *Danio rerio* according to EC_{50} values at 96 hpf. EC_{50} values at 48 hpf are included where possible for better traceability. *: As all test concentrations showed effects of more than 50 %, the value had to be less than 1 mg SEQ/ml.

4. Comprehensive evaluation of bioassays

Evaluation of environmental samples such as sediments requires a certain amount of bioassays to guarantee comprehensive conclusions on their quality. The following chapters give a broad characterization and weight-of-evidence-based classification of the examined Jordanian watercourses through a thorough evaluation of the results of the different bioassays and test systems used in this study. Furthermore, recent environmental and chemical studies on the investigated area will be taken into account for a better understanding and potential explanation of specific effects as determined in the various bioassays. An overview of all bioassay results is given in Tab. 34.

Tab. 34: Results in terms of the different endpoints applied for bioassays used in this study. Colors indicate toxicity level according to Keiter et al. (2009b) and as explained in Ch.2.6: red = strong toxicity, yellow = moderate toxicity, green = minor to no toxicity.

Sampling Site		Bioassay				
		Neutral red assay NR_{50} [mgSEQ/ml]	FET LC_{50} [mg SEQ/ml]	Comet assay [CDI]	Micronucleus assay [IF]	EROD assay [pmol BNF/mg SEQ/ml]
Jordan	1	34.1	13.8	1.3	3.3*	0.21
	2	87.6	33.9	0.5	1.3	0.03
	3	95.9	n.d.	0.3	1.6	0.05
	4	105.8	n.d.	0.3	3.4*	0.09
	5	135.1	n.d.	0.2	1.3	0.04
King Abdullah Canal	1	45.4	n.d.	1.0	2.5*	0.06
	2	n.d.	n.d.	0.2	3.2*	0.01
Wadi Mujib	1	144.7	n.d.	0.2	1.9*	0.02
	2	16.4	9.9	6.1	3.5*	0.05
	3	n.d.	n.d.	0.1	2.4*	0.01
Yarmouk River	1	80.0	n.d.	0.6	4.1*	0.16
	2	46.5	n.d.	2.1	1.6	0.19
	3	126.9	n.d.	0.4	1.5	0.09
	4	51.5	n.d.	1.1	2.4*	0.05
Zarqa River	1	16.5	48.5	1.7	3.9*	0.12
	2	38.1	n.d.	0.8	1.8	0.11
	3	39.3	n.d.	2.1	2.4	0.17
	4	79.8	n.d.	1.2	3.1*	0.15
	5	160.7	n.d.	0.3	3.5*	0.06
	6	75.8	19.8	0.6	3.1*	0.14

N.d.: not detectable within the concentration range tested. * significantly different from negative control.

4.1.1 Comparison of acute cytotoxicity in the neutral red assay with RTL-W1 cells and acute toxicity in the fish embryo test with zebrafish

In recent years, acute toxicity tests with fish have aroused considerable ethical concern, since they are conducted with juvenile or adult animals. Following the European Directive 2010/63/EU, the principle of the "3Rs" (replacement, reduction and refinement) originally described Russell and Burch (1959) has again found its way into ecotoxicological assessment and regulation. Two alternatives to acute toxicity testing with adult fish were used in this study: the neutral red assay (NRA) with RTL-W1 cells and the fish embryo test with zebrafish (FET). While the later has recently been validated and accepted as an OECD guideline (OECD TG 236) and since it is generally accepted that fish possess necessary characteristics for the use in detection of hazardous environmental pollutants such as comparability to higher vertebrates (Embry et al. 2010), there is still no validation available for the use of fish cell lines in toxicity assessment, although this has repeatedly been advocated (Fent 2001a, Schirmer 2006, Tollefsen et al. 2006). One of the remaining problems of toxicity assessment using fish cell lines is the heterogeneity in terms of data correlation with acute fish toxicity. Although many studies have proven a rather good correlation of LC_{50} values not only for testing of monosubstances (Ahne 1985, Segner 1993, 2004), but also for testing of effluent waters (Castaño et al. 2000), many studies have also found striking deviations, since cell lines have a tendency to react less sensitively to pure substances (Bols et al. 1985, Castaño et al. 1996, Lange et al. 1995), and variable results have been obtained for the testing of effluents (Ahne 1985, Rusche and Kohlpoth 1993).

Results obtained in this study did not show a good correlation between NR_{50} values derived from the NRA with RTL-W1 cells and LC_{50} values derived from the FET with zebrafish (Fig. 77, Tab. 34). Only in the case of the sampling site Mujib 2, both tests indicated strong toxicity for the sediment extracts (threshold values according to Keiter et al. 2009b, Ch. 2.6). At Zarqa 1, however, only the NRA indicated strongly toxic effects, whereas in the FET assigned no toxicity to the extracts. In the cases of Jordan 1 and Zarqa 6, both tests identified a moderately toxic potential, and for Jordan 2, 3, 4 and 5, KAC 2, Mujib 1 and 2, Yarmouk 3 and Zarqa 5, little to no toxicity was determined. For the remaining seven sample sites, the NRA assigned moderate toxicity to the extracts, whereas no to little toxicity was found in the FET. Thus, toxic potential of the extracts was generally higher in the NRA than in the FET. As the endpoints used for the determination of toxicity levels and ranking of the sites were only those that had lethal effects on the embryos, an additional correlation between NR_{50} values of the NRA and EC_{50} values of the FET was also tested, but no correlation was found here either (Fig. 77).

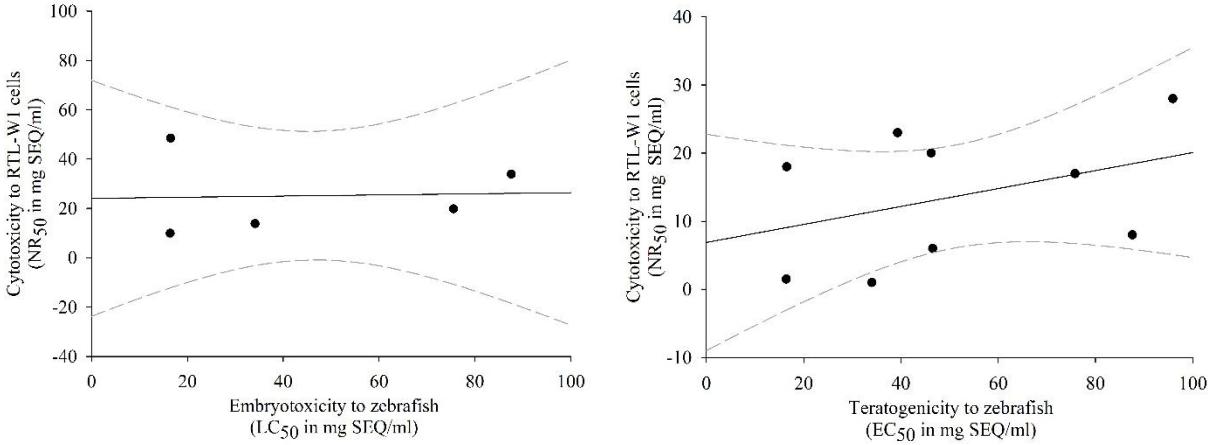

Fig. 77: Regression and correlation analysis between results for acute cytotoxicity in the neutral red assay with RTL-W1 cells and acute toxicity (left) and teratogenicity (right) in the fish embryo test with zebrafish. Dashed lines indicate 95 % confidence intervals. No correlation was detected after Spearman: $p > 0.05$.

An extensive review by Schirmer (2006) has identified a possible reason for deviations between results obtained from cell lines and acute fish toxicity tests (it should be noted, that the studies cited in the review examined correlations between cell line tests and acute fish toxicity with adult fish and not with embryos as in the present study): Cell cultures naturally offer only a limited number of target sites for hazards compared to whole organisms. However, this rather explains deviations in terms of lower sensitivity of cell cultures. In the present study, deviation was rather the other way around. The chorion has long been thought to present a protective uptake barrier especially to larger or highly lipophilic chemicals and could, therefore, prevent the embryo from being directly exposed. Electron microscopy has shown that the chorion has pores with a size of 0.17 μm^2 (Cheng 2007), and, indeed, some studies involving dechorionation and fluorescence microscopy have shown that the threshold for a nearly free passage through the chorion is approx.. 3,000 Da, and that side chains and electric charge can have an influence on the passage (Henn and Braunbeck 2011, Kais et al. 2013). Due to lack of chemical analysis, it cannot be clearly stated that such characteristics do also apply to the sediment extracts tested in this study; however, this is quite likely since an accumulation of flocculated extract was in many cases visible at the outside of the chorion as for example with Jordan 1 (Ch. 3.8.1). Furthermore, the concentrations of the solvent DMSO was not identical in the two test systems: 1 % in the NRA to 0.25 % in the FET, which was chosen due to a study by Kais et al. (2013), which proved an impact of DMSO on the chorion's permeability. Thus, solubility of the extracts and possibly also the uptake into the cells might have been facilitated in the NRA when compared to the FET and might account for the higher sensitivity. Since lack of hatching was very often recorded as a sublethal effect in the FET, e.g. for the sites Jordan 1 -3, KAC 1 and Yarmouk 2, the prolonged exposure duration to 96 h did not help to exclude the role of the chorion as barrier for sediment extracts.

Since recent studies have proven that zebrafish embryos already possess the capacity of bioactivation *via* the cytochrome P450 enzyme system (Otte et al. 2010, Weigt et al. 2011), and since the results from the EROD assay did not indicate a correlation between less toxicity in the FET and the occurrence of proteratogens either, it is not likely that a lack of bioactivation of chemicals in the samples is responsible for the deviations in results. However, it cannot be completely ruled out as a possible reason. Further studies with exogenous activation might be helpful in toxicity assessment of complex environmental samples.

Since no relationship in the sense of "if - then" or "if not - then not" could be established in this study between the NRA and FET, it is suggested that complex environmental samples address a multitude of pathways and target sites and are rather unpredictable in their mode of action. Scholz et al. (2014) also warn to extrapolate toxicity from one test to another, as they did, e.g., only find a slight correlation between embryo toxicity in the FET with zebrafish and acute mammalian toxicity. As is also postulated by Yang et al (2010), a variety of tests has to be applied to generate a full overview of their toxicological potential.

Whereas the NRA protocol does only allow a distinction between intact or dead cells, the FET also offers the opportunity to account for certain sublethal effects which might also have relevance for population development and ecology. A variety of sublethal effects were recorded in zebrafish embryos after exposure to sediment extracts of Jordanian surface waters. For instance, severe malformations of the spine/notochord and the tail were recorded for Jordan 2 and 3, Mujib 2 and Zarqa 1 and 3. Von Westernhagen (1988) found that gross malformations such as skeletal deformations are caused by numerous types of pollutants and are not pollutant-specific. However, more recent studies suggest that polycyclic aromatic hydrocarbons (PAHs) can disturb a multitude of processes during early developmental stages of fish and, thus, also lead to malformations (Barron et al. 2004). Especially a mixture of different PAHs is known to produce spinal curvature which was described as prominent effects for sediment extracts from Zarqa 1 (Incardona et al. 2004). Hardly any information could be obtained about PAH contamination of Jordanian rivers and their sediments. Tahboub et al. (2014) found low concentrations of acenaphthalene (0.025 µg/L), naphthalene (0.055 µg/L), phenanthrene (0.015 µg/L) and anthracene (0.008 µg/L) in water samples of the Zarqa River and King Talal Dam with the PAH concentrations being all within the guideline of the European Directive 2008/105 (European-Commission 2008). These finding are in accordance with results presented by Batarseh (2003) in his doctoral thesis. He was able to detect all 16 PAHs listed by the US EPA in various sediment samples from the Zarqa River which were taken very close to the sampling sites of this study. The total concentrations ranged from 69 to 234 µg/kg dry weight and were, thus, rather low compared to other studies as for example of the harbor of Braunschweig, Germany (Kolb 1994). Among the most frequently found PAHs were phenanthrene, pyrene, napthtalene, fluoranthene, benzo[b]flouranthene and chrysene. It might,

therefore, be likely that PAHs are at least partly responsible for the teratogenic effects recorded in this study, especially for those of the Zarqa River sediments.

Apart from malformations and interferences with the hatching process, the cardiovascular system of zebrafish was another sphere of action addressed by Jordanian sediment extracts. As recent studies suggest, PAHs play an important role in the causation of effects like yolk sac or pericardial edemata and disruption of cardiac function (Barron et al. 2004, Billiard et al. 2008, Billiard et al. 2006). However, it is most unlikely that only one or two groups of organic pollutants are responsible for the various effects of different sampling sites. It is far more likely that a mixture of toxins added to a mixture of effects and toxicity (Karlsson et al. 2008, Mayer and Reichenberg 2006). Tahboub et al. (2014) also found high concentrations of phenol (18.5 µg/L) which suggest also the occurrence of phenolic substances Reports about the toxicological effects of pure phenol toward zebrafish is rare since it is mostly phenolic substances that induce toxicity. LC_{50} values for acute toxicity with adult zebrafish were set at 0.16 mg/L. As the concentration of phenol is believed to be even higher in the sediments due to bad water solubility, and Tahboub et al. (2014) describe a decrease of concentration with increasing distance from the water treatment plant Khirbet As-Samra and then an increase at in the deep parts of King Talal dam, the findings correlate very well with the results from the FET and NRA in this study (Tab. 34).

Tiehm et al. (2011) were, furthermore, able to detect rather high concentrations – if compared to those found in the USA and Europe (van den Brandhof and Montforts 2010) − of the antiepileptic drug carbamazepine (240 – 1600 ng/L) and the pain reliever diclofenac (240 ng/L) in the waters of the Jordan River. Since both substances are badly soluble in water, their concentrations in sediments may be expected to be even much higher. Diclofenac was found to have an LC_{50} of 6.11 mg/L after 144 hrs an zebrafish embryos (Praskova et al. 2011) and an EC_{50} of 5.3 mg/L after 72 hrs including sublethal effects on tail malformation and edemata formation (van den Brandhof and Montforts 2010) as were also observed for the first three sampling sites at Jordan River. As Weigt et al (2011) were able to show in their study, carbamazepine leads to spine/notochord and tail malformations in zebrafish embryos already at concentrations of 31.25 µM with an EC_{50} of 222 µM (≈ 52,5 mg/L) after 72 hrs of exposure. A study by van den Brandhof and Montforts (2010) confirms these findings with an EC_{50} of 86.5 mg/L. Apart from malformations, reduced hatching success was furthermore recorded as sublethal affect and could, thus, also account for the effects observed for Jordan 1, 2 and 3.

Pollution with heavy metals is a major problem in Jordan. Several studies have detected elevated concentrations as would result from the geological structure for all areas investigated in this thesis. Besides lead, especially cadmium and mercury are characterized by persistence and toxicity. They arise from a variety of anthropogenic activities such as industrial emissions and agricultural fertilization and are distributed *via* the atmosphere (Umwelt-Bundesamt 2013).

It is, therefore, not surprising that they were found in all watercourses, especially in those that either have a big catchment area for run-off water (Wadi Mujib, Zarqa River, Yarmouk River) or many tributaries (Jordan River). Among the most frequently detected and highest concentrated heavy metals were lead, cadmium and mercury. Lead is especially enriched in the sediments of the Zarqa River (Abderahman and Abu-Rukah 2006b, Ghrefat 2012) with a tendency to decrease with further distance from the waste water treatment plant Khirbet As-Samra. Cadmium was found in the sediments or the surrounding soils of all surface waters (Banat 2005, Ghrefat 2012) with enrichment factors of up to 100 for the Yarmouk (Abu-Rukah and Ghrefat 2001, Batayneh 2010), up to 35 for Wadi Mujib (Banat and Howari 2003, Manasreh 2010), and up to 15 for the Jordan River (Howari and Banat 2001), when compared to natural occurrences. Although embryo toxicity and teratogenicity (Cheng et al. 2000, Dave 1985, Dave and Xiu 1991, Meinelt et al. 2001) and also acute toxicity on *Danio malabaricus* embryos (Saxena 1982) has been proven for cadmium, mercury and lead, no real correlation could be found between concentrations or concentration differences found in literature and the results from neither the cytotoxicity test with RTL-W1 cells nor the FET with zebrafish. However, it might as well be that the heavy metals found in Jordanian surface waters add to or influence the toxicity of various sampling sites.

According to several studies (Billsson et al. 1998, Westerlund et al. 2000), polychlorinated biphenyls (PCBs) are well known to have severe influence on the hatching process. However, information on pollution of Jordanian surface waters with PCBs is scarce. Tarawneh at al. (2012) did not find any PCBs during their study in soils in the vicinity of Zarqa River. In his doctoral thesis, Batarseh (2003) was able to detect 6 PCB congeners in the sediments of the Zarqa River. The predominant PCB was found to be PCB 28, and the total concentration ranged from 2 to 8 µg/kg dry weight at the same sampling sites as investigated in this study. It has, furthermore, been described for sludge from several water treatment plants (Batarseh 2011), and it can, therefore, be assumed to be also of environmental concern in waste water effluent streams as the Zarqa River, Yarmouk or Jordan River.

4.1.2 Genotoxicity

To date, there is not yet an overall consensus on the ideal bioassay for the assessment of sediment genotoxicity (Chen and White 2004). Two different bioassays were applied in this study to obtain information on the genotoxic potential of sediment extracts from Jordanian surface waters: the comet assay (CA) with the permanent fish cell line RTL-W1 and the micronucleus assays (MNA) with mammalian V79 cells. The applicability of the CA has been proven in many studies (Chapman et al. 2002, Devaux 1997, Klaude et al. 1996, Mitchelmore and Chipman 1998, Møller 2006, Nehls and Segner 2001), also precisely for the use with RTL-W1 cells (Boettcher et al. 2010, Braunbeck et al. 2009, Kosmehl et al. 2004, Nehls and Segner

2005, Rocha et al. 2009, Seitz et al. 2008). However, lack of standardization of protocols for the comet assay may lead to or at least explain some variations between laboratories or various studies (Collins et al. 2008). The micronucleus assay with V79cells, on the other hand, has already been validated as OECD guideline 487 (2010).

As can be seen in Tab. 34, both bioassays that screened for genotoxicity were the most sensitive ones compared to the other specific (NRA and EROD) and unspecific tests (FET). Except for Jordan 5, KAC 2 and Mujib 1 and 3, all of the samples indicated genotoxic effects in terms of the comet assay with RTL-W1 cells and only 7 out of 20 did not induce a micronuclei rate that was determined as moderately or strongly toxic according to the applied rating further explained in Ch. 2.6. This suggests a considerable contamination of Jordanian surface waters with genotoxic compounds. Other publications have also clearly proven genotoxic activity in sediments, and many have linked this to the presence of PAHs. As was already stated above, low concentrations of PAHs were found in the water and sediments of Zarqa River (Batarseh 2003, Tarawneh 2012). However, other components may play a role in inducing mutagenicity as well (Chen and White 2004). Mutagenicity of municipal and industrial wastewater has been determined in several studies (Gauthier et al. 1993, Malik and Ahmad 1995, Meier et al. 1987, Rappaport et al. 1979), and could, thus, be also the source of contamination for Zarqa River, Yarmouk River and the lower parts of Jordan River (Ministry for Water and Irrigation 2009). Non-point sources such as urban runoff during flash floods are also known to be a possible source to enter particle-bound combustion by-products as for example from the Jordan Petroleum Refinery (Tarawneh 2012) into watercourses, which could especially be the case in Wadi Mujib and Yarmouk River as they have rather large watersheds (Ministry for Water and Irrigation 2009). The landfill Mafraq, where urban waste is being incinerated, is in the proximate vicinity of Zarqa River. Alawi et al.(1996) were able to detect very high concentrations of polychlorinated dibenzodioxins and polychlorinated dibenzofurans in the surroundings of this landfill. Both compound groups are known to exhibit a highly mutagenic potential (Mc Gregor 1998, Safe 1986). It cannot be excluded that run-off and fly ash does not contribute to the genotoxic results from Zarqa River, since five out of six samples showed strong genotoxicity. Furthermore, Batarseh (2003) was able to detect the PCBs 52, 101, 138, 153, 180 and most prominently PCB 28 in sediments samples of the Zarqa River. Although not all PCB congeners are rated mutagenic, the lower chlorinated PCBs such as PCB 28, 52 and 101 are known to induce single strand breaks detected in the CA in fish cells (Ludewig and Robertson 2013, Marabini 2011, Schilderman et al. 1999) and could, thus, account for the strong effects found for the CA with extracts of the Zarqa River. Since micronuclei and DNA strand breaks are also induced by phenolic compounds (Barale et al. 1990, Li et al. 2005, Robertson 1991, Yager et al. 1990), contamination of the Zarqa River and King Talal Dam with phenolics as determined by Tahboub et al. (2014) could also account for the genotoxic potential of the corresponding sites.

As has already been stated above, pollution with heavy metals is a major problem in Jordan. Especially lead, cadmium and mercury were found at elevated concentrations in all surface waters studied with the exception of KAC, where no literature was found despite thorough research (Abderahman and Abu-Rukah 2006b, Banat 2005, Banat and Howari 2003, Ghrefat 2012, Howari and Banat 2001, Manasreh 2010). Although some mechanisms of their toxicity remain unclear (Risso-de Faverney et al. 2001), mutagenicity has been proven for cadmium and mercury, since they, for example, have induced micronuclei in eel (Sanchez-Galan et al. 2001). Micronuclei induction of mercury has also been shown in carp (Al-Sabti 1994, Nepomuceno et al. 1997) and in different fish erythrocytes (Porto et al. 2005). An intensive study of heavy metals in the sediments of Yarmouk and Jordan River by Howari and Banat (2001) reveals a striking correlation between the contamination with mercury and results of the micronucleus test conducted in this study (Tab. 34). The highest induction factors were obtained for Jordan 1 (3.3) and 4 (3.4) and for Yarmouk 1 (4.1) and 4 (2.4). Likewise, the concentrations of mercury were among the highest, e.g. approx. 6.5 and 8.0 ppm for Jordan 1 and 4, and 7.5 and 5.0 for Yarmouk 1 and 4, respectively. For the other sampling sites, mercury concentration as well as induction factors were notably smaller, e.g. 1.8 ppm at Jordan 2 (IF 1.3) and 1.5 ppm at Yarmouk 3 (IF 1.5). It is, therefore, suggested that mercury is at least partly responsible for the micronuclei induction of sediment extracts from the Jordan and Yarmouk River, but probably also the other surface waters, and does also account for regional differences in the induction factors.

As has been mentioned above, genotoxic results were the most prominent findings of this study. However, when comparing the results of the two different test systems, only Jordan 1, Mujib 2, Yarmouk 1 and Zarqa 1 did react strongly positive in both test and were, thus, identified as hot spots in terms of genotoxic potential. The rest of the sites did not show concurrence in the results since in most of the cases the comet assay was more sensitive (Jordan 2, 3, 5, KAC 1, Yarmouk 2, 3,4, Zarqa 2, 3, 4, 6). Only at Jordan 4, KAC 2, Mujib 3 and Zarqa 5 did the MNA show a stronger response. A regression and correlation analysis is shown in Fig. 78 and confirms weak correlation between the two bioassays.

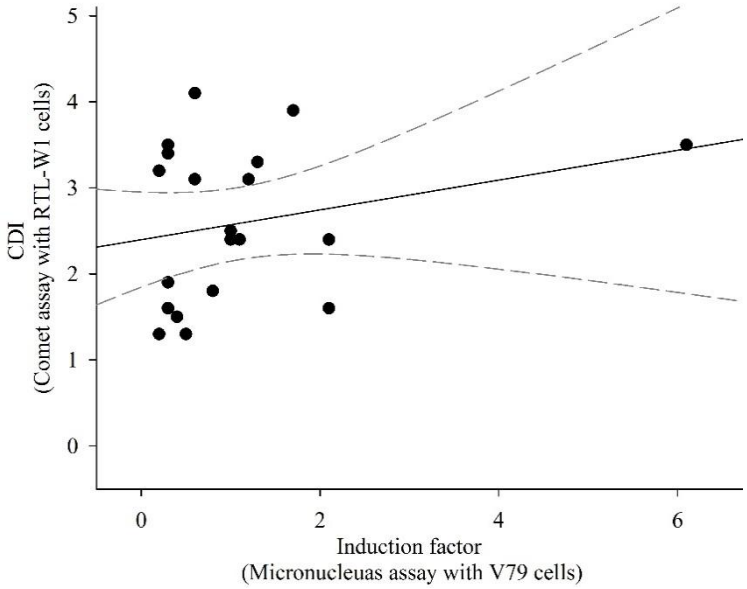

Fig. 78: Regression and correlation analysis of genotoxic effects as results from the comet assay with RTL-W1 cells (CDI) and the micronucleus assay with V79 cells (induction factor). Dashed lines symbolize 95 % confidence interval. No correlation was detected after Spearman: $p > 0.05$.

In most of the cases, the comet assay was more sensitive than the MNA. This was even more evident when comparing the LOEC values (Ch. 3.6).There might be different reasons for the deviation between these two assays. First of all, they detect different types of DNA damage. The comet assay detects single-strand breaks that might result from alkali-labile sites or arise during excision-repair of damaged DNA (Hartmann et al. 2001b) up to one break per 10^{10} Da (Gedik et al. 1992). These broken DNA strands are then released in the course of lysis and unwinding stages of the comet assay process and then produce the comet tail upon electrophoresis. The MNA detects formation of micronuclei during mitosis that might result from chromosomal breakage or from adverse effects on the spindle apparatus during the anaphase (Fenech 2000). Thus, the MNA does also account for aneugenic effects, whereas the comet assay does not (Dhawan 2009). This would suggest the MNA being more sensitive than the comet. However, the MNA is only capable of detecting established chromosomal aberrations, whereas the CA does also account for effects that could be repaired in intact cells through repair systems. Nevertheless, the present of aneugenic hazards in the samples that reacted more sensitive in the MNA could be a possible explanation for the deviation, although very few purely aneugens are known (Aardema et al. 1998). Furthermore, the MNA with V79 cells was conducted under external metabolic activation with Aroclor 1245 induced rat liver S9 fractions and might, therefore, respond to mutagens that need bioactivation. However, intrinsic bioactivation capacity of RTL-W1 cells for at least a broad range of mutagens in complex environmental samples has been proven (Bols et al. 1999, Kosmehl et al. 2004, Nehls and Segner 2001).

Secondly, two different cell lines were used: the permanent fish cell line RTL-W1 and the mammalian fibroblasts V79. This became necessary because the MNA with RTL-W1 cells did not prove to be suitable in this study (Ch. 3.5). Since fish cells in general typically express comparatively low amounts of DNA repair enzymes and a lower activity of the DNA polymerase (Walton 1983, 1984a, 1984b), they hold a potential to be generally more sensitive (Frenzilli et al. 2009, Kim and Hyun 2006, Nikoloff et al. 2012). Babich and Borenfreund (1991) have found that fish cell culture mainly respond to the same chemicals as mammalian cells such as benzo[*a*]-anthracene or benzo[*b*]fluoranthene, and other studies have also found fish cell lines to be more sensitive to, for example, sodium arsenate and arsenite (Raisuddin and Jha 2004), and also for the antibiotic trimethoprim (Papis 2011). Possible reasons suggested are a lower metabolic rate and low activities of DNA repair enzymes (Raisuddin and Jha 2004). These findings do also correlate with the results of this study, since in most cases the comet assay was at least as sensitive as the MNA. Nevertheless, especially rainbow trout cells are known to also possess repair mechanisms (Espina and Weis 1995, Walton 1983, 1984b, Wirgin and Waldman 1998).

Since genotoxic effects have high ecotoxicological and toxicological relevance as generation of DNA damage can be a predecessor to carcinogenesis also in humans (Møller 2006), it is crucial to neither underestimate nor overestimate results. The risk of overestimation does mainly apply to the comet assay, since several studies suggest that DNA fragments resulting from apoptosis instead from genotoxins could also be detected in the assay (Choucroun et al. 2001). However, other scientific experts have clearly excluded this possibility (Collins et al. 2008). Since terminal apoptotic and necrotic cells have a very low molecular weight of DNA, Vasquez and Tice (Tice 1999) suggest that the DNA of many of these cells is expected to be lost from the gels under the typical electrophoretic conditions. Furthermore, no correlation between ghost cells and apoptosis inducing chemicals has been found by Meintières at al. (2003). Another study also tested cytotoxic concentrations that did not result in positive effects in terms of DNA migration (Hartmann et al. 2001b). Since apoptotic cells are characterized by internucleosomal DNA fragmentation and appear as large fan-like cells with small head known as hedgehogs during examination (Frenzilli et al. 2009), they can easily be distinguished from real comets. Such hedgehog-like cells were at no time integrated into scoring of comets during this study. Furthermore, no concentration inducing more than 20 % of cytotoxicity was used for the comet assay, and since exposure duration was only 24 hrs compared to 48 hrs in the NRA, this source of possible overestimation of the comet assay can be suspended. Kirkland et al. (2007) suggest that false positive results may be generated through exogenous bioactivation *via* S9 mix, however, this does not account for the protocol of the comet assay with RTL-W1 cells used in this study. Furthermore, the comet assay without bioactivation was even more sensitive than the MNA with exogenous activation.

Overall, results of the present study suggest that the comet assay is a useful tool for screening of genotoxic potentials (Mitchelmore and Chipman 1998), especially because a good correlation of *in vitro* results with RTL-W1 cells and *in vivo* results from the European Barbel has been demonstrated for environmental samples (Boettcher et al. 2010). However, to prevent sediments from being underestimated in their genotoxic potential and to secure the screening for aneugenic compounds, the comet assay should be combined with a follow-up testing in the MNA (Kim and Hyun 2006).

4.1.3 EROD induction

The EROD assay was the most specific test applied in this study, as it determines the response of a gene-regulating system to the exposure of organic hazards (Ch. 3.7). Due to this high specificity, it is not surprising that the results of the EROD assay were found to be the least sensitive when compared to other more unspecific tests as the NRA or FET (Tab. 34). Because EROD expression is induced by highly persistent organic compounds such as (co)planar polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), polychlorinated dibenzodioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) and because many of those compounds are also known to have mutagenic potential, it is also not surprising that the correlation seemed to be best with the bioassays screening for genotoxicity (Tab. 34). Only little data is available on EROD-inducing compounds in Jordan. As already mentioned above, Batarseh (2003) has detected the PCBs 52, 101, 138, 153, 180 and most prominently PCB 28 in sediments samples of the Zarqa River during a sampling campaign in 2000 and 2001. However, due to their non-planar structure, all of these PCBs are characterized as non-dioxin-like and have been found to not induce EROD activity (Marabini 2011). Nevertheless, because these congeners were the only PCBs scanned for in the study by Batarseh, other PCBs might be likely to occur in the sediments as well and might be responsible for the rather high results obtained for the EROD assay with extracts of the Zarqa River.

The second well-studied group of EROD-inducing compounds are PAHs. Among those found in the vicinity and also in the sediments of the Zarqa River (Batarseh 2003, Tahboub 2014, Tarawneh 2012), only chrysene and benzo[b]fluoranthene are known to induce EROD activity in RTL-W1 cells (Behrens et al. 2001, Bols et al. 1999). In other studies, EROD induction could also only partly be correlated with the total amount of PAHs; thus, it seems necessary to include the EROD bioassay into environmental risk assessment and monitoring to gain an insight into the actual dioxin-like potential of a river (Hollert et al. 2002a).

Few studies are concerned with the pollution with combustion by-products such as PCDFs and PCDDs in Jordan. Alawi (1996) detected a total amount of up to 85 and 26 mg/kg, respectively, originating form a landfill close to the Zarqa River. Al-Dabbas (2010) also identified landfills with waste combustion apart from medical waste and transport as the main source for PCDDs

and PCDFs in Jordan. Furthermore, many substances that induce EROD activity emerge during industrial combustion or herbicide production. This presents a good correlation with the findings of this study, as the Yarmouk River and Zarqa River were the two sites that showed, apart from Jordan 1, the strongest effects in the EROD assay and are also the rivers mostly affected by industry or waste combustion (Abu-Rukah and Al-Kofahi 2001, Al-Dabbas 2010). The Zarqa Governorate hosts among others the Jordanian Petroleum Refinery, three steel factories, the chemical factories Industrial Commercial & Agriculture, Mafraq Agricultural Co. and Sulphochemical Co., Arab White Cement Industries and Jordan Industries for Bricks. Concerning the remaining areas of investigation of this study, no information was found about pollution with those substances indicating that a screening is not yet integral part of the environmental monitoring in Jordan.

The recently improved new test design of the EROD assay including the use of ß-napthoflavone as reference substance and the normalization of EROD activity against MTT reduction, which was applied in this study, proved to be a most promising alternative to conventional protein-based normalization (Heinrich et al. 2014). Reasons for the frequently observed decline in EROD activity after cell treatment with complex environmental samples, chemical mixtures or even monosubstances have long been the object of scientific discussion (Chen 1996, Hahn et al. 1993, Petrulis and Bunce 1999, Petrulis et al. 2001, Rodman et al. 1989, Schirmer et al. 2000). Integrating a cytotoxicity test simultaneously into the measurement of EROD activity allows to distinguish between EROD inhibition as caused by high concentrations of xenobiotics or competitive inhibition and inhibition caused by cell death or hormesis and therefore adds to the scientific discussion. In this study, all sampling sites showed, if only a very small, induction of EROD activity. A conventional saturation curve was never to be observed, whereas EROD activity declined at higher extract concentrations (Tab. 34). This was, however, in all cases linked to hormesis in terms of increasing cell viability or to reduced cell viability, both of which were measured by the incorporated MTT assay (Ch. 3.7). However, although inhibitory effects of a sample itself at higher concentration were basically excluded by that, the presence of inhibitory compounds as such inhibiting already at small concentrations could not be excluded. Despite the knowledge of inhibiting substances being present in complex samples (Mahadevan et al. 2007, Shimada and Guengerich 2006), this aspect has widely been ignored in routine monitoring. Inhibition of EROD activity can follow various pathways: 1) A whole variety of ligands is capable of binding to the Ah-receptor; however, these vary in their ability to initiate the production of CYP1A1 m-RNA (Chen 1996) Hence, there is a competition between the ligands for a limited number of AhR-binding sites (Petrulis et al. 2001). 2) Ligands may exert competitive inhibition of CYP 1A1 while simultaneously inducing gene expression. Competitive inhibition occurs since the ligand as original substrate competes with the synthetic substrate 7-ethoxyresorufin for the catalytic capacity of EROD (Behrens et al. 2001, Petrulis and Bunce 1999). 3) Direct inhibitory effects of ligands or other substances on CYP1A1, which

is well known for heavy metals, especially cadmium, in fish cells. Brüschweiler et al. (1996) proved that heavy metals do not block AhR binding sites, but interfere with EROD, because the EROD content was found to be the same with a combined exposure with heavy metals and a strong EROD inducer as without heavy metals. Especially the fish EROD is known to be extremely sensitive to heavy metal concentrations (Oliveira et al. 2004, Viarengo et al. 1997). In summary, there is a high risk of underestimating induced EROD activity in complex samples. Thus, a lack of EROD induction cannot truly be an indication for the absence of dioxin-like substances, whereas, induction can clearly indicate their presence. The observed effects depend on both the inherent properties of the inducers and inhibitors and their concentrations.(Petrulis et al. 2001). The interpretation of the responses remains a challenge and further research on inhibition and its effects on the organisms have to be conducted.

Inhibitory effects are also believed to play a role in the exposure of RTL-W1 cells to sediment extracts from Jordanian surface waters. Especially at sites where two or more bioassays showed moderate to strong toxicity, thus indicating a high content of hazardous chemicals some of which may have inhibition capability, it is most likely that inhibition of EROD is responsible for the low induction (e.g. Mujib 2 or Zarqa 1, Tab. 34). On the other hand, the results did not seem to correlate well with the findings of heavy metals such as mercury being present at rather high concentrations e.g. at the sampling sites Jordan 1 and Yarmouk 1 as detected by Howari and Banat (2001) and as already discussed in Ch. 4.1.2, because these sites were among the strongest to induce EROD activity. However, it is also possible that the effects would have been even higher without the presence of heavy metals.

In summary, the EROD assay with RTL-W1 cells showed a strong EROD-inducing potential for the sampling sites Jordan 1, Yarmouk 1 and 2 and Zarqa 3. Moderate induction was found for Jordan 4, Yarmouk 3, Zarqa 1, 2, 4 and 6. It could not be determined whether this moderate toxicity and the even lower induction at the remaining sampling sites was due to the absence of dioxin-like compounds or caused by inhibitory effects. However, for Jordan 4 and Wadi Mujib 2, the correlation with the other bioassays indicating likewise no toxicity suggests a good status in terms of contamination.

4.1.4 Acceptability of the bioassays applied

As has been shown in various studies, hazard-based chemical analysis of environmental samples alone cannot provide evidence for the toxicological consequences in organisms and ecosystems. Likewise, risk-based effect observations in bioassays do not have the power to identify the causative agents (Burton 2002, Calmano 2001, Castaño et al. 1996, Fent 2001a). It is therefore essential to include toxicity testing into sediment quality assessment (Carlsson et al. 2014). Alternative methods for the risk assessment and evaluation of chemicals have become

more and more important within the scope of the European chemical policy REACH (Registration, Evaluation and Authorization of Chemicals) and the EU Directive 2010/63/EU. The effort of reducing animal testing has also found its way into environmental screening and monitoring for a long time (Höfer et al. 2004). However, especially in sediment ecotoxicology, various fields of research remain the object to scientific discussion. For example, how realistic and relevant are ecotoxicological scenarios based of extraction methods or can results obtained from *in vitro* methods with cell lines be extrapolated to whole organisms and populations?

Sediments are a well-known sink for particle-sorbed hazardous substances in aquatic systems and can serve as a reservoir of toxic contaminants that continuously threaten the health and viability of aquatic biota. Hydrophobic characteristics of, e.g., mutagens lead to exclusion from water and to adsorption to particulate material, and this material is likely to be incorporated into bottom sediments (Chen and White 2004). This is also postulated by the results from the present study, since extracts obtained from solid phase extraction methods of water samples did not results in any effects concerning general toxicity, whereas the sediment extracts did so very well. Sediments can pose a long-term hazard to benthic biota (Baumann 1995, Marvin et al. 1999, Marvin et al. 2000), which becomes especially relevant during the changing availability of bound contaminants during flood events (Eggleton and Thomas 2004, Wölz et al. 2010, Wölz et al. 2011). Thus, there is general agreement that assessment of sediment quality should be an integral part of environmental studies (Wölz et al. 2009) and is recently found its way into the regulatory framework such as the European Water Framework Directive (2000).

Except for *in situ* studies, there is the need to collect and process samples for further use in *in vivo* or *in vitro* bioassays. It is only natural that sampling, transport and storage already effect the chemical and biological characteristics of a sediment sample (Chen and White 2004, Hjorth 2004). Extraction of sediments becomes especially important for bioassays with cell lines as testing of whole sediments cannot be applied. Therefore, various extraction techniques have been proposed, since obtaining an appropriate sample is obviously the first crucial step for analytical procedures and bioassays (Hjorth 2004, Marvin et al. 1992, Raynie 2006, Seiler et al. 2006) and for standardization (Rönnpagel et al. 1998). The procedure of dissolving compounds and of adding them to cell cultures can be the source of several problems. Unfortunately, these are difficulties that cannot readily be solved, but are, nevertheless, important to consider for date interpretation (Dayeh et al. 2013). Other than extraction techniques based on semipermeable membranes (Karacık et al. 2013), solvent-based extraction does always result in a more or less broad elution of compounds based on the solvent used and does, thus, depict a worst-case-scenario rather than an image of the natural condition. The inability to properly incorporate *in situ* exposure and bioavailability when using organic extraction to assess the toxic hazards of contaminated sediments has been criticized (Jha et al. 2000). It is suggested that extraction and *in vitro* assays should be followed by *in vivo* tests; however, they are very costly, complex and time-consuming. Likewise, it is also well known

that the portion of a chemical that is either bioavailable or bioaccessible in a given soil or sediment environment can differ substantially between organisms (Semple et al. 2004). Therefore, also the alienability of testing whole sediments or semipermeable membrane extracts depend on the test organisms used and can only add one share of toxicity assessment.

Extraction offers a broad insight into the contaminant spectrum of a sample. Various solvents have been studied for the ability to elude contaminants from sediments, and there is the consensus that there is no ideal universal solvent (Chen and White 2004). In this study, acetone was used for soxhlet extractions since being intermediately polar it is capable of extracting both lipophilic and slightly hydrophobic substances (Banjoo and Nelson 2005, Hollert et al. 2000), thus covering major contaminants as PCBs or PAHs. Furthermore, soxhlet extraction requires addition of heat for the evaporation of the solvent, which may add to the alterations of samples, as temperature may effect especially volatile substances (Wang et al. 2010). With 56° C, acetone has a rather low boiling point compared to, e.g., n-hexane (69° C), ethanol (78° C) or even dimethylsulfoxide (189° C). Finally, acetone closely mimics the spectrum of extractants found in the lipid fractions of biological samples. Many environmental studies have thus made use of acetone as solvent in soxhlet extraction, and the comparison of results is therefore rendered possible and facilitated (Boettcher et al. 2010, Hollert et al. 2002a, Keiter et al. 2009b, Kosmehl et al. 2008a, Seitz et al. 2008). In fact, many studies have proven, that soxhlet extraction being the basic and conventional method for extraction of sediment is not inferior to more modern approaches such as membrane dialysis extraction or microwave extraction (Schulze et al. 2012, Wang et al. 2010, Zielke et al. 2011). As the aim of this study was to screen for potential ecotoxicological effects caused by Jordanian surface water sediments, alterations of samples caused by sampling, storage, processing and extraction (worst-case scenario) are acknowledged and accepted.

In addition to the discussion of the chapters above, it should be mentioned that short-term exposure of single cells can of course only imitate long-term effects on whole organisms and ecosystems to a certain extent (Rönnpagel et al. 1998). Because of their practicability, simple laboratory tests are, however, irreplaceable tools for hazard assessment (Segner 1998a). As has already been discussed above, the use of primary fish cell lines is a well-studied and approved technique as alternative to whole animal testing in ecotoxicology (Bols et al. 2005, Dowling and Mothersill 2001, Fent 2001a, Schirmer 2006, Tollefsen et al. 2006). However, current *in vitro* methods for acute aquatic toxicity are neither standardized nor validated, which also applies for the other bioassays applied in this thesis involving cell lines, except for the micronucleus assay with V79 cells (OECD 2010). Inter-laboratory comparison and evaluation of results, therefore, remains a challenge. Furthermore, as has been shown for the EROD assay by Rodman et al. (1989), bioassays tend to vary in sensitivity and applicability depending on the test organism used. At the same time, however, they allow testing for specific endpoints

such as mutagenicity and, therefore, contribute to a better understanding of individual characteristics of samples concerning the spectrum of toxicity.

As can be seen from the results obtained in this study (Tab. 34), a stepwise proceeding in toxicity assessment cannot be recommended, since there is not necessarily a relationship between the different bioassays for toxicity assessment in terms of "if-then" or "if not-then not". Rather, a complete biotest battery should routinely be incorporated into thorough assessment studies (Carlsson et al. 2014). Nevertheless, toxicity tests should not be regarded as ultimate predicting models for environmental conclusions. They provide information for what might happen under certain conditions (Chapman 2002). A certain extrapolation of results and consequences to the field, however, is necessary due to the multitude of chemicals in the environment, species diversity and complex ecological processes and influencing factors that can impossibly be all accounted for under laboratory conditions (Fent 2001a). Although studies of sediment toxicity are often stimulated by a concern for human welfare, the likeliness of adverse human effects from contaminated sediments is unclear. Humans are rarely in intimate contact with aquatic sediments and exposure to sediment toxins appears unlikely (Chen and White 2004); nevertheless, effects on fish and cell cultures can serve as a warning of possible impacts on human health (Bols et al. 2005, Kirsch-Volders et al. 2011). Although ecological and toxicological relevance of *in vitro* bioassays is still being challenged, it is concluded that a test battery as applied in this study is a useful tool for potential toxicity assessment of sediments, especially as a first-time screening and survey as it is − to the best of our knowledge − the case in Jordan.

4.2 Classification of sediment toxicity

Assessment of water and sediment toxicity is not yet standardized and far from being straightforward. It still lacks universally valid or applicable assessment standards, and the variety of test systems used is still very broad (Keiter et al. 2009b). Furthermore, there is often no direct correlation between the concentration of a biohazard in the sediment and the toxicological effects caused by the sediment due to complex mechanisms for bioavailability of organic compounds (DiToro 1991). Apart from the need to combine different exposure paths and test organisms into a comprehensive evaluation to meet the ecological and biological diversity of any watercourse itself, there is an increasing demand to consider ecological relevance of results obtained from different bioassays. For the classification of the Jordanian watercourses studied within the scope of this thesis, the results of three bioassay were rated according to the toxicity threshold values established within the framework of a fuzzy logic-classification approach by Keiter et al. (2009b): 1) the neutral red assay with RTWL-W1 cells, 2) the comet assay with RTWL-W1 cells, 3) the fish embryo toxicity test with zebrafish. Following this approach, the dataset of each bioassay is divided into three toxicity levels (non-

toxic, moderately toxic and strongly toxic) to cover the entire response range of test systems. To gain a location-independent insight into the response ranges, data from various studies were integrated into this calculation: Danube (Keiter et al. 2009a, Keiter et al. 2008, Keiter et al. 2006, Seitz et al. 2008), Rhine (Kosmehl et al. 2004) and Neckar (Braunbeck et al. 2009, Hollert et al. 2000, Hollert et al. 2002b). Among other methods, the empirical method was used and proved to be the most suitable by Keiter et al. (2009b) as explained in Ch. 2.6.

Threshold values were furthermore established by Keiter et al. (2009b) for the EROD assay, however, since the EROD assay in this study was conducted according to a new protocol (Heinrich et al. 2014), the same threshold values could not be applied. Therefore, a rank-sum based classification was applied according to Canfield at al. (1994) and Hollert et al. (2002b). The same was applied for the micronucleus assay, since no sufficient data base was available for the micronucleus assay with V79 cells exposed to sediment extracts. Thus, data for each individual result was scaled proportionally between 1 % and 100 % (e.g. an IF of 1.3 being the lowest and an IF of 4.1 being the highest observed effect). Scaling of data results in a relative ranking of results. The ranked data is then classified into three groups equivalent to the toxicity levels of Keiter et al (2009b): non-toxic < 33.3 %, moderately toxic for 33.3 to 66.6 % and strongly toxic > 66.6 %. Results of the rank-sum-based classification for the EROD assay and micronucleus assay is shown in Fig. 79. It has to be noted that the rank-sum-based analysis for the classification of toxicity data is location-dependent, as it does only take data obtained in this study into consideration (Ahlf and Heise 2005). However, due to the lack of comparable data for the EROD assay conducted under the protocol of Heinrich et al. (2014), it seems to be sufficient to gain at least insight into the relative toxicity according to EROD induction of the sampling sites analyzed in this study. Data obtained from the micronucleus assay with RTL-W1 cells in similar studies suggest good correlation with the rank-sum analysis applied for this test (Boettcher et al. 2010, Keiter et al. 2009a, Rocha et al. 2009).

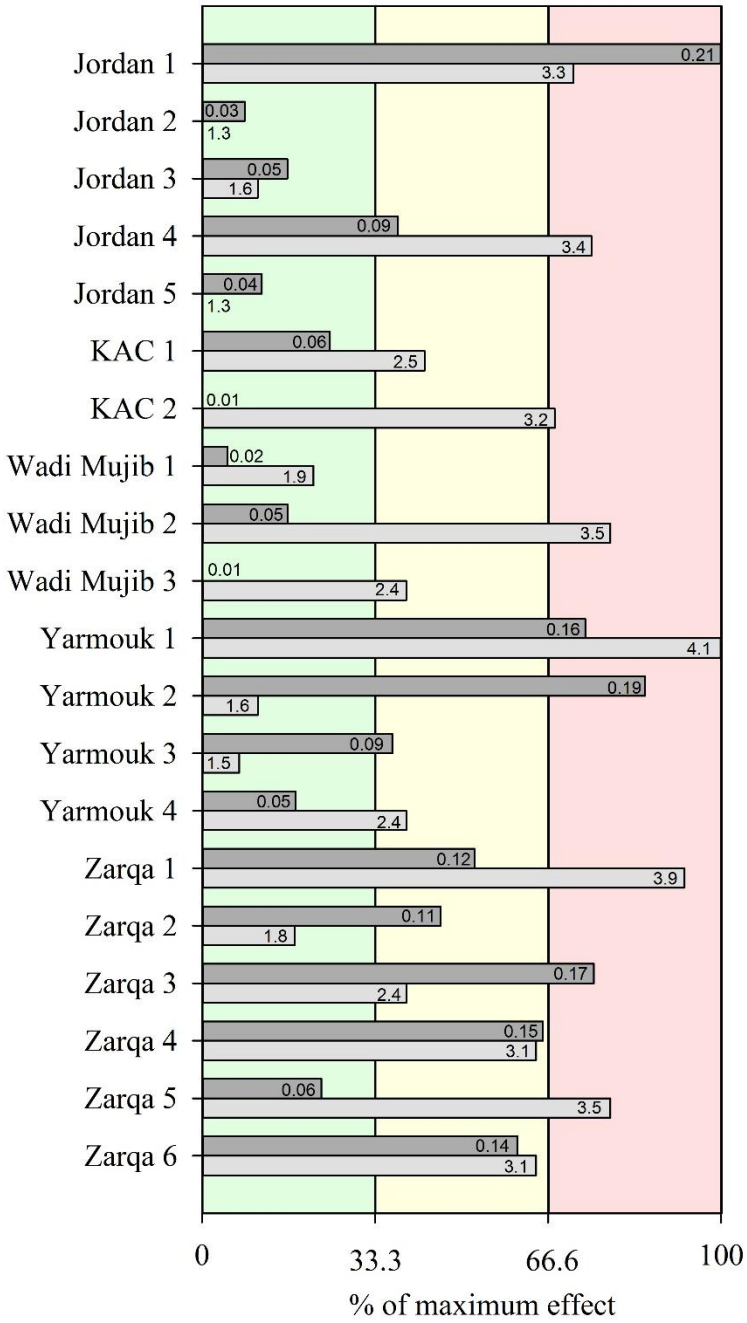

Fig. 79: Rank-sum-based analysis with scaling of all results between 1 and 100 % according to Canfield at al. (1994) and Hollert et al. (2002b) for the EROD assay (dark grey) based on the quotient of BNF induction equivalents in mg SEQ/ml and for the micronucleus assay (light grey) based on the maximum induction factors. Numbers show the corresponding results of the bioassays for each sampling site. Colors indicate toxicity level: red = strongly toxic, yellow = moderately toxic, green = non-toxic.

The results obtained from the rank-sum analysis and allocation to toxicity levels *via* threshold values are then transferred into quality classes in accordance with the classification criteria for physical and chemical parameters after Graw and Borchardt (1999), which complies with the EU Water Framework Directive 2000/60/EC (EU-WRRL 2000). Thus, class 1 stands for no contamination, class 2 for moderate contamination, class 3 for considerable contamination,

class 4 for increased contamination and class 5 for high to very high contamination. The combination of the three different toxicity levels obtained from the five bioassays define the quality classes according to Keiter et al (2009b; Tab. 35).

Tab. 35: Definition of quality classes in following the classification system after Graw and Borchardt (1999) and the EU Water Framework Directive 2000/60/EC (EU-WRRL 2000) according to Keiter et al. (2009b) in terms of toxicity levels obtained from the various bioassays.

	Toxicity level		
Quality class	non-toxic	Moderately toxic	Strongly toxic
I	5	-	-
	4	1	-
	4	-	1
II	3	1	1
	3	2	-
	2	3	-
III	3	-	2
	2	-	3
	1	3	1
	2	2	1
	2	1	2
	1	2	2
	1	4	-
	-	4	1
	-	5	-
IV	-	3	2
	1	1	3
	-	2	3
V	1	-	4
	-	1	4
	-	-	5

Following the suggestion of Henschel et al. (2001), a certain weighting is applied to test of high ecological relevance. Thus, the fish embryo toxicity test was granted a higher ecological rank than the cytotoxicity test, because it studies the development of an *intact* living organisms after exposure to sediment extracts rather than effects at the single cell basis (Keiter et al. 2009b). However, the comet and micronucleus assay were also assigned a higher rank, since genotoxic substances may affect viability of gametes and, therefore, reduce reproduction and can thus affect a whole population (White 1999, Zorita 2007). Therefore, if at least one sampling site was rated strongly toxic in either the fish embryo toxicity test, the comet assay or the micronucleus assay, it was automatically downgraded by one class. The same was applied when

one sampling site showed moderate toxicity in all three of the tests just mentioned. Furthermore, a sampling site could not be rated class 1, when at least one of the tests just mentioned indicated moderate or strong toxicity. This system of ranking, however, is not final and needs further investigation and discussion. The comprehensive characterization of Jordanian surface waters is therefore based on a weight-of-evidence approach, which draws conclusions based on available data. There remains the need for a continued scientific discourse of toxicity assessment of sediments (Chapman 1995). It has further to be noted that all tests were conducted under laboratory conditions and can, therefore, not be directly applied *in situ* and a direct extrapolation to field and ecological effects cannot be made without further investigation (Chapman et al. 2002). The results of the classification for all Jordanian surface waters investigated in this study are shown in Tab. 36.

Tab. 36: Results of the classification of toxicity of the sediment samples from Jordanian surface waters with and without consideration of ecological relevance. Numbers and colors indicate the quality classes: I, blue (no contamination) – V, red (very high)

	Sampling site	Weihting of ecological relevance	
		without	with
Jordan River	1, Baqura	IV	V
	2, Sheik Hussein Bridge	I	II
	3, Damiya Bridge	I	II
	4, Allenby Bridge	III	IV
	5, King Abdullah Bridge	I	I
KAC	1, Deir Allah	III	IV
	2, Confluence Zarqa	I	II
Wadi Mujib	1, Reservoir inlet	I	I
	2, Reservoir outlet	V	V
	3, Mouth to Dead Sea	I	II
Yarmouk River	1, Unity Dam	IV	V
	2, Wadi Raqab	III	IV
	3, Diversion to KAC	II	II
	4, Gesher	III	IV
Zarqa River	1, Khirbet As Samra	IV	V
	2, Confluence Zarqa	IV	V
	3, Seil Jerash	III	IV
	4, Jerash Bridge	III	IV
	5, Inlet King Talal Dam	II	III
	6, Outlet King Talal Dam	III	IV

4.2.1 Overall classification of the Jordan River

The Jordan River as the largest and longest river in Israel and Jordan suffers massively under anthropogenic pressure and water deprivation (Ch. 1.3.1). An estimation on the gradient of sediment quality based on the effective classification of five sampling sites alongside the Lower Jordan River is shown in Fig. 80.

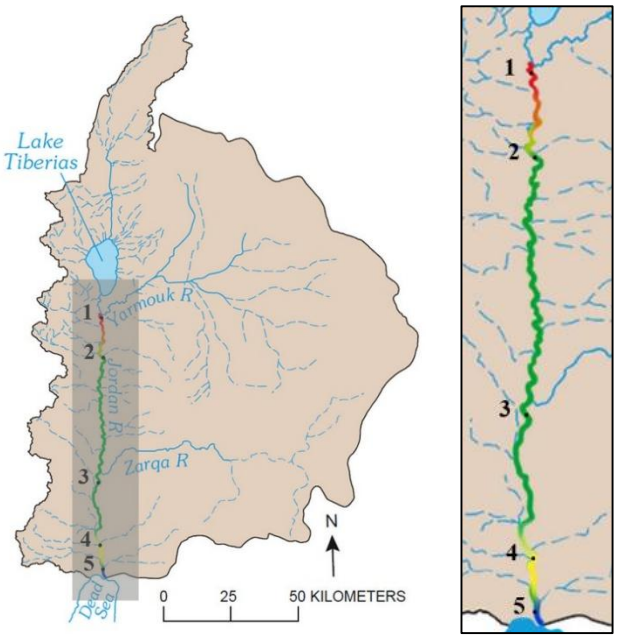

Fig. 80: Classification of the contamination of the Jordan River in terms of toxicity assessment with consideration of ecological relevance of the applied bioassays. Sampling site Jordan 1 was identified as contaminated hot spot and there seems to be a self-purification process alongside the river flow direction. An effective classification was only conducted for the five sampling sites; thus, the gradient of quality classes is only an estimation. Map modified according to EXACT (1998).

The results of this study clearly identified the northern Lower Jordan River as contamination hot spot being rated quality class V, as extracts did not only induce strong genotoxic effects and EROD activity, but also showed cytotoxic, embryo-toxic and teratogenic potentials (cf. Tab. 34). The sampling site Jordan 1 at Baqura was located shortly after the inlet of effluents from the Bitania waste water treatment plant and the Saline Water Carrier. The waste water effluents are therefore regarded as the main source of contamination, and detailed investigation of the efficiency und sufficiency of water treatment is strongly recommended. The results from the two sampling sites further downstream suggested a self-purification capacity of the Jordan River, since the quality class improves to class II and even to class I for Jordan 5 at the King Abdullah Bridge, where none of the bioassays showed moderate or even strong effects. This was also in concordance with a slight improvement of nutrient levels as e.g. NH_4^+, PO_4^{3-} and NO_2^-, indicating that additional discharge of agricultural sewage plays a minor role in sediment

contamination. Nevertheless, especially almost constantly high levels of NO_3^- indicate eutrophication of the River. The overall classification according to the LAWA standards (1998) was class III-IV for Jordan 1 and 2, class III for Jordan 3 and 5 and class II-III for Jordan 4. Although, despite thorough literature research, no point source could be identified, Jordan 4 at Damiya Bridge was found to be another hot spot of contamination and was rated toxicity quality class IV mainly due to genotoxic effects. Precariously, genotoxic substances were identified as main hazard in the sediment of the Lower Jordan River, as for all sampling sites except Jordan 5 positive results in either the comet assay or the micronucleus assay were obtained. As discussed in detail in the chapters 4.1.1, 4.1.2 and 4.1.3, available data on chemical analysis of e.g. pharmaceuticals and heavy metals (Howari and Banat 2001, Tiehm et al. 2011) correlate quite well with the findings of this study. However, in terms of the ecotoxicological test battery applied in this study, sediment contamination and toxic potential did not seem to be as bad as has been estimated from extrapolation of non-point sources of contamination (Holtzman et al. 2005, Vengosh 2003). Nevertheless, salinity, ecological consequences for the Dead Sea and the critical results indicating genotoxicity may prevent the water of the southern parts of the Lower Jordan River from use for further irrigation (Farber et al. 2005).

4.2.2 Overall classification of the King Abdullah Canal

Due to limited access, only two samples could be collected at the King Abdullah Canal. However, site 1 at Deir Allah is among those being of highest interest to Jordanian water management as it corresponds with the intake for drinking water pumping to the capital Amman. An estimation on the gradient of sediment quality based on the effective classification of the two sampling sites alongside the King Abdullah Canal is shown in Fig. 81. The overall classification of physical and chemical analysis suggested a rather high eutrophication of the KAC, which is not surprising considering the Yarmouk being the main tributary. According to the LAWA standards (1998), the canal was rated quality category II-III. The results of the bioassays indicated considerable contamination with genotoxic substances, since for KAC 1 both the comet and micronucleus assay showed strong and moderate effects, as did the micronucleus assay for KAC 2. This is of special concern, as the water from the KAC is used for drinking water supply in Amman. Screening for possible mutagens should, therefore, be thoroughly conducted. Furthermore, the hatching rate of zebrafish embryos was severely influenced by extracts from KAC 1. Sediment quality improved after the confluence with discharges of the King Talal Dam, because cytotoxicity in the neutral red assay was only detected for KAC 1, and also the results from the EROD assay improved. However, thus, in terms of toxicity KAC 1, was rated class IV and KAC 2 was rated class II.

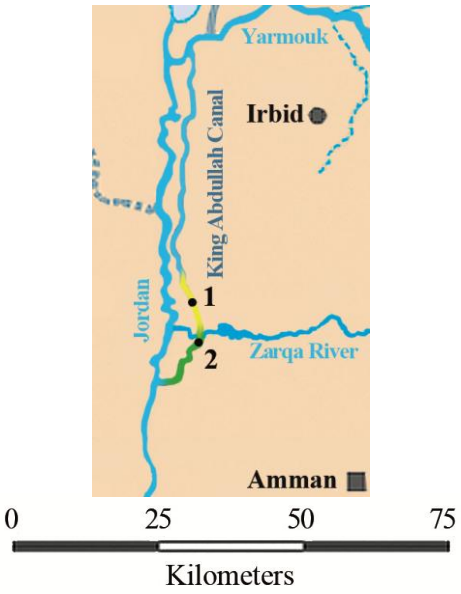

Fig. 81: Classification of the contamination of the King Abdullah Canal in terms of toxicity assessment with consideration of ecological relevance of the applied bioassays. Results suggested an increase of sediment quality after confluence with the Zarqa River. An effective classification was only conducted for the five sampling sites; thus, the gradient of quality classes is only an estimation. Map modified after UNEP/DEWA/GRID-Geneva.

4.2.3 Overall classification of Wadi Mujib

Despite receiving domestic, industrial and municipal wastewaters, Wadi Mujib as one of the largest water reservoirs is primarily used for drinking and irrigation water supplies. Tow samples were taken at the inlet and outlet of the reservoir itself and another one at the mouth to the Dead Sea close to the extraction place for drinking water. Except for PO_4^{3-}, nutrient levels are within the threshold values of the LAWA, and the sites were thus rated between II and II-III according to LAWA standards. Since no bioassay applied in this study indicated any contamination for Mujib 1 at the inlet of the dam, this site could be rated toxicity class I like Jordan 5 (Fig. 82). However, water levels at this site depend strongly on rainfall and the sediment might not be covered with water throughout the year. This could naturally result in less binding of contaminates to the sediment. The opposite applies for Mujib 2 at the outlet of the dam, where there is a constant flow of water and thus a higher passage of substances that potentially bind to organic matter. Furthermore, the particle size of sediments from Mujib 2 was only 200 µm compared to 630 µm for all other samples, thus offering a larger surface and more binding sites. Except for the EROD assay, all bioassay showed high effects indicating the presence of genotoxic and embryo-toxic contaminants. Mujib 2 was therefore rated class V. The gradient of sediment classification of the following stream could only be estimated, as the next sampling site was approximately 26 km further downstream. There, the micronucleus

assay screening for mutagenic potential indicated moderate toxicity resulting in an overall rating as class II.

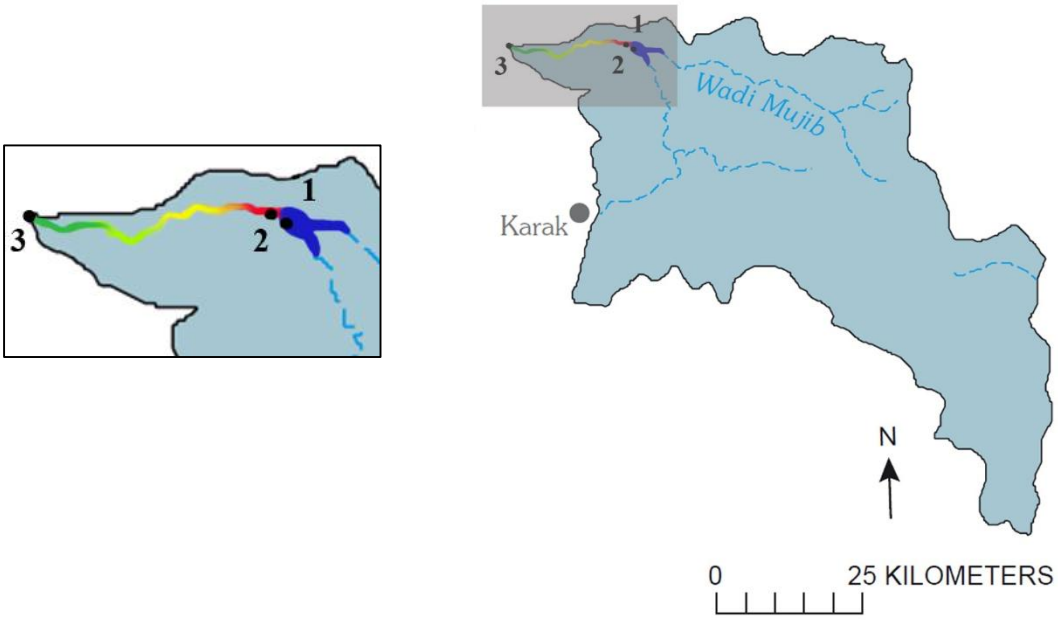

Fig. 82: Contamination classification of Wadi Mujib in terms of toxicity assessment with consideration of ecological relevance of the applied bioassays. An effective classification was only conducted for the five sampling sites; thus, the gradient of quality classes is only an estimation. Map modified according to EXACT (1998).

4.2.4 Overall classification of the Yarmouk River

Being the major tributary to the Jordan River and the King Abdullah Canal, the Yarmouk River can be considered as the most important water resource of Jordan in terms of drinking water and irrigational water supplies. The analysis of the physical and chemical parameters, however, suggested considerable problems with eutrophication as especially high levels of PO_4^{3-} and NO_3^- were found resulting in class II-III according to LAWA (1998). Considering the fact that the river receives untreated effluents from two waste water treatment plants during flood events occurring regularly during the winter season, this did not seem to be surprising. More concerning are the results from the bioassays applied, since all of them except the fish embryo toxicity test indicated at least at one site moderate or even strong contamination. An estimation on the gradient of sediment quality based on the effective classification of four sampling sites alongside the Yarmouk River is shown in Fig. 83. The strongest effects were found for Yarmouk 1 at the outlet of the Unity Dam, which was identified as a hot spot of contamination. Results suggested elevated presence of genotoxic and dioxin-like substances in the sediments. The neutral red assay also indicated moderate general toxicity, and the overall classification for

this site was category V. Overall, there was a slight improvement of sediment quality during the river flow direction. Nevertheless, genotoxic contaminants played a major role in toxicity assessment and are of precarious concern especially in terms of drinking water supply for the King Abdullah Canal. The sampling site at the diversion to the King Abdulla Canal received the best category within the Yarmouk River system and was rated class II. However, this site is especially known for flash floods and mixing/remobilization of sediments, which may account for the rather good quality (Abu-Rukah and Ghrefat 2001). The suitability of the Yarmouk River for irrigation purposes cannot be ensured on grounds of sediment toxicity assessment, but rather supports a study by Al-Taani (2013) who identified the river as being not acceptable for irrigation due to high nutrient levels.

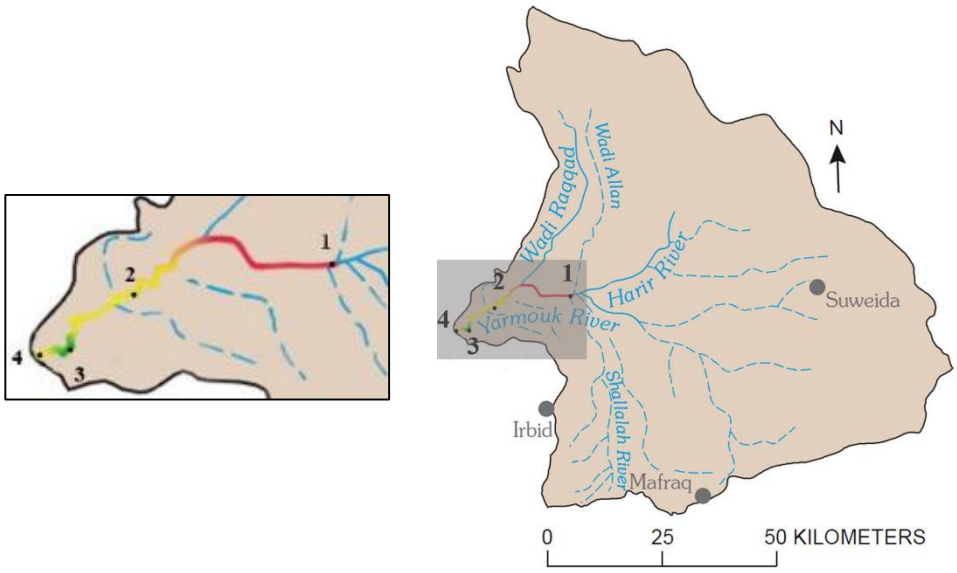

Fig. 83: Classification of the Yarmouk River in terms of quality classes as assessed by various bioassays with consideration of their ecological relevance. The outlet the Unity Dam could be identified as hot spot, whereas results from the other sampling sites suggest a certain rate of self-purification alongside the river's flow direction. An effective classification was only conducted for the five sampling sites; thus, the gradient of quality classes is only an estimation. Map modified according to EXACT (1998).

4.2.5 Overall classification of the Zarqa River

Approximately 65 % of the Jordanian population live in the Arman-Zarqa basin, and it is also the center for principal industries, since industrial sites are located on the banks of the Zarqa River. Since, furthermore, during summer the river carries solemnly treated and untreated sewage waters, it is a highly anthropogenicly influenced surface water. Findings of this study add to other studies identifying the Zarqa River as being highly polluted and suffering from eutrophication. LAWA-based classification resulted in no better than class III and even

class III-IV for Zarqa 1 and 2, located shortly after the inlet of the Khirbet As Samra treatment plant. In terms of results obtained from the bioassays, Zarqa 1 was also identified as hot spot of contamination indicating that sewage treatment is not yet sufficient. Severe sublethal effects on zebrafish embryos could be observed at Zarqa 1 and 6, the latter accompanied by lethal effects resulting in moderate embryo toxicity. However, especially the presence of genotoxic and dioxin-like compounds make the usage of water for irrigation questionable. As has also been described for the Jordan and Yarmouk Rivers, sediment quality of the Zarqa River seemed to improve slightly alongside flow direction. Zarqa 5 at the inlet of King Talal Dam could be rated class III due to no effects observed in the FET, EROD assay and NRA. However, similar as for Mujib 1, water levels are subject to strong fluctuations, and sediment coverage is not guaranteed throughout the year. This could account for lower contamination found for this site compared to the remaining Zarqa River. An estimation on the gradient of sediment quality based on the effective classification of four sampling sites alongside the Zarqa River is shown in Fig. 85.

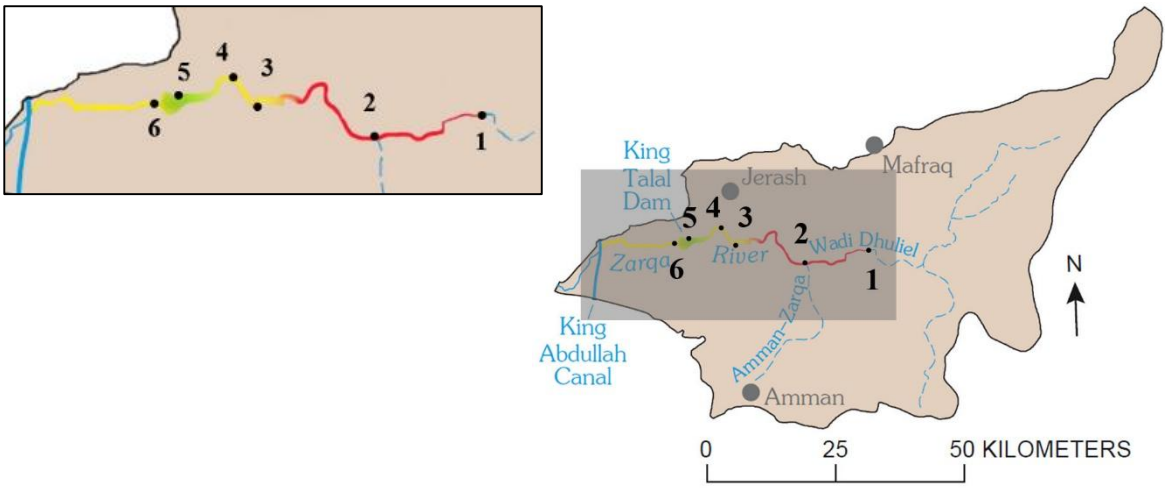

Fig. 84: Classification of the Zarqa River in terms of quality classes as assessed by various bioassays with consideration of their ecological relevance suggesting a high contamination of the whole river. The effluent of As Samra treatment plant could be identified as hot spot of contamination, whereas a slight rate of self-purification alongside the river's flow direction seemed to occur. An effective classification was only conducted for the six sampling sites; thus, the gradient of quality classes is only an estimation . Map modified according to EXACT (1998).

4.3 Contribution to Integrated Water Resources Management

Water scarcity is a major challenge in Jordan which is believed to even further increase under the rapidly growing population and due to climate change as was for example modelled for the Zarqa basin (Abdullah and Al-Omari 2008). Water managers are challenged to meet multiple and often conflicting demands, such as to provide a sufficient quantity of water used for various needs and at the same time ensuring satisfactory water quality and support healthy and diverse ecosystems. The fact that water is an integral part of the ecosystem, a natural resource and a social and economic good is today recognized in the concept of Integrated Water Resources Management (IWRM). It is only natural, however, that in semi-arid to arid regions suffering from water deprivation quantity of water seems to have higher priority to quality issues. It is therefore even more necessary to prevent the existing water resources from being polluted and overexploited. Whereas assessment of sediment quality is already an integral part of holistic IWRM in countries like the USA and the European Union (Apitz and Power 2002, MacDonald 1994, Netzband 2007), sediments have so far been neglected in Jordanian routine monitoring. However, sediments play a critical role in determining the fate and effects of environmental contaminants in addition to providing important habitats for aquatic organisms. Hence, sediment quality questions and concerns receive more and more attention in water management (MacDonald 1994).

The results of this study suggested a certain discrepancy between regular monitoring programs conducted by the Royal Scientific Society (RSS) and the Water Authority of Jordan (WAJ) or studies of e.g. Tahoub (2014) which assign an overall good water quality to Jordanian surface waters as the Zarqa River or Wadi Mujib. After an incident in January 1998, when drinking water derived from the KAC had an unpleasant odor and created algae blooms as a consequence of insufficient treatment at the ZAI treatment station, quality monitoring was extended (Alkhoury et al. 2010). Nowadays, the parameters monitored in surface water by the WAJ program include pH, NO_3^-, PO_4^{3-}, TOC, NH_4, odor and heavy metals (once every three to twelve months), chlorinated pesticides (once a year), chlorination byproducts (daily) and fecal coliforms (five times/week; (JISM 2001). This analysis, however, disregards contamination with PAHs, PCBs, PCDDs or PCDFs, and ignores the role of sediments as a sink and possible source for contaminants that can be made available again especially during events of flash floods (Eggleton and Thomas 2004, Wölz et al. 2010, Wölz et al. 2011) occurring on a regular basis in Jordan. Hazardous compounds can therefore easily enter the food chain *via* irrigation or even get access to the drinking water supply system.

All recommendations made in the following have to be regarded as fragmentary, as they constitute only one aspect of sediment quality analysis and are based on results obtained from bioassays that are themselves based on a worst-case scenario as discussed in Ch. 4.1.4. Extrapolation of the results from the bioassays applied in this study to human welfare and the

ecosystem can of course not be readily conducted; nevertheless, a certain risk can by no means be waived (Chen and White 2004, Keiter et al. 2006). Then again, no chemical measurement alone reliably predicts toxicity (Carlsson et al. 2014, O'Connor and Paul 2000).

Generally, based on the findings of this study, it is strongly recommended that water quality monitoring should be expanded. Water quality monitoring should cover the entire spectrum of pollutants: standard organic, inorganic and nutrient-related substances. It should, thus, be extended to routine monitoring of mutagens as for example PCBs and PAHs, especially at drinking water abstraction sites such as KAC 2 or Wadi Mujib 3. Additionally, monitoring should include at least chemical analyses of sediments for major contaminants. Moreover, quality assessment should be conducted more frequently, and should take possible alterations of water quality and changes of sediment mobility after flash floods into consideration. In order to evaluate pursued long-term improvement of sediment quality, it is suggested to establish a database on sediment quality based on chemical analysis. An extension to toxicity assessment in terms of ecotoxicological bioassays would be desirable, but requires extensive education and training of laboratory staff and upgrading of laboratory facilities. Furthermore, the ecotoxicological knowledge acquired through this study needs to be supplemented by studies on bioavailability, on benthic communities that might be adversely affected by sediment contamination, and it requires strong support from other disciplines that monitor e.g. site stability and the potential for sediment mobility and overlying water chemistry (Ahlf and Förstner 2001).

As the major hotspots of contamination detected in this study were found to be in strong correlation with the effluent of sewage water, it is also implied that sewage water treatment is not yet sufficient particularly regarding mutagenic and dioxin-like compounds. Currently, only 63 % of the Jordanian population are connected to public sewerage (Bonn 2013). It is therefore strongly recommended in accordance to already existing plans (Hashemite Kingdom of Jordan and GTZ 2008b) to further upgrade and enhance existing water treatment plants, implement secondary treatment, support the continuation of construction programs and to further develop the sewerage network. To achieve improvement of sediment quality, it seems indispensable to stop discharge of untreated sewage into rivers and wadis. Since flash floods are a major source of untreated sewage, investigation of flash floods management as for example in the form of overflow or catchment basins are recommended. This is especially advisable for the Yarmouk River and the Wadi Mujib, since both water courses have rather large catchment areas and because their water serves either directly as drinking water source in the case of Wadi Mujib or *via* diversion to the King Abdullah Canal in the case of the Yarmouk River. Furthermore, other potential sources of contamination such as industrial effluents or surface run-offs have to be identified and removed as far as possible.

Since sediments could be identified as sinks for contaminates, caution is advised for the usage of water for drinking purposes as well as for irrigation. Filtration has to be an integral part not only for drinking water treatment, but should also precede irrigation to purge suspended matter (Capra and Scicolone 2004). Results obtained for sediments from the King Talal Dam and Wadi Mujib indicate that storage of waste water effluents in reservoirs does not lead to significantly improved quality and are in accordance with other studies investigating water quality aspects (Al-Harahsheh and Al-Amoush 2010, Al-Taani 2013, Shatanawi and Fayyad 1996). So far, most studies that investigated the consequences of the re-use of wastewater for irrigation in Jordan have only been concerned with nitrogen or heavy metal enrichment in soil (Abderahman and Abu-Rukah 2006b, Abdulla et al. 2009, Jiries et al. 2002, Shatanawi and Fayyad 1996). However, in terms of human welfare, it might be even more important and advisable to study the accumulation of persistent organic pollutants such as PAHs and PCBS in soil as well as their uptake into crops, which has also been suggested by Batarseh (2011). Accumulation of PAHs is a well-known problem linked to irrigation with wastewater (Chen 2005, Wang 2010) and should, therefore, also be monitored in relevant areas of Jordan. Furthermore, Jordanian studies and guidelines suggest only the re-use of domestic wastewater for irrigational purposes. However, in practice, a distinction between domestic and industrial effluents seems hardly possible due to uncontrolled discharges especially into the Zarqa River (IUCN et al. 2006) or the collective treatment in wastewater plants.

Considering the hot spots of contamination identified in this study, namely Zarqa River 1 and 2 after the effluent of Khirbet As Samra, Yarmouk 1 at the outlet of Unity Dam, Jordan 1 at Baqura and Mujib 2 at the outlet of the dam, it might be worth taking sediment remediation actions into account. However, remediation of sediments is very complex and cost-intensive due to mixture of contaminants (Jacobs and Förstner 2001) which might entail disposal difficulties. Additionally, dredging can affect a river's ecosystem especially in terms of its benthic community. Comprehensive studies on site-specific characteristics such as the benthic community, bioaccumulation and biomagnification, site stability in terms of the sediment's potential for mobility and physicochemical sediment properties are necessary. Several management strategies are currently available to remediate sediment contamination. However, as sediment remediation actions are beyond the author's expertise, further discussion is left to pointing out reference studies and methods. Those include 1) monitored natural recovery, 2) *in situ* containment, in which sediment contaminants are isolated from their target organisms, but the sediments are left in place, 3) *in situ* treatment, 4) dredging or excavation followed by *ex situ* treatment, disposal and/or re-use (Apitz and Power 2002), or 5) capping of contaminated areas (Förstner et al. 2001, Förstner and Salomons 2008). For rivers with a long pollution history as the Zarqa River, resulting in a basin-wide contamination, flexibility in management may be required, allowing transition to longer term objectives (Netzband 2007). Following the suggestions of Krantzberg et al. (2000), an evaluation of both short-term adverse effects and

long-term beneficial results of contaminated sediment management should be undertaken. It is imperative that source control will be achieved to a level that will forbid recontamination.

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