Ecotoxicology of traffic related Platinum in the freshwater environment

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Contents

List of Figures

List of Tables

Abbreviations

ACSV	Adsorptive cathodic stripping voltammetry
AEF	Anthropogenic enrichtment factor
BCF	Bioconcentration factor
BCR-723	Name of a reference material, which consists of tunnel dust
CF	Condition factor
COI mtDNA	Cytochrom oxidase I sequence in the mitochondrial DNA
DIN	German Institute for Standardization (Deutsches Institut für Normung)
DNA	Deoxyribonuclei acid
Dorm-2	Name of a reference material, which consists of of dogfish muscle
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
ET-AAS	Electrothermal Atomic Absorption Spectroscopy
EQS	Environmental quality standards
GIEMSA	Name of a stain, named after Gustav Giemsa, consisting of Azur-Eosin-Methylenblue solution in methanol
GOT	Glutamic oxaloacetic transaminase
H_2SO_4	Sulphuric acid
HCl	Hydrogen chloride
HF	Hydrofluoric acid
HMDE	Hanging mercury drop electrode
HNO_3	Nitric acid
HPA	High pressure ashing
HSI	Hepatosomatic index
IAEA-407	Name of a reference material which consists of fish muscle material
ICP-MS	Inductively coupled plasma mass spectroscopy
INBO	Research Institute for Nature and Forest
LAWA	Bund/Länder Arbeitsgemeinschaft Wasser (German Working Group on water issues of the Federal States and the Federal Government)
LCD glass	Liquid crystal display glass
LOD	Limit of detection
LOQ	Limit of quantification
MEM	Minimum essential medium
MD	Microwave digestion
MMS	Methyl methanesulfonate
MN	Micronucleus
MPU	Maximum possible Platinum uptake
Pacs-2	Name of a reference material, which consists of of marine sediment

PBS	Phosphate buffer saline
PE	Polyethylene
PGE	Platinum group elements
PM-10	Particulate matter, consisting of particles on the order of 10 micrometers or less
PP	Polypropylene
R	Rank correlation coefficient
rDNA	Ribosomal DNA
RR	Recovery rate
RRs	Recovery rate surrogate
RSD	Relative standard deviation
SD	Standard deviation
T	Transect (like T-1 for Transect 1)
TBT	Tributyltin
UBA	German environmental protection agency (Umweltbundesamt)
U-Test	Mann-Whitney U-Test
USA	United States of America
USEPA	United States Environmental Protection Agency
WFD	Water framework directive

List of used element abbreviations - Aluminium (Al), **Antimony (Sb)**, Barium (Ba) **Cadmium (Cd)**, Cerium (Ce), **Chromium (Cr)**, Cobalt (Co), **Copper (Cu)**, Iridium (Ir), Iron (Fe), **Lead (Pb)**, Lithium (Li), Molybdenium (Mo), **Nickel (Ni)**, Osmium (Os), Palladium (Pd), **Platinum (Pt)**, Ruthenium (Ru), Rhodium (Rh), **Silver (Ag)**, Strontium (Sr), Thullium (Th), Vanadium (V), Yttrium (Y), **Zinc (Zn)**

Chapter 1

Background and scope of the thesis

Since the 1970s, the heavy metal pollution in German rivers has been reduced considerably. The construction of effective sewage treatment systems and the technical modernization of industrial processes have mainly contributed to this improvement in the quality of rivers (Luoma & Rainbow, 2008). According to Arle et al. (2010) the highest pollutant reductions were achieved for Mercury (99%), Lead (89%) and nickel (47%). Nonetheless, rivers in Germany are still polluted today. Davis et al. (2001) as well as Scherer et al. (2003) found that in contrast to the period of 1980 to 1990, today's emissions of heavy metals into German rivers are predominately generated by diffuse sources. According to their analysis, "paved urban areas" and "erosion" are the main contributors to the heavy metal load of aquatic systems. Only 23% of the river systems monitored in Germany meet the environmental quality standards for heavy metals (Arle et al., 2010). Those environmental quality standards (EQS) were defined by the EU in the Directive 2008/105/EC, commonly called the Water Framework directive (WFD), in order to protect the aquatic environment. In Germany they were ratified in 2011 (Bundesregierung Deutschland, 2011). The main aim of the WFD is the achievement of good chemical constitution for all water bodies by 2015 (Arle et al., 2010). In order to achieve this goal, a further reduction of metal pollution in German rivers is necessary.

Within the WFD the European Commission has identified 33 priority pollutants (including, Cd, Pb, Hg and Ni) which should be monitored to assess the chemical status of a aquatic ecosystem. Further 162 specific substances (including Ag, Cr, Cu, Tl and Zn) are also monitored, if they are emitted in a significant quantity, in order to asses the ecological status of the river (Bundesregierung Deutschland, 2011). All other heavy metals are not subject to mandatory monitoring program and there distribution and concentrations in freshwater systems are undiscovered. However, regularly, the implementation of new technologies causes changes in heavy metal sources and fluxes. These might have positive effects like the banning of Pb in gasoline, which resulted in a huge decrease of Pb in aquatic systems (Rosenbaum-Mertens, 2003). But it also does have

negative effects, especially when the emission of heavy metals increases. This might be the emission of a specific heavy metal, or a metal in a specific chemical speciation. An example for the latter case is the introduction of Sn in the form of Tributyltin (TBT), which was introduced as antifouling paint for ships in the 1960s. It took 20 years to identify that TBT is acute toxic for fish and aquatic invertebrates and might negatively affect humans. Then it took another 20 years to ratify a moratorium of the use of TBT in antifouling paint (EPA, 2008). More recent examples of trace metal flux changes are the release of Platinum Group Elements (PGE) and heavy metals in the form of nanoparticles. In both cases, research on the effects on aquatic systems is still at the beginning. Especially, as both heavy metals are not included in the regular monitoring programs. This thesis aims to fill some of the knowledge gaps, which prevent a thorough assessment of the risks that Platinum (as a reference metal for PGE) bears for aquatic ecosystems.

Platinum belongs to the Platinum group elements (PGE). This is a group of six metals: Iridium (Ir), Osmium (Os), Palladium (Pd), Platinum (Pt), Ruthenium (Ru), and Rhodium (Rh). They belong to the rarest elements on earth as their abundance in the upper Earth crust is about 10^{-6} to 10^{-7}% (Hoppstock & Sures, 2004). Nowadays, these metals are of high interest for industrial processes due to their special characteristics with regard to corrosion resistance and high melting points, especially in their metallic form. The global demand in particular of Pt, Pd, and Rh is steadily rising, due to new applications in the industry (i.e. organometallic chemistry, surfaces-, materials- and crystal engineering, photo- and electrochemistry, catalysis and organic synthesis), whereas the demand for Ir, Os and Ru remains lower (Hoppstock & Sures, 2004). Consequently, the extended usage of noble metals resulted in an increase of PGE amounts emitted into the environment (reviewed in Ek et al. (2004); Hoppstock & Sures (2004); Ravindra et al. (2004); Zimmermann & Sures (2004); Rauch & Morrison (2008) and Dubiella-Jackowska et al. (2009)). In contrast to their economic significance, the distribution of PGE in urban aquatic ecosystems and their effects on biota, has gained relatively little attention.

As for most heavy metals, the pollution sources for Pt into aquatic system are diverse. 30% of the total annual Pt production are used in the industry, for jewelry, and for the production of automobile catalyst converters, respectively (Johnson Matthey, 2011). Due to its use Pt is emitted via industrial point sources, where it is used as a process catalyst for manufacturing chemical products (e.g. nitric acids, hydrogen peroxide), in the glass industry for producing glass fibres or LCD glass, in the electronic industry and in the chemical industry for the production of chemical fibres (IWW, 2004; Johnson Matthey, 2011).

A further source of Pt emission is traffic. Pt is the main catalytic element in diesel vehicles and is also used to a smaller amount in catalytic converters of gasoline vehicles (Johnson Matthey, 2011). Pt catalyses the oxidation of nitrogen oxides, hydrocarbons and carbon monoxides, and thus reduces their emission. However, it is itself emitted within the exhaust fumes. Due to mechanical abrasion, Pt separates from the catalyst converter and is emitted into the environment as particles attached to aluminium oxides (Artelt et al., 1999). Emission rates of an individual car

range from a few ng/km to 100 ng/km (Artelt et al., 1999; Moldovan, 2007). Similar to other traffic related heavy metals, also Pt enters river systems by different pathways. It is transported via the atmosphere and is eventually deposited on the streets. During a precipitation event, it is washed into the road runoff. Within or near cities, road runoff is often collected in central stormwater drainage systems and municipal sewer systems (Uhl et al., 2006). Outside cities road runoff is frequently treated decentralized: It is infiltrated locally, or different technical treatments are performed before road runoff is discharged into a receiving water body (Ceko & Waltz, 2011).

Other possible contaminations sources like hospitals, where Pt is used in cancer therapies, and the jewelry industry tend to be of minor global importance (Kümmerer et al., 1999; IWW, 2004), but may have serious effects on a local scale.

While discharges of the industry can easily be monitored and controlled (see also IWW, 2004), discharges from traffic, especially that occurring outside of urban centers are difficult to control or even predict, as no statistical data for the discharge of road runoff is available (Hillenbrand et al., 2004). However, more than half of the annual mileage is driven outside of cities, on regional, national or international roads (Hillenbrand et al., 2004; Ceko & Waltz, 2011). Consequently, this category of roads is considered to play an important role in the analysis of traffic related discharge. In Germany 231,000 km of roads are attributed to this category and a further expansion of the road networks to approximately 240,000 km until 2015 is predicted to be necessary to meet the requirements of increasing traffics (Ceko & Waltz, 2011). This implies that also the discharge of traffic related heavy metals will increase and more aquatic ecosystems will be affected. However, the knowledge of the longterm effects of road runoff on stream systems is scarce (Uhl et al., 2006). Especially for Pt, which is not well investigated yet.

Laboratory studies performed with aquatic organism like gammarids, mussels, snails and fish revealed the acute toxicity and sublethal effects of Pt on aquatic animals. Borgmann et al. (2005) tested the acute toxicity of Pt on *Hyalella azteca* and found that it is lower than the acute toxicity of Cd, Cr, Hg and Pb. Sublethal effects were tested on several aquatic animals. The studies observed histopathological effects on liver and intestinal tissues in fish and on the hepatopancreas, gill and epidermal tissues of snails (Jouhaud et al., 1999a,b; Osterauer et al., 2010a). Studies including the effects of Pt on the embryonic development revealed that the hatching success of zebra fishes and paradise snails decreased due to the exposure with Pt (Osterauer et al., 2009). Further, malformations were detected in the development of the snails (they did not develop an outer shell) (Osterauer et al., 2010b). *Daphnia* reacts with growth reduction and smaller protein content as well as a reduction of glutamic oxaloacetic transaminase (GOT) production when exposed to Pt (Biesinger & Christensen, 1972) and Singer et al. (2005) could show that mussels do induce the production of heat shock proteins (hsp70).

However, most of these results are based on Pt concentrations in the range of µg/g in tissues. It remains to be investigated, whether effects can still appear under *in situ* conditions in the envi-

ronment. In addition, potential genotoxic effects of Pt on aquatic animals are still a subject of controversial discussion in the literature. For example, genotoxic effects were found in *in vitro* cell experiments, caused by specific chemical Pt speciations (Bünger et al., 1996; Gebel et al., 1997; Migliore et al., 2002). DNA damage was observed for aquatic invertebrates, like snails, but not for vertebrates (i.e. fish) (Osterauer et al., 2011).

Thus, further research is necessary in order to evaluate the potential risk of Pt in environmentally relevant concentrations.

The German Advisory Council on the Environment addressed Pt in its penultimate report 2004 as a new health related environmental risk emitted by traffic (SRU, 2004). They also concluded, that PGE emissions should be continuously monitored at selected sites in order to allow for a more detailed analysis of future trends according to the PGE pollution. Also, the accumulation of PGE by fish and mussels should be monitored. However, until now no Pt monitoring program has been launched for aquatic systems in Germany (Ceko & Waltz, 2011).

This thesis follows the recommendations of the German Advisory Council on the Environment. Its objective is to extend the knowledge about the occurence, distribution and impact of Pt in river systems in order to come to conclusions about the importance of Pt as a pollutant and its toxic effects in aquatic ecosystems.

In total four different investigations were performed and presented in four different chapters of the thesis. First the analytical procedures used in this thesis are carefully validated. In the second study the actual distribution of Pt in different sample matrices of a river (i.e. sediment and biota) was analyzed at a rural discharge site (see Chapter 3). The accumulation of Pt by mussels, fish and fish parasites was further investigated under laboratory conditions (see Chapter 4 and 5). Furthermore, a possible genotoxic effect of Pt was examined (as well Chapter 4 and 5):

Chapter 2: Validation of analytical procedures The first part of the thesis, focuses on the validation of all analytical procedures used in the thesis. As Pt was analyzed in different concentration ranges and different sample matrices in the following studies and further compared to other traffic related heavy metals, the validation of several analytical procedures consisting of different digestion and detection method combinations was necessary . In order to guarantee a good analytical quality throughout the thesis, analytical procedures were proofed to deliver accurate and precise results. As field samples were analyzed also the limit of detection of each analytical procedure was investigated in order to ensure that a quantification of Pt and other traffic related heavy metals in environmentally relevant concentration ranges was possible.

Chapter 3: Introduction of traffic related Platinum into river systems - Occurrence and distribution of Platinum in sediments and biota In the second part a passive sampling was

performed in order to provide evidence about the Pt introduction into river systems. It should further deliver a detailed picture about the distribution of Pt in sediments and its uptake by biota. The chosen sampling site represented a typical discharge location: A highly frequented federal highway crossed a river and the road runoff was discharged into the river via three different inlets. The asiatic clam *Corbicula* sp. was evenly distributed in the sediment and served as bioindicator species for Pt uptake by aquatic organism. Sediment and clam samples were taken at a reference site upstream of the inlets as well as along three transects of 20 to 100 m, downstream from the inlets. All samples were analyzed in order to obtain data about the concentration ranges in which Pt can be found in river system. They should provide evidence about the distance Pt is transported, and the bioavailability of Pt for clams. In order to classify the environmental relevance of Pt in river systems, Pt concentrations in sediments and biota were compared to the concentration, distribution, and bioavailability of other traffic related heavy metals.

Chapter 4: Accumulation of different Platinum concentrations by *Corbicula* sp. and genotoxic effect *Corbicula* sp. was exposed to different environmentally relevant Pt concentrations under laboratory conditions. As *Corbicula* sp. is a wide spread organism in European river and lake systems, it was tested, if it could possibly serve as a biomonitor organism for Pt. The uptake of Pt by the clams was observed for ten weeks. It was examined, if Pt concentrations in the clam reflected the concentration in the surrounding environment and how much of the offered Pt was taken up by the clam. Furthermore, effects of Pt on the clam were investigated. Endpoints for this study were the mortality and the induction of micronuclei in the haemocytes and gill cells of the clams. The micronucleus test was used to examine, whether Pt induces genotoxic effects for *Corbicula* sp, or not.

Chapter 5: The accumulation of Platinum by *Squalius cephalus* and *Pomphorhynchus* sp. and the genetoxic effect of Platinum on fish erythrocytes In Chapter 5 some of the questions discussed in Chapter 4 were further adressed to fish and fish parasites. The accumulation of Pt was investigated for fish and its intestinal parasites and the genotoxicological potential was examined for fish. *Squalius cephalus* was exposed to Pt and the kinetics of its uptake was analyzed in muscle, liver and intenstinal tissues. As the group of acanthocephlans is already known to influence the metal metabolism of fish, the exposure study was performed with uninfected as well as experimentally infected fish. The study was focused on two different parasite species, i.e. *Pomphorhynchus laevis* and *Pomphorhynchus tereticollis*. Erythrocytes of fish from all treatment groups were analyzed for micronucleus induction in order to investigate the genotoxicity of Pt for fish.

By combining a monitoring study with laboratory experiments, this thesis will give a detailed overview of the ecotoxicology of Pt in freshwater systems. Based on data from the field, the concentration ranges, the distribution, and the transport of Pt in rivers will be characterized. In

addition the field samples will be used to determine the bioavailability of Pt for aquatic organisms while the accumulation kinetics of Pt and its genotoxicity for mussels and fish will be examined as part of the laboratory experiments. Consequently, the results generated by this thesis provide the foundation for an evaluation of the risks that Pt poses for freshwater systems.

Chapter 2

Validation of analytical procedures

2.1 Introduction

A crucial aspect of all studies investigating the environmental fate of specific elements, is the quality of the analytical procedures used in the studies. Before starting a monitoring study or an exposure study, it is necessary to choose an adequate methodology, which will be used to quantify the amount of the specific elements.

Within this thesis several sample matrices, including sediment, fish and clam samples were analyzed. While the sediment and some of the clam samples were derived from a field study, other clam and fish samples were obtained from exposure studies. In all these studies Pt was the main element analyzed. The concentration range of Pt in those samples extended from very low, environmentally relevant concentrations (low ng/g range), to high contaminations (µg/g range). Furthermore, Pt was compared to other traffic related heavy metals. An overview of traffic related heavy metals and their sources can be found in Table 2.1.

As the term "heavy metals" is used differently in the scientific literature (Luoma & Rainbow, 2008), it is defined as a term incorporating metals with a specific gravity greater than 5 for this thesis. The use of the term in this thesis does not include any implicit description of metal characteristics concerning their toxicity or to their function in the metabolism of organisms.

As some heavy metals are thought to have a greater influence on aquatic organism than others, only those elements were chosen for the analyses, which were classified as priority pollutants by the United States Environmental Protection Agency (USEPA) as cited in Breault et al. (2000). Those are: Cd, Cr, Cu, Pb, Ni, Sb and Zn. Ag was additionally included into the analyses, as it is not related to the traffic. It is used as a kind of control element, giving proof that changes in metal concentrations are due to discharges of road runoff and not from other sources.

Table 2.1: **Sources of traffic related heavy metals.**

Source	Heavy metal[a]
Exhaust fumes	Al, Ba, Cr, Co, Cu, Pb, Li, Ni, Sb, Sr, V, Zn
Tyre abrasion	Cd, Cu, Fe, Pb, Zn
Brake abrasion	Al, Ba, Cr, Cu, Fe, Ni, Mo, Sb, Sn, V, Zn
Catalytic converters	Ce, Pd, Pt, Rh
Corrosion	Cd, Cr, Cu, Fe, Ni, Zn
Road surface abrasion	Cr, Ni

[a] Information is summarized according to the studies from Breault et al. (2000); Hillenbrand et al. (2004) and Uhl et al. (2006).

The aim of this chapter is to find a combination of analytical procedures which is suitable for sediment, clam and fish samples with a wide spectrum of concentration ranges. At the same time the procedures should allow for the analysis of as many traffic related heavy metals as possible. Thus the procedures should meet the following requirements:

- give accurate and precise results,
- allow for the detection of Pt and other traffic related heavy metals within the same digestion solution (for reasons of better comparability within samples),
- offer a limit of detection low enough to gain significant results even for samples from unpolluted sites in the field and from exposure studies with very low exposure concentrations.

Figure 2.1 summarizes the samples matrices, preparation steps, digestion methods and detection methods which will be validated for traffic related heavy metals.

The analysis of Pt, especially in biotic matrices and environmentally relevant concentrations is generally considered to be challenging (Bencs et al., 2003; Dubiella-Jackowska et al., 2007; Balcerzak, 2011). Due to its low natural background level, Pt concentrations in environmental samples are still very low compared with other heavy metals. Reviews of Pt in environmental matrices reveal that Pt concentrations are usually in the range of ultra trace concentrations (low ng/g) in all environmental matrices, while all other heavy metals can be found in a µg/g range (Ravindra et al., 2004; Ek et al., 2004; Hoppstock & Sures, 2004; Zimmermann & Sures, 2004; Rauch & Morrison, 2008; Dubiella-Jackowska et al., 2009; Haus et al., 2009b; Kalavrouziotis & Koukoulakis, 2009). In addition, environmental transformation processes as well as biological uptake pathways of Pt are largely unknown as the precise quantification of very low metal concentrations (<1 ng/g) in biological materials requires very sophisticated analytical techniques (Haus et al., 2007a). For

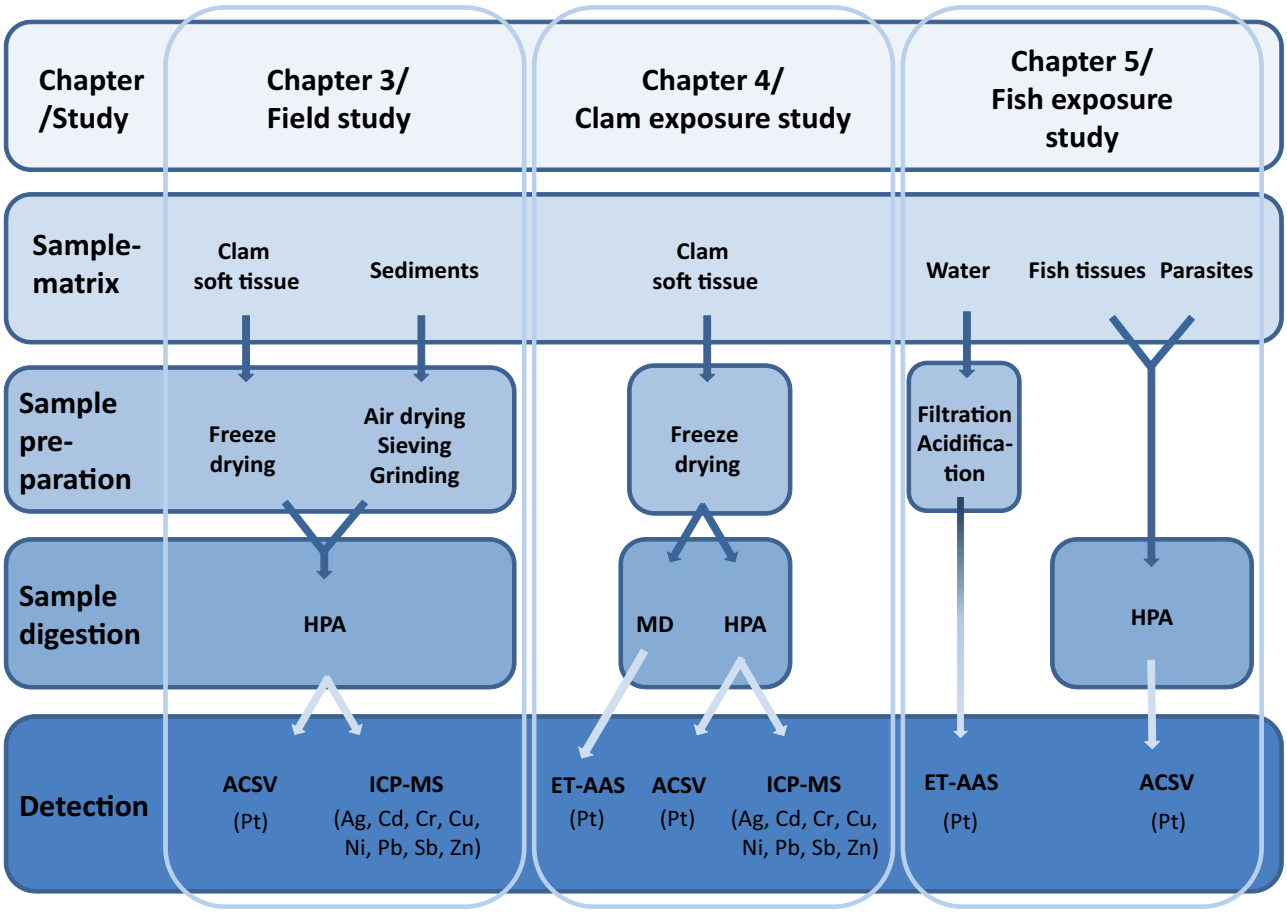

Figure 2.1: **Overview of the used analysis procedures for the analysis of traffic related heavy metals in this thesis.**

higher concentration ranges, which can be obtained in exposure studies, the ICP Atomic Emission Spectrometry (ICP-OES) or Electrothermal Atomic Absorption Spectrometry (ET-AAS) can successfully be employed, as has been shown in the studies of Zimmermann et al. (2003); Singer et al. (2005); Sures & Zimmermann (2007); Tsogas et al. (2008); Osterauer et al. (2010b); Petrova et al. (2010). In this study ET-AAS will be validated in order to use this method to quantify samples with high concentrations of Pt (µg/g range), which are obtained in exposure studies.

For low concentration ranges of heavy metals, mostly ICP-MS is the detection method of choice. However, interferences prevent a correct analysis of Pt. Hafnium oxides HfO^+ which are formed in the plasma can interfere with the detection of all Pt isotopes . This problem can be overcome with a high resolution ICP-MS technique or a variety of matrix separation steps and/or mathematical corrections (Hees et al., 1998; Müller & Heumann, 2000; Bencs et al., 2003; Balcerzak, 2011). To avoid these problems the analysis of Pt with ICP-MS is neglected for this thesis. In 1986, Zhao and Freiser published an alternative procedure to analyze Pt by using an electrochemical method (Zhao & Freiser, 1986). According to this study the method allows a detection limit of up to 20 pg/g. This method was later improved by Alt et al. (1988) and Messerschmidt et al. (1992) for

biological matrices. They digested the samples with a high pressure ashing digestion method and detected Pt with the adsorptive cathodic stripping voltammetry (HPA/ACSV). It will be used to determine Pt for samples with low concentrations (ng/g) in this thesis.

Other traffic related metals will be analyzed using ICP-MS. However, as one of the aims of this chapter is to use the same digestion solution for analyzing Pt and all the other heavy metals, it has to be proved, if the digestion solution, optimized for the ACSV detection is also suitable for the ICP-MS detection.

Within this chapter the different combinations of sample digestion and detection methods will be tested for accuracy (i.e. recovery and precision of the element analysis) and the limits of detection (LOD). The aim is to identify the strengths and weaknesses of the above mentioned analytical methods in order to decide which metal, will be analyzed using which analytical procedure at what analytical quality in the following studies of this thesis.

2.2 Material and Methods

In the following studies, different sample matrices with different contamination ranges have to be analyzed. To achieve reliable results different analytical procedures have to be used. An analytical procedure in this thesis is defined as a process consisting of the following steps:

- sample preparation
- sample digestion
- analytical detection

Preparation of sediment and clam samples are described in Chapter 3 and the preparation of fish tissues is described in Chapter 5. Especially the combination of sample digestion and analytical detection has to be chosen based on the parameters, such as sample matrices, the metals which should be analyzed and the concentration range of those metals.

2.2.1 Digestion methods

For all detection methods used, heavy metals bound in the samples have to be brought into solution. To cover the different Pt concentration ranges in the samples, two different combinations of digestion and detection methods were validated. Samples with high Pt concentrations were digested by a microwave digestion (MD) followed by a detection via electrothermal atomic absorption spectroscopy (ET-AAS). Samples with low Pt concentrations were digested with the high pressure asher (HPA), followed by a detection via adsorptive cathodic stripping voltammetry (ACSV). For the analysis of further traffic related heavy metals an aliquot of the resulting HPA digestion solution was additionally analyzed by ICP-MS.

Microwave Digestion (MD) The microwave digestion was employed for those samples, where Pt concentrations were expected to be high enough for a Pt detection using ET-AAS. This is primarily the case for clam tissues samples which were exposed to high Pt concentrations (100 µg/L).

Microwave digestion enables to rapidly heat the digestion agents and the sample material due to the microwave energy and it allows for the simultaneous digestion of a multitude of samples. The specific microwave digestion method used in this study was developed by Sures et al. (1995). It was especially adapted to small sample amounts and to the consecutive detection by ET-AAS.

Approximately 70 mg of freeze dried clam tissue was weighed and placed into the digestion vessels consisting of perfluoralkoxy. Next, 1.8 ml nitric acid (HNO_3) (subboiled from 65% HNO_3

p.a., Fisher Scientific, Leicestershire, UK) were added. Twelve different samples could be digested simultaneously in the microwave system (CEM MDS 2000 Microwave Digester, CEM Corp., Matthews, North Carolina, USA). A pressure control was connected to the vessel with the highest sample mass. The pressure in this vessel was analyzed during the digestion procedure and the microwave energy was controlled based on the pressure signal. The digestion program consisted of four different steps (see Table 2.2). During step 1 to step 3, samples were heated until a specific pressure value was achieved within the vessels. For each step this pressure was held for a certain period of time. The maximum pressure achieved during this program was 3.44 bar. During the fourth step of the program, the samples cooled down and the pressure in the vessels reduced to $\leq$ 0.5 bar.

Table 2.2: **Pressure control program of the microwave digestion.**

Step	1	2	3	4
max radiation (W)	$650 \pm (50)$	$650 \pm (50)$	$650 \pm (50)$	0
max pressure (bar)	2.48	2.96	3.44	≤ 0.5
max duration (min)	12	12	12	15
Duration of pressure setting (min)	3	3	5	0

The program was ended and the vessels were opened after the pressure decreased to approximately 0.5 bar. The digestion was considered successful if the solution was transparent and no suspended solids were visible. After every digestion the vessels were thoroughly cleaned using a cleaning digestion program. In addition to the sample digestion, several digestion solutions without sample material were prepared. Those procedural blanks should provide an indication of a potential contamination of the digestion vessels and also severed as a basis for the calculation of the limit of detection (LOD) and limit of quantification (LOQ).

High pressure asher digestion (HPA digestion) For sample materials containing only low Pt concentrations the ACSV was used as detection method. For this detection method a complete destruction of organic matter is neccessary, as organic compounds can attach to the mercury drop and disturb the analysis (Haus et al., 2009a). Low Pt concentrations were found in all samples of the river Alb, in tissues from clams exposed to concentrations lower than 100 µg/L, and in fish tissues and parasites. The advantage of the HPA digestion compared to the MD is, that samples and digestion agents can be exposed to a more extreme temperature and pressure regime during the digestion process. This results in a thorough destruction of the organic matter in the samples. Furthermore, the digestion solutions were also used for the detection of Ag, Cd, Cr, Cu, Ni, Pb, Sb, and Zn by ICP-MS to allow for a correlation analysis between the different metals in the same sample aliquots.

For the digestion up to 270 mg freeze dried tissue, or 1 g fresh tissue, or 120 mg sediment sample was added to a quartz vessel (Kürner Analysentechnik, Rosenheim, Germany). Those vessels were cleaned by vapor cleaning with concentrated HNO_3 before each digestion. The vessel set used for sediment samples was additionally cleaned using hydrofluoric acid (HF), latest after three digestions. To the samples 4 ml HNO_3 (subboiled from 65% HNO_3 p.a., Fisher Scientific, Leicestershire, UK) and 0.5 ml HCl (30%, superpure grade, Merck, Darmstadt, Germany) were added. The vessels were closed using a seal ring, teflon tape and quartz caps. Five vessels can be placed into the HPA and digested simultaneously. In the HPA (HPA, Kürner Analysentechnik, Rosenheim, Germany or HPA-S, Anton Paar, Graz, Austria) samples were heated to 320 °C and 130 bar within 50 min. For another 50 min temperature and pressure conditions were maintained constant before the samples cooled down. The HPA was opened when temperature had decreased to approximately 35 °C.

After digestion, the digestion solution was levelled to 10 ml (using millipore water). If samples were to be analyzed by ACSV and ICP-MS, 2 ml of the digestion solution were taken for the ICP-MS analysis and stored at room temperature in 2 ml vessels (PP-Tubes, 2 ml, Sarstedt, Nümbrecht, Germany).

Next to organic compounds, also the presence of nitric acid has negative effects on the Pt analysis with ACSV. The detection curve (amplitude of the measurement) becomes compressed and the baseline (i.e. start of peak until end of peak) is moved to higher currents. This results in lower peak heights and higher LODs, even if the concentration of HNO_3 in the solution is very low (Zhao & Freiser, 1986; Haus et al., 2009a). Therefore, all HNO_3 had to be removed from the digestion solution. HNO_3 can be removed by evaporation and a following replacement with sulfuric acid (H_2SO_4). In accordance with Alt et al. (1994), 0.3 ml H_2SO_4 (96%, suprapure grade, Merck, Darmstadt, Germany) and 0.5 ml HCl (30%, suprapure grade, Merck, Darmstadt, Germany) were added to the digestion solution. The digestion vessels were placed in an aluminium block and heated on a ceramic heating field to approximately 160 °C. The solution was evaporated until only 0.5 ml remained in the vessels. Then 0.5 ml HCl were added and again the solution was evaporated to 0.5 ml. This step was repeated until no nitric (i.e. brownish) gases resulted from the addition of hydrochloric acid. Then the solution was evaporated to a volume of 0.3 ml and added with millipore water to 5 ml. The resulting solution was stored in 15 ml PE vessels (PE-Tubes, 15 ml, Sarstedt, Nümbrecht, Germany) by room temperature and darkness until detection.

2.2.2 Detection methods

Depending on the expected Pt concentrations in the samples and the metals which should be detected, one of the following detection methods was used in this thesis:

- Adsorptive cathodic stripping voltammetry (ACSV) for Pt concentrations in the pg/g to ng/g range
- Electrothermal atomic absorption spectroscopy (ET-AAS) for Pt concentrations in the µg/g range and
- Inductively coupled plasma mass spectroscopy (ICP-MS) for all metals besides Pt at the following mass lines: ^{52}Cr, ^{60}Ni, ^{63}Cu, ^{66}Zn, ^{107}Ag, ^{111}Cd, ^{121}Sb, ^{208}Pb.

Adsorptive cathodic stripping voltammetry (ACSV) Up to now, the ACSV is considered the most sensitive method for Pt detection in biological samples (Balcerzak, 1997; Zimmermann et al., 2001).

The voltammetric analysis was performed as described by Alt et al. (1988, 1994) with a VA Processor 693 and a Voltammetric Measuring Stand 694 (Metrohm AG, Filderstadt, Austria), including the following instrumental set-up:

- working electrode: hanging mercury drop electrode (HMDE)
- auxiliary electrode: glassy carbon
- reference electrode: Ag/AgCl/KCl (3 mol/L)

The volume of the digestion solution used for the analyses depended on the expected Pt concentrations in the sample. The lower the Pt concentrations, the more digestion solution had to be used. The added sample volumes ranged from 20 µl to 2 ml. Those were transferred to 15 ml electrolyte (98 ml 0.36 mol/L sulphuric acid, mixed with 1.5 ml hydrazine sulfate (0.01 mol/L) and 0.05 ml formaldehyde solution (37 vol.%, suprapure grade, Merck, Darmstadt, Germany)).

According to Alt et al. (1994) Pt in the electrolyte builds a Pt-formazone complex (see Figure 2.2). This complex was adsorbed at a mercury drop (size 3) at a potential of 0.6 V (against 3 mol/l Ag/AgCl) for 1 minute. Subsequently, a potential range of -0.5 V is applied in the differential pulse mode to measure the catalytic current of the hydrogen wave at about -0.85 V. The quantitative determination was performed by the standard addition method, which ideally consisted of five measurements (see Figure 2.3).

After a de-aeration with N_2 for 5 minutes, Pt is analyzed in the electrolyte to ensure that the electrolyte is not contaminated with Pt. For the second measurement, the digestion solution is added. Afterwards, a standard solution with known Pt concentration is added three times and subsequently analyzed. After the standard addition, the peak heights of every analysis will be plotted against the amount of added platinum. Following linear regression analysis, the Pt concentration in the sample can be calculated. The regression coefficient should be as high as possible, preferred values should be higher than 0.99.

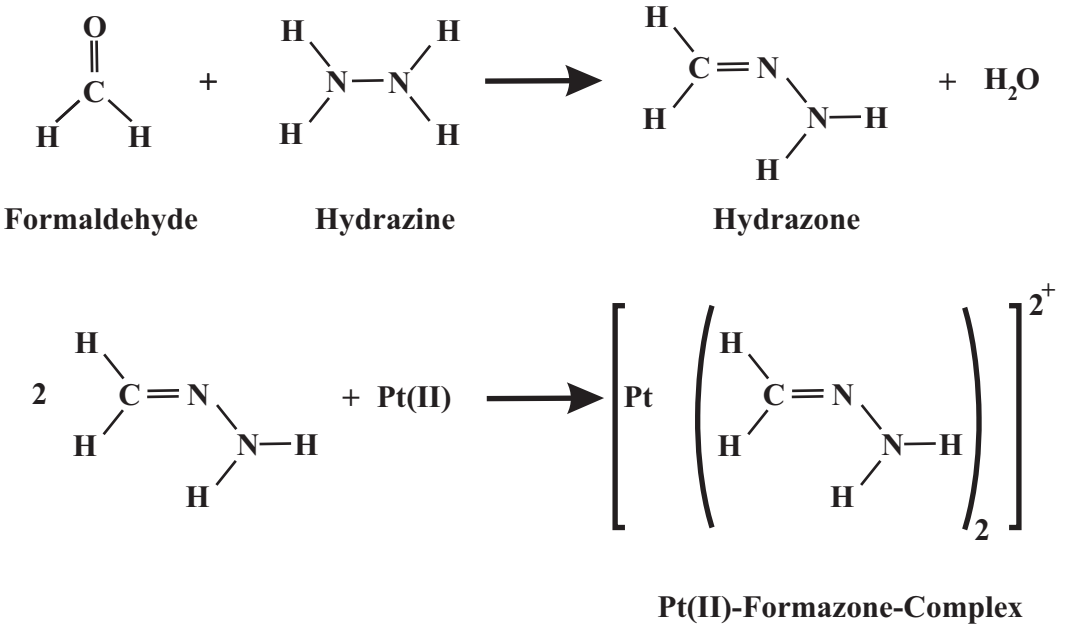

Figure 2.2: **Formation of the Pt(II)-Formazone-Complex (adapted from Metrohm (2003)).**

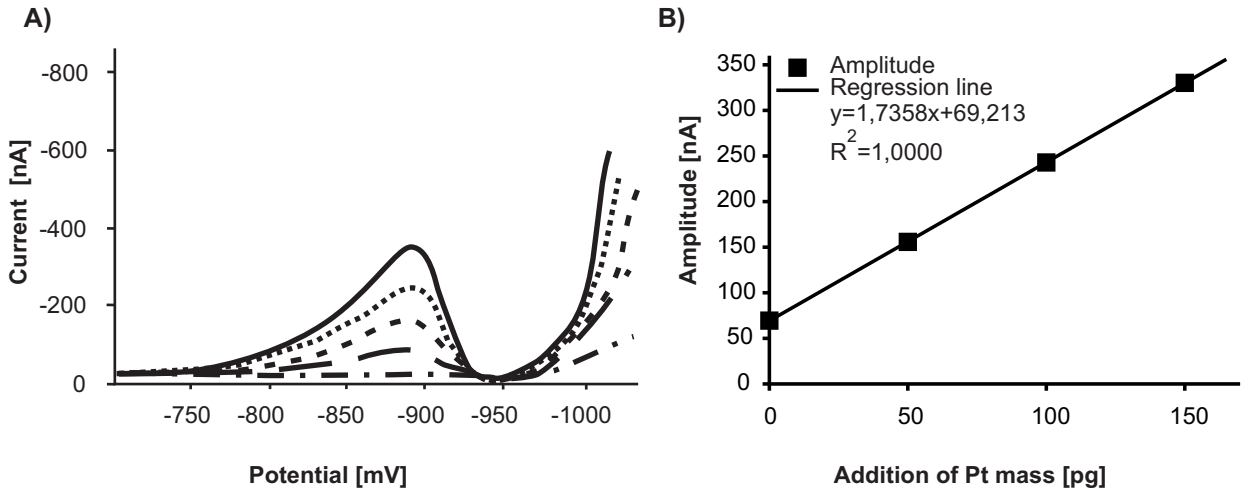

Figure 2.3: **Characteristic curves (A) of a standard addition and the resulting plot with regression line (B) after ACSV analysis.**

Electrothermal atomic Absorption Spectroscopy (ET-AAS) ET-AAS was used for clam samples exposed to 100 µg/L Pt (see Chapter 4) and water samples of the fish exposure study (see Chapter 5). In contrast to the other detection methods described in this chapter, ET-AAS does not require laborious sample preparation steps.

Throughout this study only Pt was analyzed via ET-AAS. The Atomic Absorption Spectrometer 4100 ZL (Perkin Elmer, Überlingen, Germany) equipped with a Zeeman effect background correction system was used. The method used was optimized for animal samples by Zimmermann

et al. (2003). Water samples were analyzed without any digestion of the sample and treated identically to the digestion solution of clam samples. Both sample matrices were diluted with millipore water. The dilution factor depended on the Pt concentration in the sample. This resulted in a dilution range from factor 1 to factor 5. Then 40 µL of diluted sample solutions were introduced into the graphit tube by an autosampler system (AS 70, Perkin Elmer, Überlingen, Germany). The sample was heated to different temperature plateaus (see Table 2.3).

Table 2.3: **Temperature program of the ET-AAS analysis.**

Step	Time to ramp (s)	max Temperature (°C) /Duration (s)
1	1	110/5
2	10	140/60
3	20	600/1
4	30	1300/20
5	0	2500/3
6	1	2600/3

For the detection, a light of a Pt specific wavelength (265.9 nm) was sent through the atomized sample. Calibration was carried out as a matrix adapted calibration approach. Six different solutions of a Pt standard solution (1000 mg Pt/L, Ultra Scientific, Wesel, Germany) were added to a digestion solution of a clam control sample, or to a water control sample, depending on the matrix of the samples to analyze. A linear regression line was fitted with the peak area results of the analyses and Pt concentrations of samples were calculated. The correlation coefficient of the linear regression line was always higher than 0.99.

Inductively coupled plasma mass spectroscopy (ICP-MS) The ICP-MS combines two advantages in one detection method: High sensitivity and the possibility of multi element analysis (Thomas, 2001). Within this thesis it was used for the detection of Ag, Cd, Cr, Cu, Ni, Pb, Sb and Zn in sediment and clam samples using the following mass lines: ^{52}Cr, ^{60}Ni, ^{63}Cu, ^{66}Zn, ^{107}Ag, ^{111}Cd, ^{121}Sb, ^{208}Pb. It was, however, not used to analyze Pt, as the detection of Pt in animal samples via ICP-MS presents several difficulties, especially in case of environmentally relevant concentrations, due to spectral interferences (Bencs et al., 2003).

The ICP-MS used in this study was a Perkin Elmer Elan 5000 Quadrupole ICP-MS. For the analyses, samples were digested with the HPA digestion as described above. Parameters used for the ICP-MS analyses are listed in Table 2.4.

Prior to the detection, digestion solutions were diluted with a solution of 1% HNO_3 (subboiled) by

Table 2.4: **Parameters used for the ICP-MS analysis.**

Parameter	Settings
Power	1070 W
Peristaltic Pump Speed	8.3 ml/min
Scan Mode	Peak Hop
Torch	Quarz Torch
Spray chamber	Scott Double Pass Chamber
Cones	Nickel
Plasma Gas	Argon 14.4 L/min
Auxiliar Gas	Argon 0.55-0.65 L/min
Nebulizer Gas	Argon 0.8-0.85 L/min
Temperature of spray chamber	room temperature (20 °C)
Sweeps/Reading	25
Readings Replicate	1
Replicates	3
Points/Spectral Peak	1
Resolution	normal

the factor 1:10. Yttrium (Y) and Thulium (Th) were used as internal standards for clam samples with a concentration of 10 µg/L each. A quality standard solution (QS) (ICP Multielementstandard IV solution, Merck, Darmstadt, Germany) was analyzed after every tenth sample to control the accuracy and the stability of the measurements. A series of eleven dilutions of a multielement standard solution (ICP Multielementstandard IV solution, Merck, Darmstadt, Germany) were prepared and analyzed for the calibration. The regression line of the calibration (r^2 >0.99) was used to calculate the element concentrations.

2.2.3 Validation experiments - Recovery, precision, limit of detection and limit of quantification

To validate the different analytical procedures three different quality measurements were determined. These are the accuracy, the precision and the limit of detection. The accuracy of an analytical procedure is defined as the closeness of agreement between the result of a measurement and the true value of the measurand (Danzer, 2007). Whereby the precision is defined as the closeness of agreement between independent test results obtained under stipulated conditions (Danzer,

2007). To test the sensitivity of the analytical procedures, the limit of detection (LOD) and the limit of quantification (LOQ) were also determined.

Recovery experiments To investigate the accuracy of the analytical procedures, recovery experiments were performed. For this purpose certified reference materials were digested and analyzed using the different combinations of digestion and detection methods. As the matrix of a sample may have great influence on the analytical results, recovery experiments were carried out for sediment and biotic samples, separately (see summary in Table 2.5).

Table 2.5: **Validation experiments.**

	Digestion method	Detection method	Sample matrix	Analyzed metal(s)	Certified standard material[a]
1	HPA	ACSV	clam, fish, parasites	Pt	none
2	HPA	ACSV	sediment	Pt	BCR-723
3	HPA	ICP-MS	sediment	Ag, Cd, Cr, Cu, Ni, Pb, Sb, Zn	Pacs-2
4	HPA	ICP-MS	clams	Ag, Cd, Cr, Cu, Ni, Pb, Sb, Zn	Dorm-2 and IAEA-407

[a] Certified standard materials are described in detail later in the text and in Table 2.6.

The results of the recovery experiments are presented as the recovery rate (RR). To calculate this ratio, reference materials were analyzed, and the subsequent results were compared to the concentration certified for the reference material.

Recovery rate (RR):

$$RR = \frac{C_a}{C_r} * 100\% \tag{2.1}$$

$with$
$C_a = analyzed\ concentration\ of\ the\ reference\ material$
$C_r = certified\ concentration\ of\ the\ reference\ material$

The certified reference materials are described in Table 2.6.

Table 2.6: **Certified values for the reference materials (means ± 95% confidence interval).**

Reference material	BCR-723	Pacs-2	Dorm-2	IAEA-407
No of analyses	11	7	8	4
Mass used for digestion (mg)	50-100	70-110	70-200	45 -115
Certified values				
Ag (µg/g)	n.c	1.2 (± 0.14)	0.04 (± 0.013)	0.04 (± 0.004)
Cd (µg/g)	n.c.	2.1 (± 0.15)	0.19 (± 0.004)	0.19 (± 0.004)
Cr (µg/g)	n.c.	91 (± 4.6)	35 (± 5.5)	0.73 (± 0.006)
Cu (µg/g)	n.c.	310 (± 12)	2.3 (± 0.16)	3.3 (± 0.08)
Ni (µg/g)	n.c.	40 (± 2.3)	19 (± 3.1)	0.6 (± 0.05)
Pb (µg/g)	n.c.	183 (± 8)	0.07 (± 0.007)	0.1 (± 0.02)
Pt (ng/g)	81 (± 2.5)	n.c.	n.c.	n.c.
Sb (µg/g)	n.c.	11 (± 2.6)	n.c.	0.01 (± 0.001)
Zn (µg/g)	n.c.	364 (± 23)	26 (± 2.3)	67 (± 0.8)

n.c.- not certified

BCR -723 consists of ceiling dust collected from the Tanzenburg Tunnel, Austria in 1998. It was produced and certified by the Institute for Reference Materials and Measurements (IRMM) of the European Commission (Sutherland, 2007).

Pacs-2 is a reference material consisting of marine sediment collected from Esquimalt harbour, British Columbia, Canada. It was produced and certified by the National Council of Canada (National Research Council Canada, 2004).

DORM-2 and IAEA-407 were used to validate the analytical procedures for animal samples. DORM-2 consists of dogfish muscle (*Squalus acanthias*). It was produced and certified by the National Council of Canada (Itoh et al., 2011). IAEA-407 is fish homogenate material (mostly herring) of the North Sea. The material was not certified, but analyzed by 105 laboratories of 47 countries and described by the International Atomic Energy Agency, Marine Environmental Laboratory in Monaco (Wyse et al., 2003). The certified values are listed in Table 2.6.

Recovery experiment without certified reference material As no suitable reference material for Pt analysis in animal samples is available, the RR of Pt in clam tissue had to be analyzed by a surrogate recovery experiment. Therefore, samples of freeze dried clam tissue were divided into four aliquots and spiked with four different dilutions of a Pt standard solution (1000 mg Pt/L, Ultra Scientific, Wesel, Germany) prior to and after the digestion. The measuring solution contained 0 to 100 pg Pt. Two regression lines were fitted to the points defined by the analyzed concentrations prior to and after digestion. The surrogate recovery rate was calculated as the ratio between the gradients of the regression lines of the samples spiked before and after the digestion.

Surrogate recovery rate (RR_s):

$$RR_s = \frac{G_b}{G_a} * 100\% \tag{2.2}$$

$with$
$G_b = gradient\ of\ Pt\ concentration\ spiked\ before\ the\ digestion$
$G_a = gradient\ of\ Pt\ concentration\ spiked\ after\ the\ digestion$

No recovery experiments were conducted for the combination of microwave digestion and ET-AAS detection. This combination was already shown to be highly reliable and has been used frequently for Pt analysis in the same instrumental setting (Zimmermann et al., 2003; Singer et al., 2005; Sures & Zimmermann, 2007; Osterauer et al., 2010b). Zimmermann et al. (2003) reported an recovery of 95% and a precision of 3-9%.

Precision Next to the accuracy also the precision defines the validity of an analytical procedure. It describes to which degree repeated analyses of the same sample vary from each other. For HPA digestion followed by ICP-MS analysis the above mentioned certified reference materials were used (Pacs-2, Dorm-2, IAEA-407) to investigate the precision. The precision of the Pt analysis was determined by multiple analyses of BCR-723 for sediment samples and multiple analyses of the same sample of homogenized freeze dried clam tissue. The clam sample was obtained from the river Alb (see Chapter 3) and contained 0.63 ng/g Pt.

The precision was calculated as the relative standard deviation (RSD) of those repeated analyses of the same sample (using Equation 2.3).

Relative standard deviation (RSD):

$$RSD = \frac{SD}{\bar{x}} 100\% \tag{2.3}$$

$with$
$SD = standard\ deviation$
$\bar{x} = mean$

Limit of detection (LOD), limit of quantification (LOQ) LOD and LOQ were determined in accordance to DIN 32645 (DIN, 1994). The "direct method" was performed. This method uses the uncertainty of procedural blank measurements to quantify the LOD and the LOQ. Consequently, digestion solutions, called procedural blanks were prepared. Those procedural blanks were treated like samples: they had the same acid composition during digestion and were analyzed in the same way samples were analyzed. However, no sample material was added. The procedural LOD was calculated as the three-fold standard deviation of the concentrations found in the blanks. The LOQ was calculated as the nine-fold standard deviation of the concentrations found in the blanks.

For the combination of HPA digestion and ACSV detection 45 procedural blanks, for the combination of HPA digestion and ICP-MS detection 28 procedural blanks were analyzed. 15 procedural blanks were analyzed for the combination of microwave digestion and ET-AAS analysis.

Those blanks were prepared next to the preparation of samples, digestion and analyses of blanks accompanied the digestion and analyses of samples. Next to the purpose of calculating the limit of detection and the limit of quantification, procedural blanks had also the purpose to verify that digestion vessels and other instruments used during the analytical procedure were thoroughly cleaned for ultra trace analysis.

2.2.4 Classification of the different analytical procedures

The determination of the recovery rates and precisions of the different analytical procedures does give values according to the accuracy of an analytical procedure. However, the values alone do not evaluate, if the analytical quality is sufficient for the analytical purpose. In literature several guidelines are available which suggest limits for RR and precisions for different analytical purposes. In the studies of this thesis it is important to know the analytical quality for the analyzed heavy metals. To validate the differences in the analytical quality of the procedure used for a specific element, three different quality classes are defined for this thesis. The criteria for these three classes were defined as follows:

- Class A: The recovery rate for a specific element does overlap the certified range of the certified reference material (mean value ± 95% confidence interval) according to the guideline of the german environmental protection agency (UBA) (Wellmitz & Gluschke, 2005). For analytical procedures, which are validated without a certified reference material, class A is defined by a recovery rate between 95 and 105%. For both cases, the precision is 15% or below (for elements which are analyzed close to the LOD: 20% or below).

- Class B: The recovery rate is between 80% and 110%, the precision is 15% or below (for elements which are analyzed close to the LOD: 20% or below). These criteria are used by European Medicines Agency (2009) and Haedrich (1994) to define a good analytical quality of an analytical procedure for a specific element.

- Class C elements: Metals which show an accuracy between 70 and 80% or 110 and 120% and/or a precision between 16% and 25% are also incorporated in the data analyses of the following chapters. However, during the interpretation of the results, it should be taken into consideration, that those concentrations might be severely under- or overestimated. This criterion is not uncommon in environmental monitoring studies, as elements with comparable RRs and precisions were already included in the data analysis of other monitoring studies (e.g. Perdikaki, 1999; Seco-Gesto et al., 2007; González et al., 2008).

Heavy metals which do not refer to the above mentioned classes due to a lower analytical quality, were not considered in the following chapters of this thesis.

2.3 Results

2.3.1 Recovery rates of the different analytical procedures

The recoveries of the experiments are presented as recovery rates. Mean concentrations analyzed can be seen in Appendix A.1.

HPA and ACSV The combination of HPA digestion and ACSV detection was used for Pt analysis in sediment and freeze dried clam tissue samples. As described in Section 2.2.3 , the RR for sediment samples was analyzed using the certified reference material BCR-723, while the RR_s of Pt for clam tissue had to be calculated using the results of a surrogate recovery experiment (see Figure 2.4).

For the analysis of Pt in sediment samples, eleven aliquots of the BCR-723 were digested and analyzed individually. The analyses resulted in a mean concentration of 73 ng/g with a standard deviation of 14.6 ng/g. As the material is certified to contain 81.3 ng/g, the RR results in 88%.

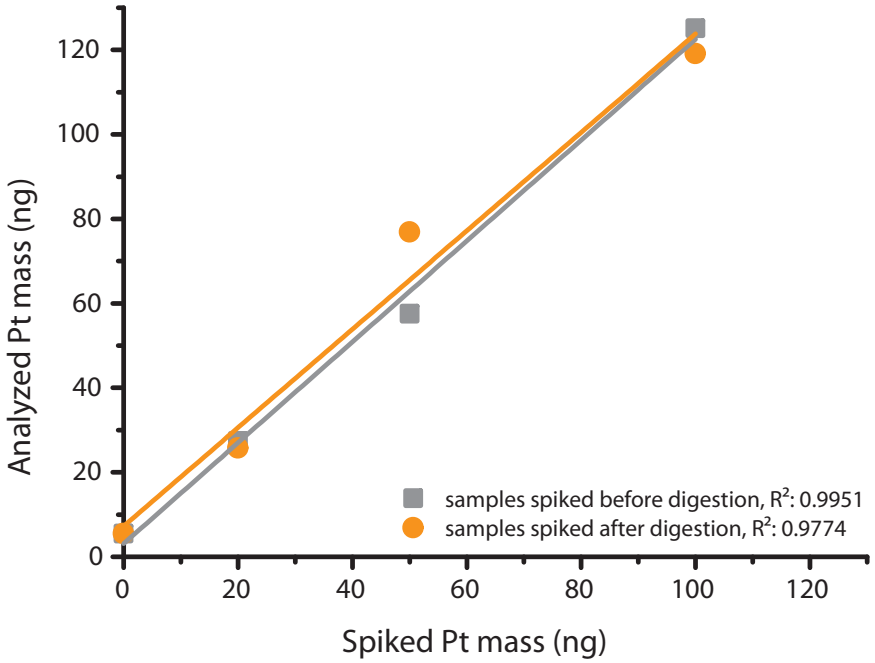

Figure 2.4: **Surrogate recovery experiment for the Pt analysis in clam tissue with ACSV after HPA digestion.**

For the RR_s of the clam tissue sample, four aliquots of the clam sample, taken before the digestion as well as four aliquots of a digestion solution of the same clam sample were spiked with different

amounts of Pt. The regression lines of Pt concentrations in samples spiked before and samples spiked after the digestion can be seen in Figure 2.4. The gradient of the regression line for Pt concentrations spiked before the digestion is 1.19, for Pt concentrations spiked after the digestion 1.17. This results in a RR_s of 102%.

HPA and ICP-MS The combination of HPA digestion and ICP-MS detection was validated for the analysis of Ag, Cd, Cr, Cu, Ni, Pb, Sb, and Zn in sediment samples and animal samples.

Figures 2.5 and 2.6 present the RR, obtained from the analyses of three different reference materials. For a better valuation of the trueness of the results, the certified concentration range (i.e. certified value of the reference material ± 95% confidence interval) is indicated by the gray area. In Figure 2.5 the RR of elements in the reference material Pacs-2 are presented.

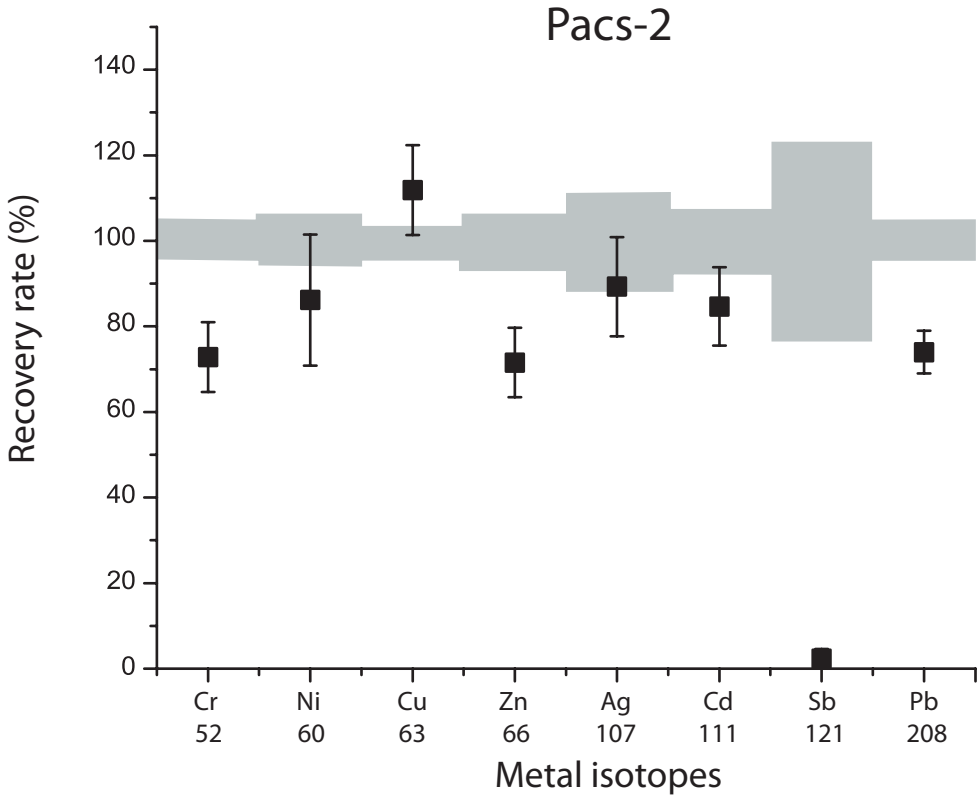

Figure 2.5: **Recovery rates for heavy metal analyses in sediments using HPA digestion and ICP-MS detection. Seven aliquots of the reference material Pacs-2 were used to analyze the recovery rate with a ± 95% confidence interval. The certified range of the elements in Pacs-2 is indicated by the gray area.**

Figure 2.5 reveals that for some metals the 95% confidence interval of the obtained RR meets the 95% confidence interval of the certified concentration range. These are: Ni, Cu, Ag, as well as

Cd. The RR for Sb and Pb is below the certified range. However, Pb has an RR above 70% of the certified reference value in Pacs-2. Only Sb showed an RR below 70%. For the animal samples two reference materials were used. The RR of the elements is plotted in Figure 2.6.

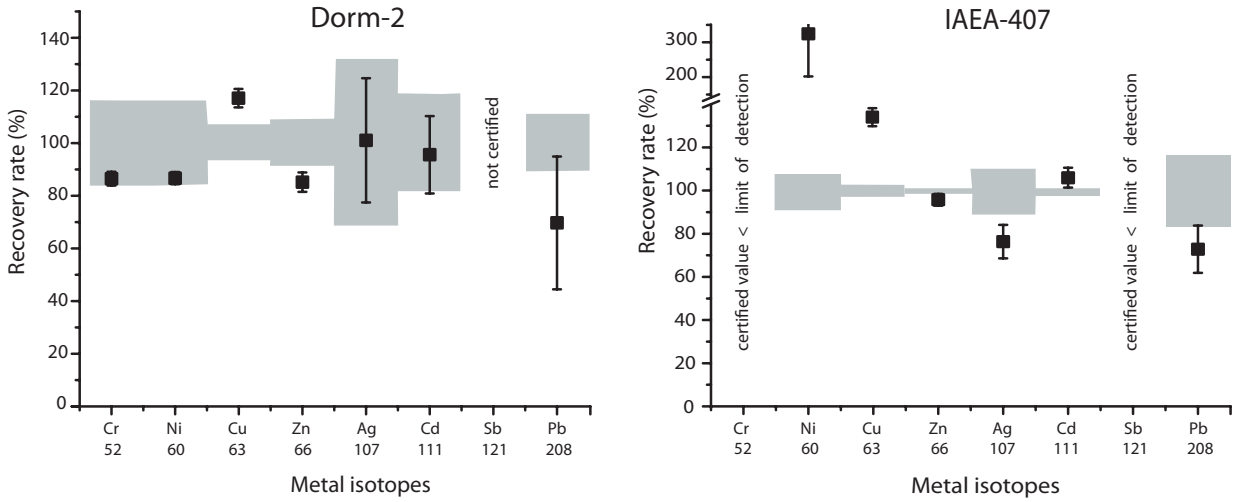

Figure 2.6: **Recovery rates for heavy metal analyses in animal samples using HPA digestion and ICP-MS detection. Eight aliquots of the reference material Dorm-2 and four aliquots of the reference material IAEA-407 were used to analyze the recovery rate with a ± 95% confidence interval. The certified range of the elements in Dorm-2 and IAEA-407 are indicated by the gray areas.**

For both reference materials, RR are found in the certified range for Cd. Ag, Pb, and Zn are found in concentrations below the certified range of one reference material, while they are found within the certified range of the other material. Ni is found to be higher than certified for IAEA-407 but within the certified range of Dorm-2. The RR of Cu does not overlap the certified range of both materials, they were found to be higher than 100% (117% for Dorm-2 and 134% for IAEA-407). The RR for Cr is in the certified range of Dorm-2, while the concentration of Cu certified for IAEA-407 is lower than detection limit. Sb could not be analyzed as the concentration in the reference material is below the detection limit for IAEA-407 and not certified at all in Dorm-2.

2.3.2 Precision of the different analytical procedures

For all analytical procedures used within this thesis the precision was determined. The results are presented in Table 2.7 as RSD (in %).

As can be seen, Cr, Cu, and Zn reveal an RSD $\leq$ 15% for each analytical procedure. Some metals exhibit a higher deviation than 20% for one or several analytical procedures (Cd, Ni, Pb,

Table 2.7: **Precision of analytical procedures (in RSD).**

	HPA/ACSV	HPA/ACSV	HPA/ICP-MS	HPA/ICP-MS	HPA/ICP-MS
	sediment	fauna	sediment	fauna	fauna
	BCR-723	clam sample	Pacs-2	Dorm-2	IAEA-407
	n=11	n=8	n=7	n=8	n=4
^{52}Cr	n.a.	n.a.	15%	4%	<LOD
^{60}Ni	n.a.	n.a.	24%	4%	38%
^{63}Cu	n.a.	n.a.	15%	4%	3%
^{66}Zn	n.a.	n.a.	15%	6%	3%
^{107}Ag	n.a.	n.a.	18%	14%	10%
^{111}Cd	n.a.	n.a.	15%	21%	4%
^{121}Sb	n.a.	n.a.	108%	n.c.	n.c.
Pt	20%	16%	n.c.	n.c.	n.c.
^{208}Pb	n.a.	n.a.	9%	52%	15%

n.a. = not analyzed

n.c. = not certified

<LOD = certified value is below the detection limit

and Sb). Especially, Sb shows very high variances with RSDs of up to 112%, when analyzed in sediments by ICP-MS, following HPA digestion . Ni shows high RSDs in sediments (24%) and in fish homogenate material IAEA-407 (36%). Analyzed in dogfish tissue (Dorm-2), however, only an RSD of 4% is observed for Ni. Also Pb has high variances when analyzed in Dorm-2 (43 to 52%), but not in sediments or IAEA-407.

2.3.3 Limits of detection and limits of quantification for the different analytical procedures

The limit of detection (LOD) and the limit of quantification (LOQ) were calculated as the 3- and 9- fold standard deviation of the analyses of independently analyzed procedural blanks. The values of the LOD and LOQ are given in µg/L in the measurement solution. Sediment and animal samples were digested in different sets of digestion vessels resulting in different blank values. Consequently, sediment and animal samples are listed separately, even if they were analyzed with the same analytical procedure.

Pt was analyzed in different sample matrices with different analytical procedures. For the analysis of Pt in sediments, 7 procedural blanks were prepared and digested by HPA and detected by ACSV. This results in an LOD of 13.6 ng/L in the digestion solution or 0.9 ng/g for 100 mg sediment and an LOQ of 40 ng/L in the digestion solution or 2.6 ng/g for 100 mg sediment. Further, 38 procedural blanks were run during the analyses of clam and fish tissue by HPA/ACSV, resulting in an LOD of 5.2 ng/L in the digestion solution or 0.01 ng/g for 200 mg freeze dried clam tissue and an LOQ of 16 ng/L in the digestion solution or 0.16 ng/g for 200 mg freeze dried clam tissue.

Clam tissue was also analyzed with ET-AAS after microwave digestion. During those analyses 15 procedural blanks were run, resulting in an LOD of 5.6 µg/L and 0.08 µg/g for 0.7 g freeze dried clam tissue and an LOQ of 16.8 µg/L in the digestion solution or 0.2 µg/g for 0.7 g freeze dried clam tissue.

All heavy metals other than Pt were analyzed with ICP-MS after HPA digestion. LODs and LOQs for the sample solution are summarized in Table 2.8.

Table 2.8: **Limits of detection (LOD) and limits of quantification (LOQ) for sediment and biota samples analyzed by HPA/ICP-MS in the measurement solution (µg/L).**

	LOD sediment n=12	LOQ sediment n=12	LOD clam tissue n=16	LOQ clam tissue n=16
^{52}Cr	2.2	6.6	5.6	17
^{60}Ni	2.0	5.9	1.4	4.0
^{63}Cu	0.35	1.0	0.51	1.5
^{66}Zn	6.5	19	14	28
^{107}Ag	0.03	0.08	0.03	0.10
^{111}Cd	0.34	1.0	0.02	0.05
^{121}Sb	0.12	0.35	0.09	0.26
^{208}Pb	0.06	0.19	0.14	0.43

2.3.4 Classification of the different analytical procedures

According to the results obtained in the recovery experiments and the experiments to determine the precision, the analytical procedures for each metal are classified. The results of the classification for the Pt analysis are summarized in Table 2.9 and Table 2.10.

Table 2.9: **Classification of the analytical procedures used for the Platinum analysis.**

HPA & ACSV Biota				
Element	RR (%)	RSD (%)	LOD (ng/L)	Classification
Pt	102	16	5.2	A

MD & ET-AAS Biota				
Element	RR (%)	RSD (%)	LOD (µg/L)	Classification
Pt	95	3-9	5.6	A

HPA & ACSV Sediment				
Element	RR (%)	RSD (%)	LOD (ng/L)	Classification
Pt	88	20	13.6	C

Even though, the RSD of Pt analyzed by HPA/ACSV is above 15% the analytical quality of the procedure is rated as A. The Pt content of the sample used to determine the precision is 0.6 ng/g which is close to the LOD of 0.5 for clam samples.

Table 2.10 summarizes the classification results for the analysis of traffic related heavy metals, other than Pt. It can be seen that for all traffic related heavy metals, except Sb, the analytical procedures for all metals can be classified, due to the classification system defined in section 2.2. For animal samples however, two different reference materials were used which result in different classifications for some metals. The classifications for the analyses of Ag, Cu and Ni are better when the reference material Dorm-2 is analyzed, while the classifications of Pb and Zn are better when the reference material IAEA-407 is analyzed.

Table 2.10: **Classification of the analytical procedures used for the analysis of traffic related heavy metals.**

HPA & ICP-MS Sediment				
Element	RR (%)	RSD (%)	LOD (µg/L)	Classification
^{52}Cr	73	15	6.6	C
^{60}Ni	86	24	5.9	C
^{63}Cu	113	15	0.5	A
^{66}Zn	72	15	6.5	C
^{107}Ag	89	18	0.03	C
^{111}Cd	85	15	0.3	A
^{208}Pb	74	9	0.06	C
HPA & ICP-MS Biota[a]				
Element	RR (%)	RSD (%)	LOD (µg/L)	Classification
^{52}Cr	87	4	6.7	A
^{60}Ni	87/325	4/38	1.4	A/-
^{63}Cu	117/134	4/3	0.3	C/-
^{66}Zn	85/96	4/3	14	B/A
^{107}Ag	101/76	14/10	0.03	A/C
^{111}Cd	96/106	15/4	0.01	A/A
^{206}Pb	70/73	52/15	0.14	-/C

[a] If two reference materials were tested, the first value is given for results obtained by the analysis of Dorm-2 and the second for results obtained by the analysis of IAEA-407.

2.4 Discussion

The purpose of this chapter was the validation of the different analytical procedures used in this thesis. A major challenge in this thesis was , that a wide range of different environmental matrices as well as a wide range of heavy metals had to be analyzed. In addition some of the metals, especially Pt, were present in ultra trace concentrations. Therefore, a combination of different analytical procedures was chosen to analyze the load of traffic related metals in the different samples.

In this section the quality of the applied analytical procedures for the different metals will be discussed. This implies true and precise analytical results, as well as LODs low enough to gain significant results for samples collected from unpolluted sites or from exposure studies with very low concentrations.

2.4.1 Validation of the Pt analysis in animal samples without certified reference material

As no certified reference material for Pt in animal sample material is available, the recovery rate for the Pt analysis in clam, fish and parasite tissues by HPA/ACSV was determined using a surrogate recovery experiment. This approach is also recommended by the International Union of Pure and Applied Chemistry, if no appropiate reference material is available (Thompson et al., 1999). As the surrogate is not part of the digested sample material, a surrogate recovery experiment gives only an estimation of the recovery rate that would be obtained in a recovery experiment including the analysis of a reference material. It does show the analyte loss during the digestion procedure and the accuracy of the detection method. To strengthen the value of the surrogate recovery experiment often interlaboratory comparison studies or intermethod comparison studies are conducted. In interlaboratory comparison studies the same material is analyzed with the same analytical procedure by different laboratories, while in intermethod comparison studies the same sample material is analyzed using different analytical procedures. The latter was already done in order to validate the analytical procedure for Pt with HPA/ACSV. This analytical procedure was already compared to the analysis by MD/AAS (Zimmermann et al., 2003), MD/sector field ICP-MS (Zimmermann et al., 2001) as well as MD/ACSV (Haus et al., 2009a). They resulted in comparable results with deviations of 19%, 2-30% and 25%, respectively. The surrogate recovery experiment is therefore sufficient to validate the method for this thesis.

The results of the recovery experiment as well as for the precision obtained in this study are comparable with other studies, using the same analytical procedure. The RR was determined to be 102% for this study. Zimmermann et al. (2001), also using the surrogate recovery experiment

with freeze dried mussel tissue (*Dreissena polymorpha*) determined an RR of 95%. Alt et al. (1994) found an RR of 103% for urine samples and 95% for blood samples, also in surrogate recovery experiments.

The precision in this study was 16% for a clam sample with a Pt concentration of 0.6 ng/g. Alt et al. (1994) found comparable relative standard deviations for the analysis of a blood sample (13% for five analysis of a blood sample containing 37.5 ng/L Pt) and a lower relative standard deviation for urine (6.2% for four analysis of a urine sample containing 2.1 ng/L Pt).

The LOD determined in this study was 5.2 ng/L in the measurement solution (0.05 ng/g in 200 mg freeze dried clam tissue). This is lower than LODs obtained with other analytical procedures for biological matrices. Sector field ICP-MS studies revealed an LOD four times higher than obtained with the procedure used here (Zimmermann et al., 2001). For an analysis with MD/ET-AAS Zimmermann et al. (2003) found an LOD 1000-fold higher than the LOD found for HPA/ACSV in this study. A lower LOD was obtained using HPA/ACSV for the analysis of blood and urine by Alt et al. (1994), who found a LOD of 0.1 ng/L in the measurement solution. Pt concentrations detected in animal samples from aquatic field studies are in the range from 0.1 to 1295 ng/g (Haus et al., 2009b). Thus, it has been determined that an analysis of Pt in aquatic animals can be performed using the combination of HPA digestion and ACSV detection, described here.

It can therefore be concluded, that the Pt analysis in animal samples by HPA/ACSV does reveal good analytical quality values (which are classified as class A). They are in very good accordance to the values obtained in other studies with the same analytical procedure and were already tested to agree with results obtained by other analytical procedures.

Animal samples containing Pt concentrations in a µg/g range were analyzed by MD/ET-AAS. The validation data for this analytical procedure were not determined in this study, because they were already obtained using the identical instrumental setting by other authors (Zimmermann et al., 2003). Further, the procedure in this instrumental setting was already successfully used in other studies (Singer et al., 2005; Sures & Zimmermann, 2007; Osterauer et al., 2010b). The RR and RSD obtained by Zimmermann et al. (2003) lead to a classification of class A for this analytical procedure. The LOD obtained for this procedure in this study (5.6 µg/L) is exactly the same as that determined by Zimmermann et al. (2003).

2.4.2 Validation of analytical procedures with certified reference material

HPA/ACSV for sediment samples The results of the recovery experiment with the certified reference material BCR-721 revealed a recovery of 88% and a precision of 20%. Even though the detected concentration overlaps with the certified range of the reference material, the Pt analysis in sediments had to be rated as Class C, as the precision is >15%.

The reference material BCR-721 is the only certified reference material for abiotic sample material, which contains environmentally relevant concentrations. Therefore, it is often used in validation experiments. Those experiments were reviewed by Sutherland (2007). The median precision found in the reviewed studies was 5.3%, while it was 20% in this study. Within this review Sutherland (2007) offers an explanation for the relatively high RSD found in this study. Sutherland (2007) remarks that the material tends to be inhomogeneous, resulting in Pt nuggets. This leads to poor precisions if less than 100 mg are used for the digestion. However, BCR-721 is the only environmentally relevant reference material for Pt and it is scarce. It was therefore decided, to use it carefully and only in masses between 50 and 100 mg per digestion. Also the RR of 88% is lower than that observed in other studies. Sutherland (2007) summarizes that other laboratories achieved recovery rates in the range of 91 to 119%. The lower RR in this study can also be explained by the low mass used for the digestion. Further, it is assumed that the composition of acids used for digestion also effects the RR. The main acid used in the digestion was HNO_3, accompanied by a smaller amount of HCl (4 ml HNO_3 and 0.5 ml HCl). HNO_3, however, is not able to decompose silicates that may incorporate Pt (Tsogas et al., 2008). In other studies aqua regia was frequently used for digestions. However, the use of aqua regia during the sample preparation is considered to be unsuitable for an analysis by ACSV, as high amounts of HCl in the digestion solution inhibit the voltammetric peak height and therefore effect the sensitivity of the measurement (Nygren et al., 1990; Bencs et al., 2003; Haus, 2005). In studies in which aqua regia was not used for the digestion, the decomposition of silicates was promoted by hydrofluoric acid (HF) (Zischka et al., 2002; Sutherland, 2007; Balcerzak, 2011) or perchlorid acid (Messerschmidt et al., 1992). As the HPA digestion is performed in quartz vessels, HF had to be excluded as a digestion acid. The handling of perchlorid acid does involve a higher risk for the experimenter. In order to reduce the health risks for the experimenter it was decided not to use perchlorid acids in this study. To avoid problems with the analytical detection method and to avoid higher risks for the experimenter, the composition of digestion agents was maintained and a recovery of 88% is considered as sufficient for the aims of the thesis.

The LOD for sediment samples (i.e. 13.6 ng/L in digestion solution and 0.9 ng/g sediment) is higher than observed for the clam samples in this study. As different digestion vessel sets were used for the digestion of the different matrices, the differences in LODs can be explained by higher Pt content in sediments. Those cause a higher contamination of the digestion vessels and therefore also higher LODs. For the analysis of Pt in sediments it is difficult to find LOD results in the literature. Even though quite a number of studies describe the Pt concentrations in sediment samples, there are only few references to an LOD regarding the whole analytical procedure. Those LODs that have been reported are commonly much higher than in this study. Like in Jackson et al. (2007), who report an LOD of 4 µg/g analyzing Pt in sediments with ICP-MS following a Pb fire assay, or Terashima et al. (2002) who report an LOD of 0.5 µg/g in 2 g of sediment sample by a detection with ET-AAS. Rauch & Hemond (2003) found similar LOD to the present study with 0.5 ng/g. They used an ICP-MS approach and corrected the interference of HfO^+

mathematically. Only Boulyga & Heumann (2005) reported lower LODs. They were able to achieve LODs of 0.06 ng/g with the laser ablation ICP-MS methods. Sediment concentrations reported from environmental monitoring studies are reported to be below detection limit to 85 ng/g (Haus et al., 2009b). According to Terashima et al. (2002) the geological background value for Pt is 2.7 ng/g. It can therefore be concluded, that Pt in sediments can be detected with the here used analytical procedure consisting of HPA digestion followed by ACSV detection.

In summary: For sediment samples the recovery and precision achieved with the applied methods were comparatively poorer but still in the same range than that of other studies or of other analytical procedures. The LOD on the other hand was frequently lower than in comparable studies. Furthermore, especially the combination of HPA digestion and ACSV detection is sensitive enough to analyze Pt in environmental samples. Pt analyzed in sediments by HPA and ACSV is classified as a Class C method. For the following chapter it has to be taken into account that Pt concentrations obtained from sediment samples might be underestimated, due to an incomplete digestion of the silicate fraction.

HPA/ICP-MS for sediment samples As could be seen in the results, the analytical procedures for Cd and Cu could be rated as class A, due to a recovery in good accordance with the certified range of the reference material Pacs-2 and a precision of 15%. For almost all other metals the analysis could be classified as class C. The determined RR was higher than 70% and the precision was 20% or below. These are Ag , Cr, Pb, Ni, and Zn. For Sb, however, the RR of the analytical procedure is very poor, consequently it will be excluded from analyses in the following chapter.

The main reason for the classification into class C is the low RR determined. As already discussed for Pt, also these low concentrations can be explained with an incomplete digestion. As has already been mentioned above, HNO_3, which was the main acid used in the digestion, does not decompose silicates, which then leads to low recoveries. This was also shown by Sandroni & Smith (2002). They used different combinations of digestion agents for the digestion of several sediment reference materials. They found that especially for Cr and Zn recoveries were low when HNO_3 was used alone. Best results were obtained in digestions using aqua regia or HNO_3 in combination with HF. However, the digestion used in the present study, has the advantage, that it allows two different detections methods with the same digestion solution. Implying that Pt and the other traffic related heavy metals can be determined in the same sample aliquot, which makes a direct comparison of the metals as well as correlation analyses easier. Additionally, a single digestion step for two different detection methods saves time, labour and money. The RR for Sb was extremely low. This can not be explained by an incomplete digestion. Sb is very volatile as it reacts with oxygen to Sb_2O_3 when it is heated. It can be assumed that the heating in the HPA is the main reason for the Sb loss during the analysis. The LODs obtained, are all low enough to allow for the analysis of environmentally relevant concentrations since all LODs are lower than the geological background values published by Turekian & Wedepohl (1961).

HPA/ICP-MS for animal samples For the validation of traffic related heavy metals other than Pt in animal samples, two different certified reference materials were used. The use of two reference materials resulted in different classifications for the analytical procedure of the same metal isotope.

The first difference found for the two reference materials was the concentration range of specific elements. For Cr only Dorm-2 offered concentrations above the detection limit of the used analytical procedure (HPA/ICP-MS). In IAEA-407 concentrations were too low for a detection.

The next difference was found for the RR determined in the different reference materials. While the mean concentrations ± 95% confidence interval of Ni and Ag did overlap the certified range of the reference material Dorm-2, they did not overlap the referenced range of IAEA-407. As already mentioned in the materials and methods section, IAEA-407 was not certified yet. The material has been part of a intercomparison study conducted by 105 laboratories. Due to the data report, for Ag, data was only obtained from three laboratories using the same analytical procedure. Therefore, the Ag content of IAEA-407 was classified as an informational value by the authors of the study (Wyse et al., 2003). As the values of the certified reference material Dorm-2 are more reliable, only the validation results obtained for Dorm-2 are considered for the classification of the analytical quality and the analysis of Ag in animal samples by HPA/ICP-MS is classified into class A. Like for Ni, also for Cu RR in IAEA-407 is higher than in Dorm-2. No explanation could be found for the difference according to the certification reports.

The main parameter resulting into the different classifications was the precision. While for most of the heavy metals good precision values were obtained, other, (i.e. Ag, Cd, and Pb) showed relatively poor precisions in Dorm-2, while they were lower when analyzed in IAEA-407. This can partly be explained with the different concentration ranges of the specific elements in the two reference materials. The concentrations of Pb and Cd in Dorm-2 are close to the LOD, while they are higher in IAEA-407. Wellmitz & Gluschke (2005) demonstrated clearly that the level of precision decreases, the closer the values are to the LOD. This can also be seen in the study performed here by comparing the two reference materials. As the contents of Pb and Cd are higher in IAEA-407 the variations are lower. Another explanation for the high variances found in Dorm-2 could be found in inhomogeneities of the material. For Ag and Cu this is already apparent in the wide certified range, which was documented for the material (Itoh et al., 2011). The high variances of Pb, however, were not reported in the certificate. However, other studies also reported high variances for Pb in Dorm-2. Zimmermann et al. (2002) did find an RSD of 37% and Yang & Swami (2007) reported an RSD of 44% for Pb in Dorm-2. For the classification of the Pb analysis the values of IAEA-407 should be preferred considered.

It can therefore be concluded, that the analytical quality of the HPA/ICP-MS method can be classified as A for Ag, Cd, and Cr. The analysis of Zn is classified between A and B depending on the reference material used. The analyses of Cu, Pb, and Ni should be treated with caution as the re-

sults of the validation by the two reference materials are so different, that no reliable classification can be made.

The LODs determined in this study, are sufficiently low for environmental monitoring studies. They are comparable to LODs published in other studies, which monitored heavy metals in aquatic organism samples (Haus et al., 2007b; Nachev et al., 2010).

Chapter 3

Introduction of traffic related Platinum into river systems - Occurrence and distribution of Platinum in sediments and biota

3.1 Introduction

Since the introduction of automobile catalytic converters in the 1980s the distribution of PGE and especially Pt is monitored in the environment. It is not surprising that several reviews and a book summarize the current knowledge of the increasing Pt concentrations in the different matrices (Zereini & Alt, 2000; Ek et al., 2004; Hoppstock & Sures, 2004; Ravindra et al., 2004; Zimmermann & Sures, 2004; Rauch & Morrison, 2008; Dubiella-Jackowska et al., 2009; Haus et al., 2009b). To a large extend, these reviews are focused on Pt concentration in terrestrial samples and the influence of traffic on different terrestrial ecosystems.

While there has been an extensive amount of research in the distribution in terrestrial environmental settings and sample matrices like air, road dust, particulate matter (e.g. PM-10), soil and plants along highways, there are only a few studies that investigate the distribution of Pt in aquatic ecosystems (reviewed by Haus et al., 2009b).

Only a few studies applied a systematic sampling strategy to gain a deeper understanding of the Pt distribution in aquatic ecosystems. The IWW (2004) modelled the Pt load of the river Rhine after estimating the influx of Pt by different sources. Rauch & Hemond (2003) established a historical picture of Pt loads in sediments by analyzing Pt in sediment cores of the Upper Mystic lake near Boston, USA. For river systems de Vos (2002) and Prichard & Jackson (2008) investigated the

distribution of Pt along the whole catchment area. All studies concluded that often roads, but also the discharge of the industry or municipal sewage plants are common sources of Pt in rivers and lakes. It was further shown, that Pt concentrations in sediments decrease with distance to the source, and that Pt as part of the sediment and suspended particulate matter load of the river drifts to the estuaries (Prichard & Jackson, 2008).

Up to date no study investigated the fate of Pt, directly at the discharge site of rivers. Therefore, the following questions remain to be answered:

- In which sediment fractions is Pt accumulated?
- How far is Pt transported?
- Is the discharge of Pt comparable to the discharge of other heavy metals?
- Are there major differences between the Pt transport and concentration ranges in aquatic ecosystems and the Pt transport in terrestrial ecosystems?
- Does the discharge of road runoff change the sediment quality?

Next to the question of the distribution of Pt in sediments, the question of bioavailability has to be raised. Even though Pt is a precious metal, it has been shown to be biologically available to different aquatic organism like plants (Farago & Parsons, 1994; Hees et al., 1998), gammarids (Haus et al., 2007b), asselids (Rauch & Morrison, 1999; Moldovan et al., 2001; Haus et al., 2007b) mussels (Zimmermann et al., 2002, 2005b; Singer et al., 2005; Sures & Zimmermann, 2007; Frank et al., 2008), snails (Osterauer et al., 2009) and different fish species (Hees et al., 1998; Sures et al., 2003b; Zimmermann et al., 2004a, 2005a; Sures et al., 2005; Essumang et al., 2008; Osterauer et al., 2009). However, most of the studies mentioned above, documented the uptake of Pt under controlled conditions in the laboratory and with Pt commonly offered in the form of soluble salts in high quantities. The uptake of Pt in the field, however, was only shown for asselids, gammarids and fish (Rauch & Morrison, 1999; Moldovan et al., 2001; Sures et al., 2005; Haus et al., 2007b; Essumang et al., 2008). Still, also in these studies, samples were taken at random places or only in worst case scenarios. So far none of the studies took a systematic approach in order to take a look at the transportation range of Pt and to estimate the effects on the aquatic organism. Furthermore, only Haus et al. (2007b) compared Pt concentrations to the concentrations of other traffic related heavy metals in sediments as well as biota samples.

It is the aim of this study to answer these questions for lentic systems. For a better understanding of the distribution of Pt in river systems, sediment and biota samples were taken in a small river which intersects a major road near Karlsruhe, Germany. To analyze the heavy metal distribution in different sediment grain sizes as well as in the asian clam *Corbicula* sp., samples were taken along three different transects (20 or 100 m) starting at three different inlets of road runoff. Samples were

analyzed to clarify the influence of Pt and other traffic related heavy metals on sediment qualities. It was tested up to which distance from the inlet the introduction of traffic related metals including Pt could be detected. In addition it was investigated in which grain size class they accumulate and which traffic related heavy metals are correlated with Pt. Furthermore, the bioavailability of all analyzed metals was investigated, in order to detect the extend to which Pt and other traffic related heavy metals are accumulated by clams.

3.2 Material and Methods

3.2.1 The sampling site: River Alb in Baden-Württemberg

To analyze the distribution and occurrence of Pt in an aquatic system, the river Alb was chosen as sampling site. The river Alb originates near Bad Herrenalb, located in the German state of Baden Württemberg and flows 52 km through the Black forest and the city of Ettlingen until it empties into the river Rhine near Karlsruhe. The Alb has a watershed of 457 km ^{2} (Regierungspräsidium Karlsruhe, 2009). The sampling site for this study is located between Karlsruhe and Maxau, approximately 10 km before the Alb enters into the River Rhine (see Figure 3.1A). The following aspects were taken into consideration when choosing this approximately 150 m stretch of the river. At this site:

- The river Alb is being crossed by the federal highway B10. This is the busiest road in Karlsruhe with a traffic intensity of approximately 75000 cars per day (Regierungspräsidium Tübingen, 2007)
- The roadrunoff of the B10 is collected by a drainage system and directly discharged into the river at three different inlet points (see Figure 3.1B and C).
- The bivalve *Corbicula* sp. is evenly distributed in the sediments of the river.

The drainage system of the B10 can be seen in Figure 3.1B. The rainwater of the bridge is drained via Inlet-1, directly under the bridge, on the left bank of the river. Also on the left bank, 30 m further downstream is the second inlet (Inlet-2). As can be seen in Figure 3.1, road runoff from west of the bridge is drained through Inlet-2 into the river Alb. Inlet-3 is on the right bank of the river, 5 m downstream from Inlet-2. Through Inlet-3 rainwater from the eastern part of the highway is drained into the river. Samples were taken in three transects, based on the location of the inlets. Transect-1 starts at Inlet-1 and samples were taken at 0 m, 3 m, 7 m, and 20 m downstream from Inlet-1 at the left side of the river. Transect-2 starts at Inlet-2 and samples were taken at 0 m, 3 m, 7 m, 20 m, 50 m, and 100 m downstream from Inlet-2. Transect-3 includes sampling points at 2 m, 15 m, 45 m, and 95 m downstream from Inlet-3. All sampling points are marked in Figure 3.1B and C. In this chapter the following abbreviations will be used to reference specific sampling points. The transect number will be abbreviated with T-1 for Transect-1, T-2 for Transect-2 and T-3 for Transect-3. The distance of the sampling point to the respective inlet of the transect will be given in metres after a slash. T-2/50 for example is a sampling point in Transect-2, 50 m downstream from Inlet 2. At all sampling points clam and sediment samples were taken.

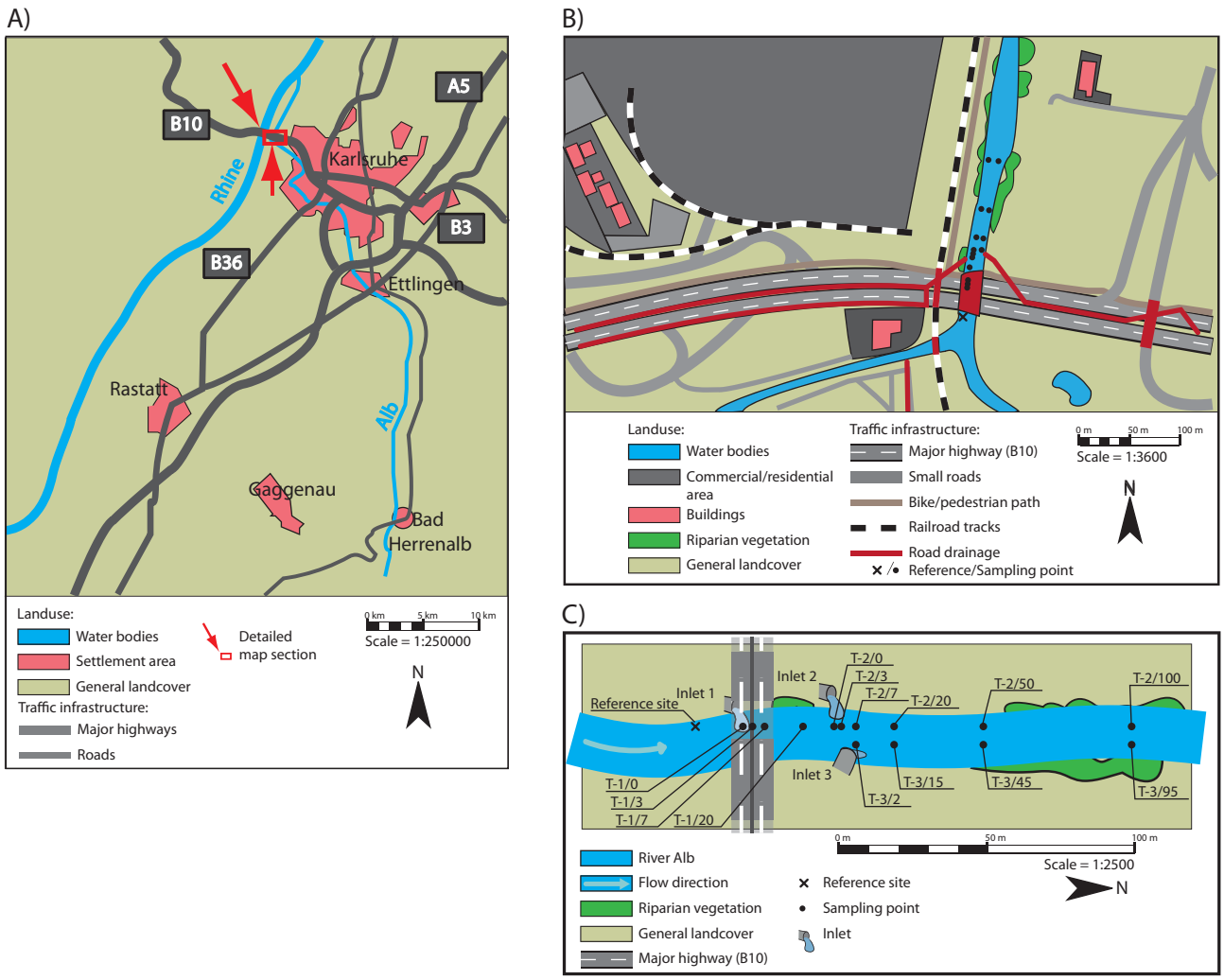

Figure 3.1: **Overview of the sampling site. A) The river Alb from the source in Bad Herrenalb to the opening into the river Rhine. The width of the river Alb is not true to scale in order to make it visible. B) The drainage system of the B10 at the sampling site of the river Alb. C) The location of the reference site and all sampling points in the river Alb in detail. Digitalization of the maps and graphic realization of this figure was performed by Markus Ruchter.**

Sediment samples Sediment samples were taken from the upper 3 cm of the sediment. Approximately 500 ml of the sediment was carefully shovelled into a plastic container. The container was closed with a lit at the bottom of the river, before transported to the water surface, in order to avoid a loss of the fine grain fraction of the sediment sample. Sediments were transferred into polyethylene bags, transported to the lab and stored at -20 °C until further processing for analysis.

Clam samples At each sampling point *Corbicula* sp. samples were taken. Approximately 80 clams were sampled using a shovel sampler at each sampling point and transferred into polyethylene bags. Clams were transported in a low temperatured cooling box without water. At the laboratory, clams were transferred to non-chlorinated and aerated tap water for a depuration of 24 h. Lee & Lee (2005) could demonstrate that 70-95% of ingested metals are egested by *Corbicula* sp. within 16 h. A time frame of 24 h should therefore be sufficient to remove metals that are part of the gut content and not accumulated by the clam. Before further analysis clam samples were frozen at -20 °C.

The collection of all samples was conducted within two weeks, whereby sediment and clam samples were taken on the same day for each sampling point. On the last day of the sampling procedure, physical and chemical water analysis was performed. Oxygen saturation, the pH-value, redox potential, conductivity and temperature were analyzed with instruments of WTW (pH-325 and LF-318 WTW, Weilheim, Germany).

3.2.2 Analytical procedures

In this study sediment and clam samples were analyzed for the following elements: Ag, Cd, Cr, Cu, Ni, Pb, Pt and Zn.

The applied analytical procedures are described in Chapter 2.2 and the respective validation can be seen in Chapter 2.3.4.

Sample preparation

Sediment preparation Sediment samples were thawed, transferred into porcellain bowls and dried in the oven at 105 °C for 24 h. After drying, the mass of the total sample was determined. Burden (2002) has shown, that the concentration of metals in sediments depended on the grainsize composition within the sediment sample . Differences in grain size composition, therefore, hamper the comparison of metal contents at the different sampling points. Several authors recommend a grain size fractionation of the total sample and the analysis of the fine fraction (i.e. <63 µm) (Burden, 2002; Luoma & Rainbow, 2008). However, traffic related heavy metals are also emitted as particles and it is thus likely that they are accumulated in a grain size >63 µm. Consequently, the sediments sample was divided into three grain size classes: Sceletal (Grainsize >2 mm), sand (Grain size: 0.063 to 2 mm) and silt/clay (<0.063 mm). The division of the grain size classes was achieved by dry sieving the samples. After fractionation, the sand and the silt/clay fraction were analyzed. Samples were ground to a size of approximately 50 µm in order to homogenize the sample for subsampling. For grinding, up to 50 g of the sample were put into a tungsten

carbide crushing mill (KH9707, Herzog Maschinenfabrik, Osnabrück, Germany) and ground for 18 s (silt/clay) or for 54 s (Sand). Until the analysis, samples were stored in acid washed glass containers. For the analysis 250 mg were weighed into the quartz pressure bombs of the HPA and were digested and analyzed as described in Chapter 2.2. Five subsamples were analyzed for each sampling point and each grain size fraction.

Clam preparation Clams were thawed at room temperature and the length, width and height as well as the total mass and the mass of the soft tissue of each clam were determined. The length of the clam is defined as the maximum distance between the anterior to the posterior side of the clam. The width of the clam is defined as the maximum distance between the dorsal and ventral side of the clam and the height of the clam is defined as the maximum distance between the right to the left side of the clam (see also Figure 3.2).

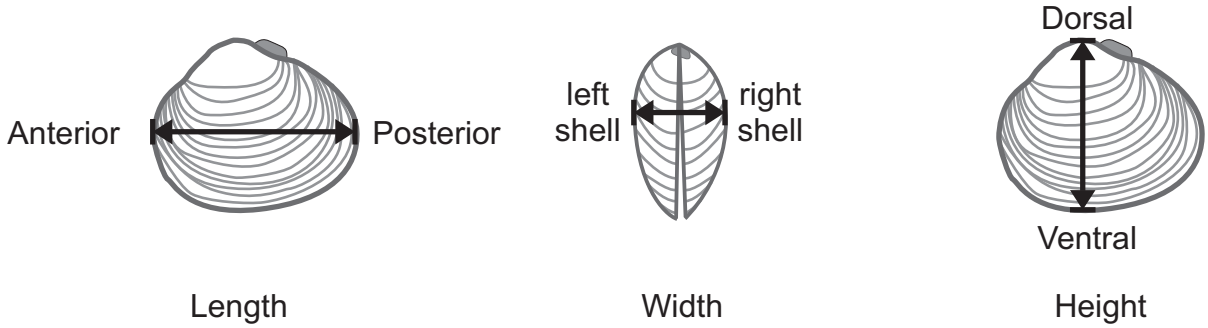

Figure 3.2: **Clam length, width and height.**

From each sampling point 30 to 45 clams of each sampling point were chosen and merged into one sample, homogenized with a dispersing tool (Ultra Turrax T25, Janke & Gunkel, Stauffen, Germany) and freeze dried (Heto Power Dry LL3000, Thermo, Langenselbold, Germany). As some studies of other authors were performed using the fresh weight of clam tissues, the mass of the sample was estimated before and after the freeze drying process to allow for a better comparison. The factor between fresh weight and dry weight was calculated to be 4.6 (with a standard deviation of 0.3). To avoid metal contamination, all of the metal instruments used were cleaned with 0.1% EDTA solution and millipore water. Each clam sample was divided into five subsamples. Up to 250 mg of each clam subsample was weighed into the 70 ml quartz vessels of the High Pressure Asher. Sample digestion procedure and analysis is described in Chapter 2.2.

3.2.3 Data analysis

Replicate handling For all samples five replicates were digested and analyzed. The results of the five replicates is presented as the arithmetic mean in most of the figures and the standard

deviation is used to express the variance of the resulting concentrations, analyzed in the different replicates. Outliers were eliminated, if the value were below or above the mean by ±2.5 fold standard deviation.

Statistical tests For each sampling point the mean concentration of the sediments (sand and silt/clay fraction) as well as concentration of clam tissue was compared to the reference site. For a statistical comparison the non parametric Mann-Whitney Test (or U-Test) was used, to test the following hypothesis for two independent groups.

H0:
Metal concentration for metal x at a certain sampling point is equal to metal concentration for the same metal at the reference site.

H1 (two sided):

Metal concentration for metal x at a certain sampling point is different from the metal concentration for the same metal at the reference site.

For analyzing dependencies in between the resulting data, the Spearman Rank correlation was applied. All tests were conducted with the statistical program Statistica (StatSoft, Tula, USA).

Concentrations in sediments <2 mm As has already been described, sediment samples were divided into different grain size fractions, which were analyzed separately. Therefore, results are given in ng/g or µg/g for the sand fraction or the silt/clay fraction, respectively. For some questions the amount of a metal in the total fraction <2 mm would be more interesting, than that counted in a single fraction. For these questions the metal concentration for the sediment sample (<2 mm) was calculated using Formula 3.1.

$$C_{sed<2\ mm} = \frac{m_{sand}}{m_{sand} + m_{silt/clay}} C_{sand} + \frac{m_{silt/clay}}{m_{sand} + m_{silt/clay}} C_{silt/clay} \tag{3.1}$$

$with$
$m_{sand} = mass\ of\ sand\ fraction$
$m_{silt/clay} = mass\ of\ silt/clay\ fraction$
$C_{sand} = metal\ concentration\ in\ sand\ fraction$
$C_{silt/clay} = metal\ concentration\ in\ silt/clay\ fraction$

It needs to be pointed out, that the sum of the mass of the two fractions, sand and silt/clay, is not the mass of the total sample. The total sample includes also the skeleton fraction (grain size >2 mm), which was not included in the metal analysis. Therefore, the metal concentration of the total sample is assumed to be higher.

Classification system for sediments For the evaluation of the analyzed sediment concentrations, the classification system published by LAWA (1998) was used. The classification system encompasses seven classification descriptions which can be seen in Table 3.1.

Table 3.1: **Sediment classification by LAWA (1998). Concentration are given in mg/kg.**

	I unpolluted	I-II very low contamination	II modest contamination	II-III considerable contamination	III increased contamination	III-IV high contamination	V very high contamination
Cd	<0,3	<0,6	<1,2	<2,4	<4,8	<9,6	>9,6
Cr	<80	<90	<100	<200	<400	<800	>800
Cu	<20	<40	<60	<120	<240	<480	>480
Ni	<30	<40	<50	<100	<200	<400	>400
Pb	<25	<50	<100	<200	<400	<800	>800
Zn	<100	<150	<200	<400	<800	<1600	>1600

Condition Factor The bioaccumulation of metals is often dependent on the physical state of an organism. For the comparison of different samples the physical state of the observed organisms should be taken into account. Therefore, the condition factor was calculated. The condition factor is used to express the nutritional state of the clams. It is deduced from the Fulton index, described in Chapter 5.2.7.

$$CF = \frac{M * 100}{L^3} \tag{3.2}$$

$with$
$CF = Condition\ factor$
$M = Mass\ of\ clam\ (g)$
$L = Total\ length\ of\ clam\ (cm)$

Enrichment Factors The metal concentration of a sediment sample is combined of the metals which were introduced by humans and the natural metal burden which derives from the local geology. As the river Alb already passed the city of Karlsruhe and several major streets prior to the sampling site, the attempt is made to estimate the already existing anthropogenic influence to the metal content in sediments at the reference site. Therefore, analyzed metal concentrations were set to relation to already published geological background data in an anthropogenic enrichment factor (AEF) (see Formula 3.3).

$$AEF = \frac{C_{silt/clay}}{C_{geological\ background\ value}} \tag{3.3}$$

$with$
$C_{silt/clay} = Metal\ concentration\ in\ the\ silt/clay\ fraction$
$C_{geological\ background\ value} = Concentration\ estimated\ as\ geological\ background\ concentration$

The background values used here were published by Turekian & Wedepohl (1961) and Terashima et al. (2002) and are listed in Table 3.2.

Table 3.2: **Geological background values for sediments.**

Metal	Geological background value[a] (mg/kg)
Ag	0.27
Cd	0.3
Cr	80
Cu	45
Ni	30
Pb	25
Pt	0.003
Zn	100

[a] Background levels are published in Turekian & Wedepohl (1961) or Terashima et al. (2002)

The list includes the values of deep sea clay samples published by Turekian & Wedepohl (1961). Unfortunately, Turekian & Wedepohl (1961) do not include background values for Pt. Terashima et al. (2002), however, analyzed Pt in 281 sediment samples containing 31 lake sediments, 189 terrigenous marine sediments and 41 marine shales. Sediment samples they analyzed had a grain

size <63 µm. They estimated that the average Pt concentration in the earth crust is 2.7 µg/kg. Both studies mentioned intend to estimate international background levels.

The second enrichment factor used in this study, helps to identify a potential relationship between metal concentrations in sediments and clams. Therefore, the Bioconcentration Factor ($BCF_{sediment}$) was calculated (see Formula 3.4).

$$BCF_{Sediment} = \frac{C_{clam}}{C_{sed<2\ mm}} \tag{3.4}$$

$with$
$C_{clam} = Metal\ concentration\ in\ clam\ sample$
$C_{sed} = Metal\ concentration\ in\ sediment\ sample$

3.3 Results

3.3.1 Chemical and physical water parameters at the different sampling points

Chemical and physical water parameters (i.e. temperature, pH-value, conductivity and oxygen saturation) were analyzed in the water column at all sampling points. Results are illustrated in Figure 3.3.

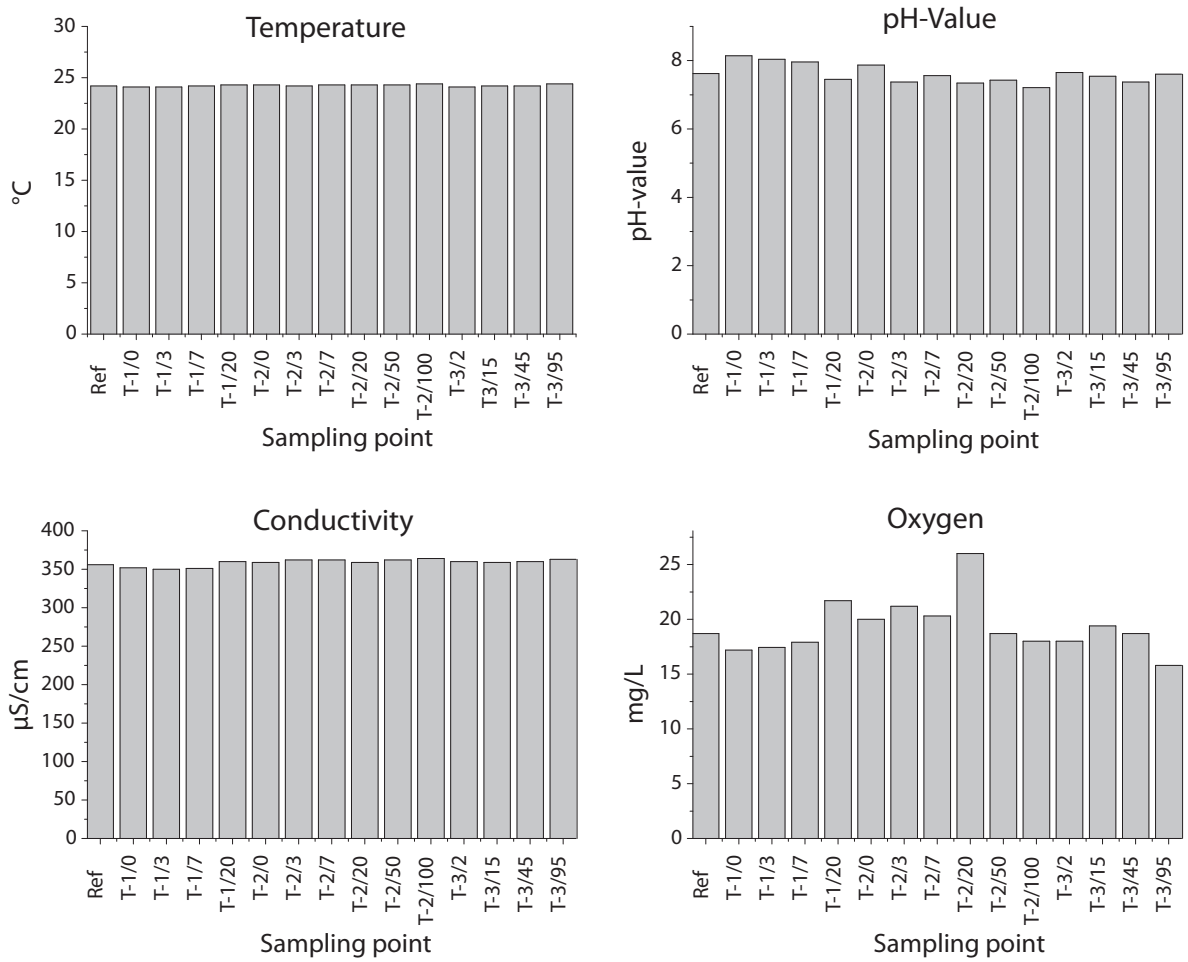

Figure 3.3: **Chemical and physical water parameters. Temperature, pH-value, conductivity and oxygen were analyzed in the water column.**

As can be seen in Figure 3.3, physical and chemical parameters are very similiar between all sampling points. The only parameter varying to a small extend is the oxygen saturation, which increases at sampling points T-1/20 and T-2/20. As these values are obtained from a single sampling event and were not monitored over a certain time period, it is not considered appropriate to

use a classification system to interpret these data (MUNLV, 2009). However, it should be noted that the parameters do not suggest an anthropogenic influence according to the background and orientation values of MUNLV (2009). An exception are the temperature values which are higher than the orientation value at all sampling points (orientation value: <21,5°C). However, the temperature regime is variable and should be monitored for a certain period of time and at different times of the day in order to gain valid information.

3.3.2 Traffic related heavy metals in sediments of the river Alb

The reference site and the anthropogenic influence on its heavy metal load

Sediments in the studied section of the river Alb turned out to consist predominantly of sandy sediments. Average grain size distribution over all sampling points was: 60% (± 22.6) sand, 33% (± 18.7) sceletal and 1.2% (± 0.97) silt/clay. Detailed data for all sampling points can be seen in Appendix A.2. The reference site used in this study is not a control site in terms of an uninfluenced site with none or only little heavy metal pollution. It lies 20 m upstream of the first inlet and therefore mirrors the heavy metal load in the sediments of the other sampling points without the influence of the road runoff of the three investigated inlets. The concentrations for all heavy metals in the different grain size categories are presented in Table 3.3.

Table 3.3: **Heavy metal concentration in sediments of the reference site.**

Metal	Unit	Sediment <2 mm mean (SD)	Sand mean (SD)	Silt/clay mean (SD)
Ag	µg/g	0.2 (0.02)	0.19 (0.02)	0.86 (0.07)
Cd	µg/g	0.15 (0.005)	<LOD	0.81 (0.05)
Cr	µg/g	14.1 (8.96)	12.3 (8.7)	148 (26)
Cu	µg/g	11.5 (2.0)	10.5 (2.0)	85 (4.2)
Ni	µg/g	9.5 (1.6)	9.1 (1.6)	36 (3.6)
Pb	µg/g	16.8 (2.7)	15.9 (2.7)	83 (4.8)
Pt	ng/g	0.88 (0.18)	0.7 (0.2)	14.4 (2.3)
Zn	µg/g	51 (3.4)	47 (3.2)	345 (14.9)

With the exception of Cd in the sand fraction, all different metals were found in values above the LOD. Furthermore, they are present in different concentration ranges. As heavy metals in sediments do not only occur due to an anthropogenic influence, this concentration difference could be the result of the geological origin of the sediments. To test this hypothesis, metal concentrations of

the sediments at the reference site were compared with geological background data by calculating an anthropogenic enrichment factor (AEF) (Figure 3.4).

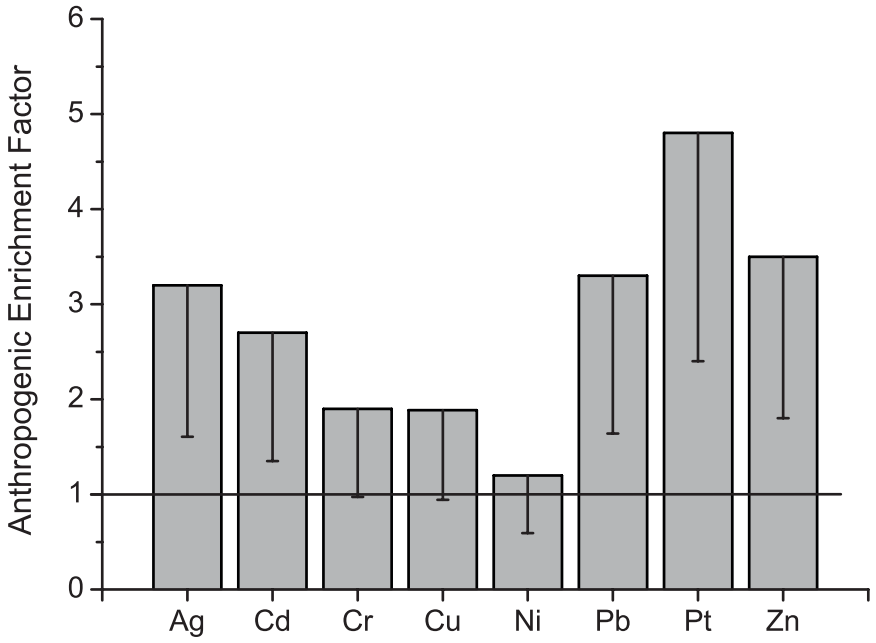

Figure 3.4: **Anthropogenic influence on heavy metal concentrations in sediments at the reference site. Error bars indicate the deviation of the background concentrations.**

As presented in Figure 3.4, the means of the analyzed heavy metal concentrations in the silt/clay fraction of sediment samples are above the geological background concentration. AEFs lie in between 1.2 (Ni) to 4.8 (Pt) times higher than expected in natural sediments. Is the deviation of the background concentrations taken into account, Cr, Cu and Ni can be considered to be in a natural concentration range. The reference site is therefore already polluted with Ag, Cd, Pb, Pt and Zn. The amount of this pollution decreases in the following order: Pt >Zn >Pb >Ag >Cd.

Platinum in sediments

As the occurrence and distribution of Pt is in the focus of this study, concentrations in the sediment categories sand (0.063 µm to 2 mm) and silt/clay (<0.063 µm) as well as the calculated combination of both fractions are investigated. Figure 3.5 gives a graphical representation of the concentration of Pt in all sediment classes.

In general it can be noted, that Pt-concentrations are higher in the silt/clay fraction than in the sand fraction for almost all sampling points. Concentrations in the silt/clay fraction are in the range of 6.8 to 45 ng/g. The only exception can be found in Transect-2, 3 m after Inlet-2. It is the only sampling point where Pt concentrations of the sand fraction exceed the Pt concentration analyzed

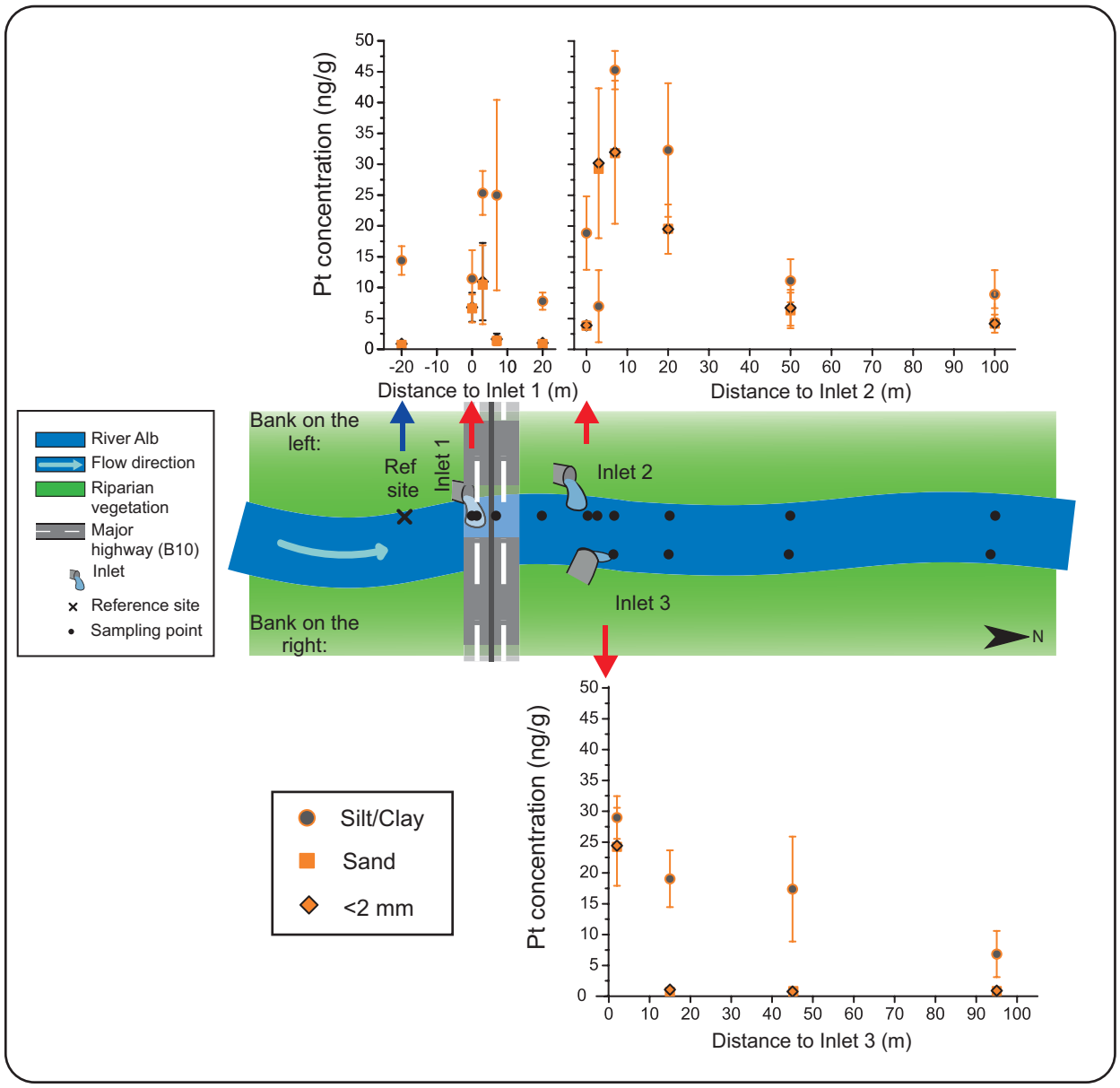

Figure 3.5: **Platinum concentrations in sediment samples of the river Alb. Platinum concentrations are shown in the sediment classes sand and silt/clay. Furthermore, concentration of Pt in the fraction <2 mm sediment samples were calculated.**

in the finer fraction. Concentrations in the sand fraction are in between 0.7 to 30 ng/g over all sampling points. As a third ”fraction” concentrations for all sediments <2 mm, were calculated (see Equation 3.1) to gain an approximate Pt load in the whole <2 mm sample . It can be seen in Figure 3.5 that the silt/clay fraction in the samples is very small and has therefore only a very small influence on the Pt concentration of the whole sample <2 mm. Thus, concentrations in the fraction <2 mm overlap mostly with Pt concentrations in the sand fraction.

Distribution of Platinum in Transect-1 In the sand fraction of sediment samples at Inlet-1 (sampling point T-1/0), Pt concentrations are already higher than in sediments at the reference site. Pt concentrations are rising at sampling point T-1/3. At these two sampling points, Pt concentration are also higher than at the reference site and the differences are statistically significant (U-test; $p<0.05$). Further downstream Pt concentrations are no longer distinguishable from Pt concentrations at the reference site. A similar pattern can be observed for the silt/clay fraction of the sediment. Compared to the reference site, higher Pt concentration are found 3 and 7 m downstream from the introduction of runoff water, whereby the difference to the reference site is statistically significant at 3 m. Concentrations at sampling point T-1/0 and T-1/20 are significantly lower compared to the reference site (U-test; $p<0.05$).

Distribution of Platinum in Transect-2 In Transect-2 Pt concentrations are increasing from the introduction of runoff water to sampling point T-2/7 and then decreasing again in all sediment fractions. Highest concentrations can be found at sampling point T-2/7 with 32 ng/g in the total sample <2 mm, 30 ng/g in the sand fraction and 45 ng/g in the silt/clay fraction. U-Tests provide evidence that in comparison to the reference site, the concentrations in the sand fraction are higher at all sampling points up to 50 m downstream from Inlet-2 in comparison. In the silt/clay fraction, concentrations are increased significantly at sampling point T-2/7 and T-2/20.

Distribution of Platinum in Transect-3 Also in Transect-3 Pt concentrations are elevated a few meters downstream from Inlet-3 in all size fractions (total sample <2 mm: 24 ng/g, sand fraction: 24 ng/g, silt/clay fraction: 29 ng/g). However, Pt concentration analyzed further downstream (15 to 95 m) do not exhibit a significantly statistical difference to the reference site.

In summary, Pt concentrations correspond to the discharge of road runoff water. Elevated concentration are found in all sediment fractions downstream from the different inlets. Concentrations are highest in the silt/clay fraction. After a steep increase of Pt in all fractions of samples up to 7 m downstream from the inlets, concentrations decrease exponentially. For two transects no differences to the reference site are detectable after 15 to 20 m in the sand fraction, downstream from the respective inlets. In Transect-2, however, elevated concentrations are detectable up to 50 m in the sand fraction. Highest Pt concentrations were detected for Transect-2, lowest for Transect-1.

Platinum in sediments compared to Platinum in soil samples

One aim of the study is to investigate similarities and differences between the Pt distribution in terrestrial and aquatic systems. Two recent studies were found which can be used to compare the sediments data with data of soils (Wichmann et al., 2007; Singer, 2008). Like in this study they

analyzed grain size fractions <2 mm and investigated similar distances from the Pt source. Data of these two studies and the data for all three transects of this study are plotted in Figure 3.6.

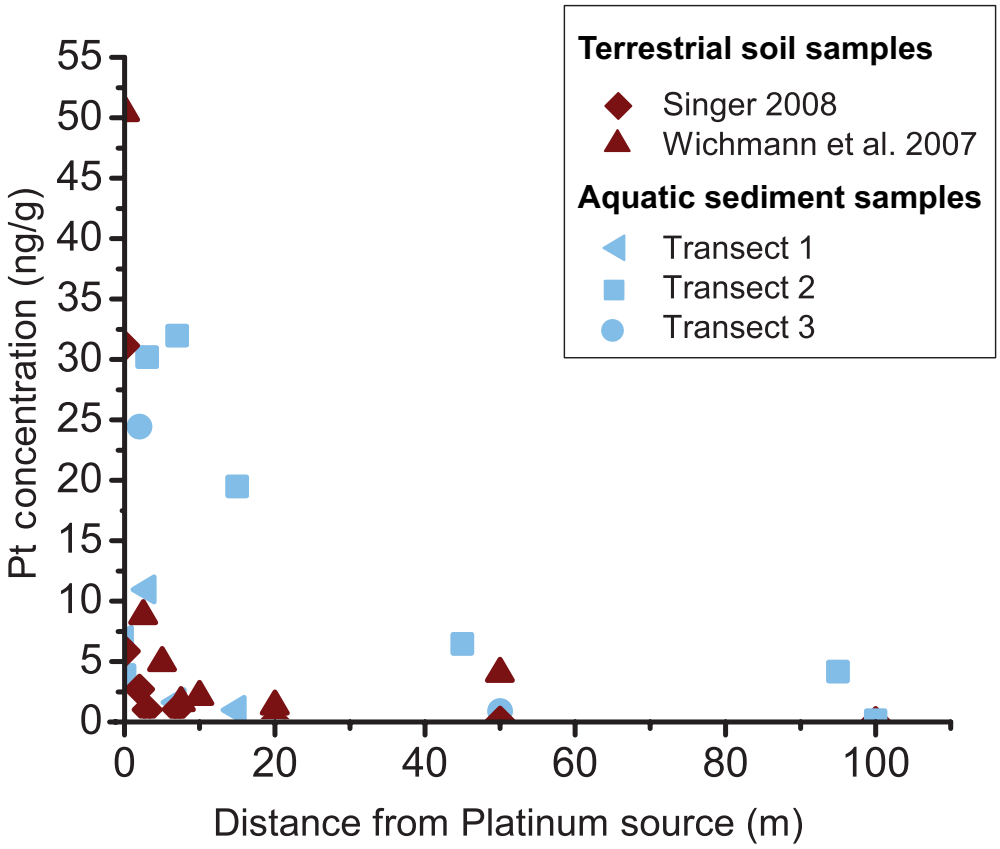

Figure 3.6: **Comparison between Platinum concentrations in sediments and Platinum concentrations in soils next to highly frequented roads. Data for Platinum concentrations in soil samples are listed in Wichmann et al. (2007) and Singer (2008). Soil samples were taken in the upper 2 cm of the soil and sieved. Fractions displayed here have a grain size <2 mm.**

Pt concentrations in soil samples are highest in direct proximity to the road (0.1 to 2 m). They exceed the Pt concentrations in sediments at the same distance downstream from the discharge point of road runoff. However, Pt concentrations in sediments are highest several meters downstream from the source (3-5 m), and not at the direct discharge situation. While 2 m away from the Pt source, concentrations in sediments of all transects are quite similar to concentrations reported by Wichmann et al. (2007), all exceed the concentrations found by Singer (2008). Especially in Transect-2 Pt concentrations are higher than those reported for soil concentration between 2 and 100 m away from the Pt source. Pt concentrations in Transect-1 and Transect-3, however, resemble the data reported for the analyzed soil samples. In general, it can be noted, that Pt concentrations in soil and sediments have been found to be in a comparable concentration range. They decrease rapidly with increasing distance to the Pt source. Whereas, in the case of greater distances from the source, the Pt concentrations in sediments are higher than those found in comparable soil

samples.

Platinum in sediments: Comparison to other traffic related heavy metals

Next to Pt, further traffic related heavy metals(i.e. Cd, Cr, Cu, Ni, Pb, Zn) and Ag were analyzed in sediment samples.

Mean concentration for all sediment samples and all sediment fractions can be found in the Appendices A.3 to A.5. The range of all heavy metals in sediment samples is presented in Table 3.4.

Table 3.4: **Minimum and maximum values of all heavy metals in sediment samples (n=5).**

Metal		Sediment <2 mm	Sand	Silt/clay
Ag	µg/g	0.16 - 0.40	0.16 - 0.4	0.54 - 1.5
Cd	µg/g	0.13 - 0.32	0.12 - 0.31	0.5 - 2.0
Cr	µg/g	14 - 177	12 - 177	128 - 224
Cu	µg/g	9.6 - 178	9.4 - 178	54 - 278
Ni	µg/g	9.5 - 33	9.1 - 33	30 - 63
Pb	µg/g	15 - 100	14 - 100	31 - 239
Pt	ng/g	0.8 - 32	0.7 - 32	6.8 - 29
Zn	µg/g	57 - 209	51 - 205	227 - 786

Pt was found in the lowest concentration range (low ng/g range), followed by Ag and Cd (high ng/g to low µg/g range). All other analyzed metals are in a concentration range of µg/g, with Ni <100 µg/g and Cr, Cu, Pb <300 µg/g. Highest concentration were found for Zn. Similar concentration ranges were already observed at the reference site.

To get an impression of the metal increase in sediments due to the introduction of road runoff, metal concentrations at the sampling points are compared to metal concentrations at the reference site (Figure 3.7 and Figure 3.8). For the figures, heavy metal concentration of the sediment fraction <2 mm at the reference site were set to 100% and the concentrations at the different sampling points were set into relation. Values above 100% therefore indicate an increase of metal content in the sediments in comparison to the reference site. As the illustration of eight different metals in the same figure is cluttered, the distribution of Cr, Cu, Pb and Pt are plotted in Figure 3.7, while the distribution of Ag, Cd, Ni and Zn are plotted in Figure 3.8.

Heavy metal distribution in Transect-1 With the exception of Cd, an increase of all heavy metal concentrations can be observed directly at the inlet of the road runoff water in comparison

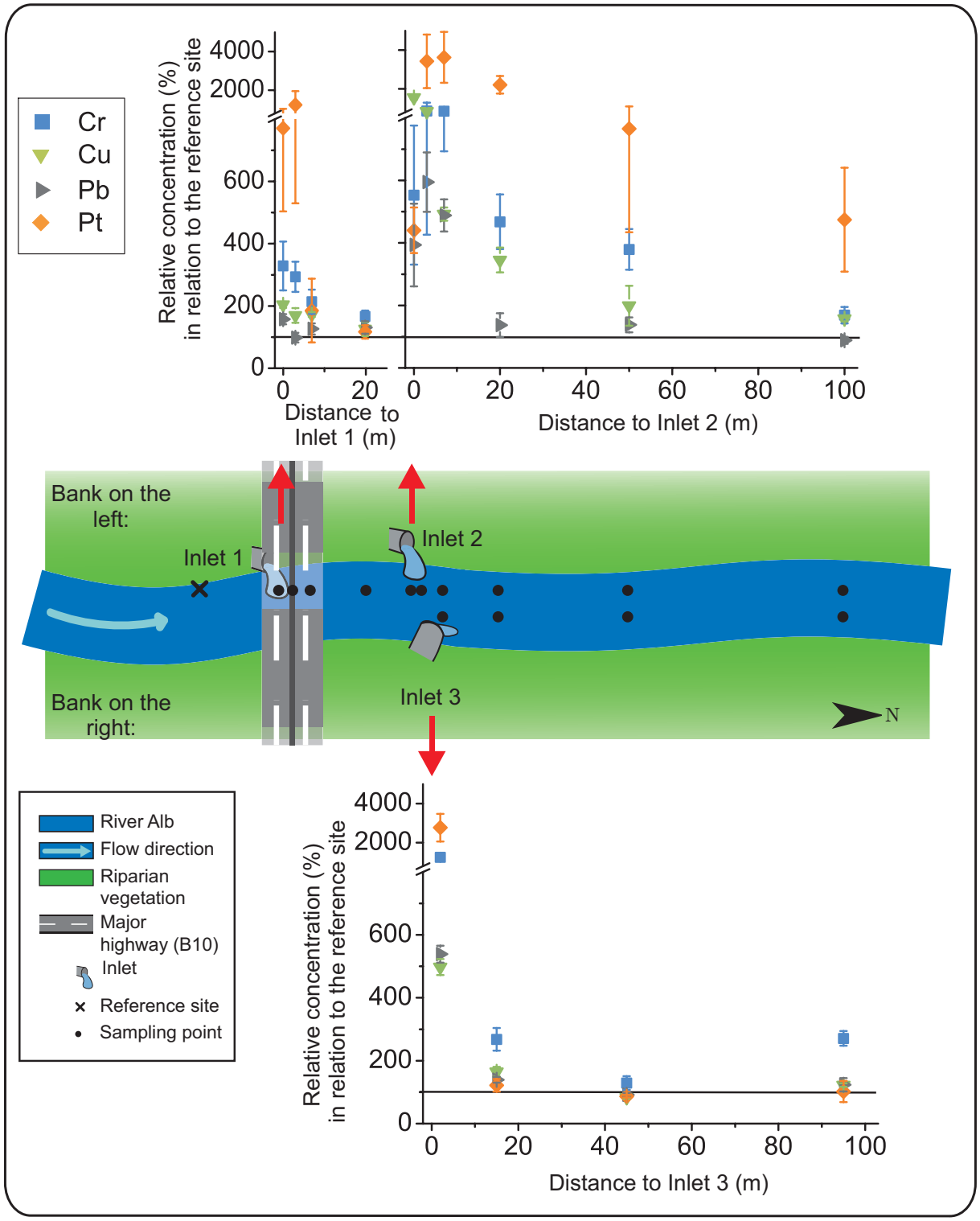

Figure 3.7: **Comparison of sediment concentrations at the reference site and the sampling points, Part 1. 100% correspond to heavy metal concentration at the reference site. Values are given for Cr, Cu, Pb and Pt in the sediment fraction <2mm.**

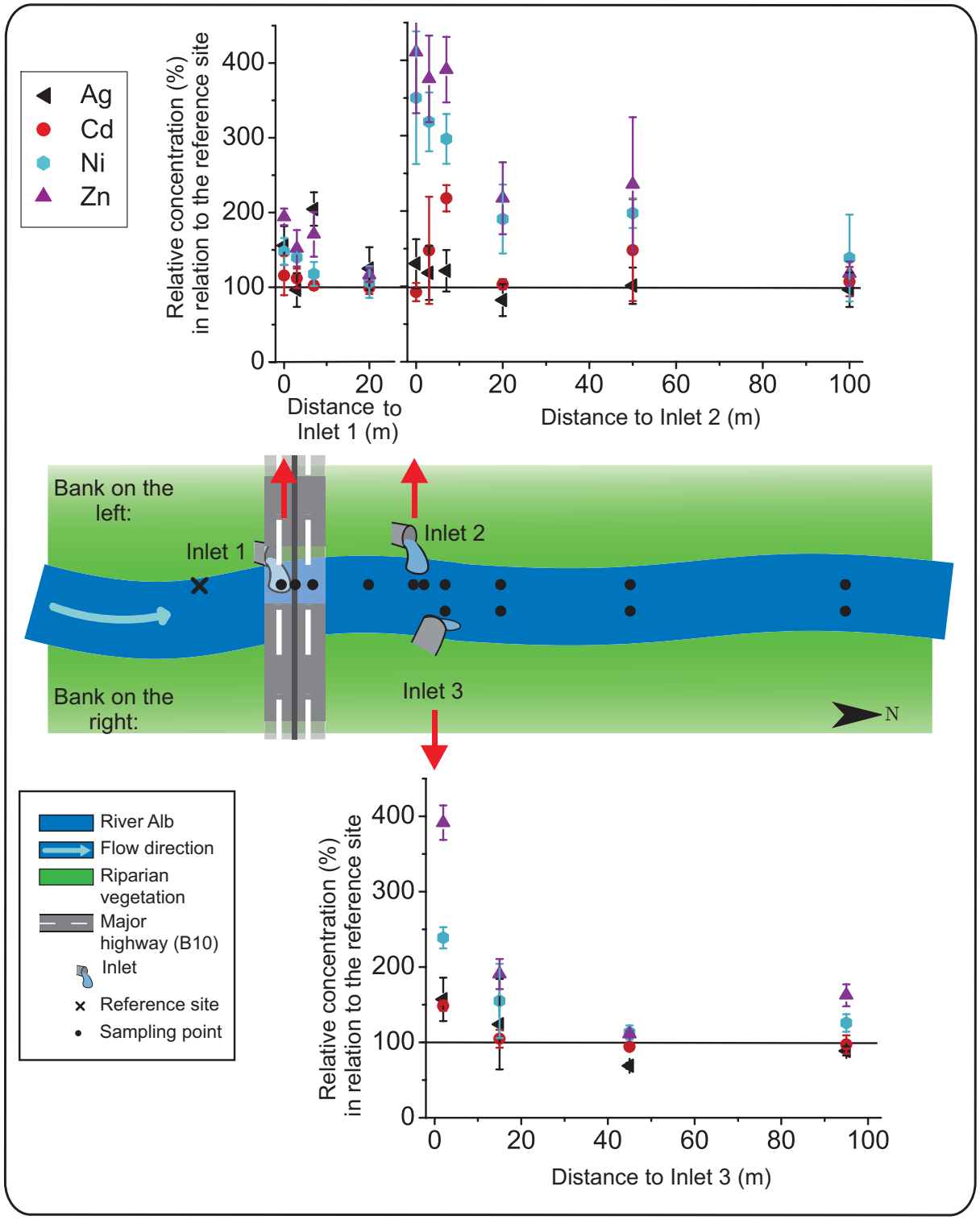

Figure 3.8: **Comparison of sediment concentrations at the reference site and the sampling points Part, 2. 100% correspond to heavy metal concentration at the reference site. Values are given for Ag, Cd, Ni, Zn in the sediment fraction <2mm.**

to the reference site. As can be seen in Appendix A.4, Cd was below the detection limit in the sand size category of all samples, nevertheless, it could be detected in the silt/clay category, in which Cd concentrations are significantly higher than in the reference sample for sampling points T-1/0 to T-1/7. Concentrations decrease immediately downstream Inlet-1. Highest relative concentrations are found for Pt 3 m downstream from Inlet-1 (12-fold). Also highly elevated in comparison to the reference site are the concentrations of Cr, which are 3-fold as high. The concentrations of other metals are also increased, like Ag and Cu (up to 2-fold), followed by Ni, Pb, and Zn an increase up to 1.8-fold compared to the reference site with 100%.

In the sand fraction at sampling point T-1/0 the observed differences between sampling points and reference site could be proofed statistically significant for almost all metals (excluding Cd, Ni, and Pb). For Cr, Cu and Zn the increase was also statistically significant up to 7 m downstream from the inlet. In the silt/clay fraction Ag and Cr concentrations do not show a significant increase compared to the reference site. Cu does show a statistically significant increase at sampling points T-1/7 and T-1/20. Cd was increased at sampling points T-1/0 to T-1/7, Ni at sampling points T-1/3 and T-1/7. Pb and Zn were increased at sampling point T-1/20.

Heavy metal distribution in Transect-2 As has already be seen for Transect-1 also at Transect-2 heavy metal concentrations in sediments increase dramatically at Inlet-2 and start to decrease again 7 or 20 m downstream. Heavy metal concentrations are higher in Transect-2 compared to Transect-1 and an increase compared to the reference site is still detectable up to 50 m downstream from the inlet. This is reflected in the results of the U-tests (p=0.05) for sand and silt/clay samples (see also Appendix A.12 and Appendix A.13). The U-tests reveal for the sand fraction, that

- 100 m downstream Inlet-2, Cu concentrations are still higher than at the reference site
- like Pt, also Cr and Ni concentrations are increased up to 50 m downstream from Inlet-2
- Zn concentrations are increased up to 20 m downstream from Inlet-2 and
- increased Pb concentrations could be found 7 m downstream from Inlet-2.

Cd and Ag only show significant increase in comparison to the reference site in the silt/clay fraction. The other metals show increased values up to 20 m (Cr, Cu, Ni, Zn) or 50 m (Pb) in the silt/clay fraction, as well. Also for Transect-2 Pt is the heavy metal with the highest increase in comparison to the reference site (approximately 36-fold, 7 m after Inlet-2), followed by Cr and Cu (up to 8.6-fold and 16-fold, respectively). In addition high concentrations can be found for Pb (up to 6-fold, 3 m downstream from Inlet-2), followed by Zn (up to 4-fold) and Ni (up to 3.5-fold). The increase of Cd and Ag is low compared to the other metals with highest relative concentrations of 2-fold for Cd and only 1.3-fold for Ag.

Heavy metal distribution in Transect-3 In Transect-3 there is a clearly noticeable increase of heavy metal concentrations 2 m after Inlet-3. While the increase is still easily detectable after 50 m in Transect-2, in Transect-3 concentrations are already comparable to the reference site 45 m after Inlet-3. The only exception is Cr, which is still increased by factor 3, 95 m downstream from Inlet-3. Even though, an increase can still be found as statistically different at sampling point T-3/15 and T-3/95 for Cr, Cu, Ni, and Zn in the sand fraction. This is also the case for Cd and Cr in the silt/clay fraction. In the silt/clay fraction Ni and Zn are elevated up to 45 m downstream from Inlet-3, Cu is elevated up to 95 m. Overall, again Pt shows the highest relative concentration (28-fold) followed by Cr (13-fold), Pb (5.3-fold), Cu (5-fold), Zn (3.9-fold), Ni (2.4-fold), Ag (1.6-fold) and Cd (1.5-fold), compared to the reference site with 100%.

In all three transects metal concentration increased directly at the inlets for the road runoff water, came to a maximum increase within 7 m downstream from the inlet and decreased afterwards, until concentrations were comparable to concentrations at the reference site. Highest relative increase could be found for Pt>Cr>Cu and Pb>Zn and Ni>Ag and Cd. While in Transect-1 and Transect-2 heavy metal concentrations decrease to the same range as those observed at the reference site within 20 m, several heavy metals are still increased 50 to 100 m after Inlet-2.

Cd, Cu, Pb and Zn in sediments compared to heavy metal concentration in soil samples

Corresponding to the analysis of Pt, concentrations of Cd, Cu, Pb and Zn in sediments were compared to concentrations in soil samples (see Figure 3.9).

Like Pt, also Cu and Zn are transported further away from the source in aquatic systems compared to the terrestrial system adjacent to a highway. Concentrations for Zn near to the highway are higher in soil samples than at the equivalent distances of the Zn source in sediment samples of the aquatic system. Concentrations of Cu in sediments are comparable to those of soil samples in Transect-2. Cd and Pb concentrations, however, are initially much higher in the soil samples than in sediment samples. Furthermore, their transportation range appear to be similar, regardless of transported by air or water.

Correlations in different sediment classes

Correlation analysis were performed to evaluate, if heavy metals originate from the same source or/and show different solution/adsorption properties. Table 3.5 present the Spearman correlation coefficients for all correlations with a p-value <0.001. The results of other significant analyses resulted in R values lower than 0.5 and are listed in Appendix A.8.

Most analyzed correlations are highly significant (p-value <0.001). Especially the group of Cr,

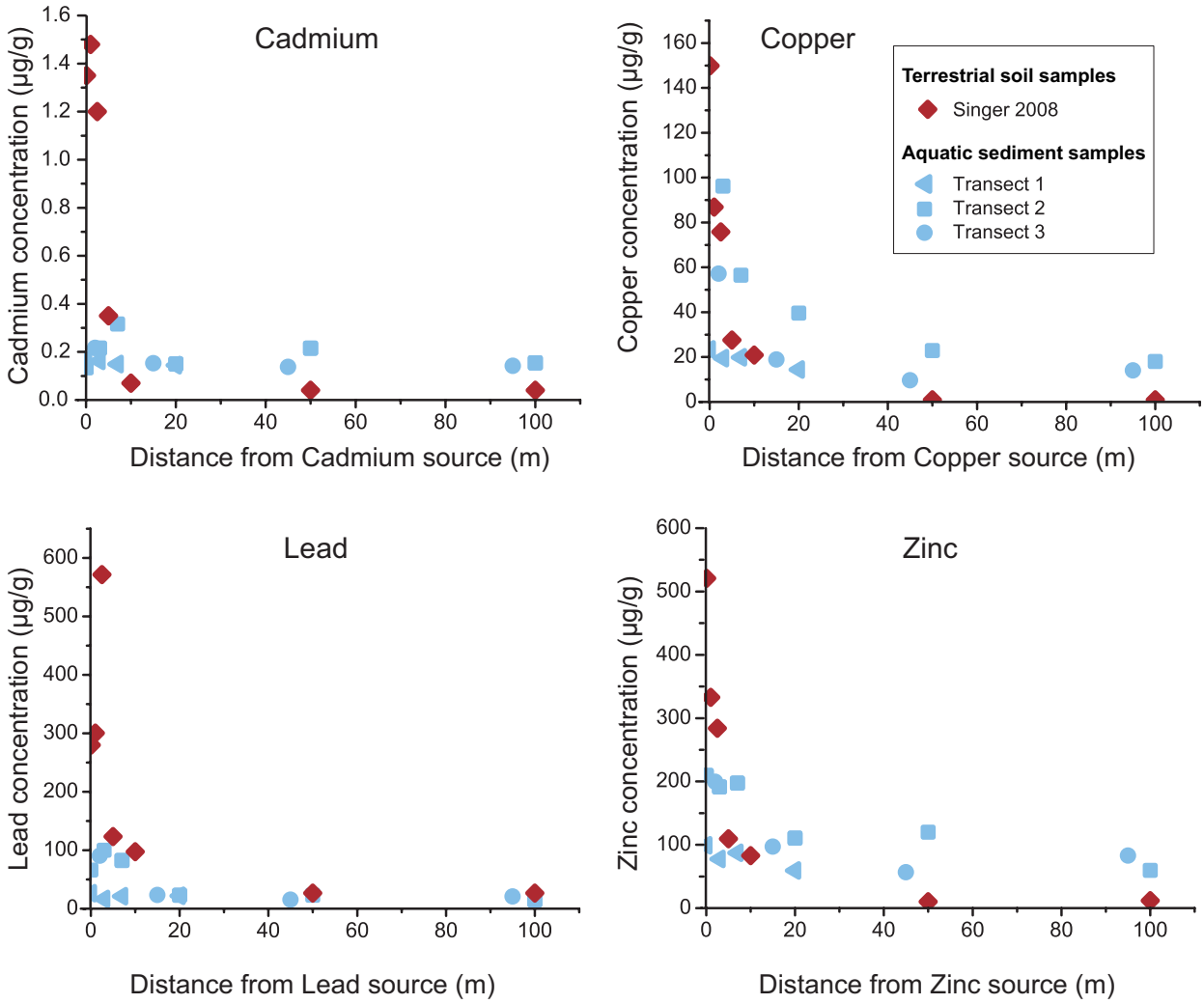

Figure 3.9: **Comparison between Cd, Cu, Pb and Zn concentrations in sediments and in soils next to highly frequented roads. Data for heavy metal concentrations in soil samples are taken from Singer (2008). Soil samples were taken in the upper 2 cm of the soil and sieved. Fractions displayed here have a grain size <2 mm.**

Cu, Ni, Pb, Pt and Zn show highly significant correlations in combination with relatively high correlation coefficients (0.5 - 0.9). As Cd concentrations were below the LOD in the sand fraction, it was not included in the correlation analysis. All significant correlations for Ag are below an Rank correlation coefficient of 0.5. Pt correlates best with Cu>Cr>Ni>Zn.

Table 3.6 illustrates correlations among heavy metals in the silt/clay fraction of the sediments with a p-value of <0.001 and a Spearman Rank correlation coefficients >0.4.

Despite the results for the sand fraction, Cd concentration were above LOD in the silt/clay fraction and correlated with most of the other heavy metals in the silt/clay subsample of the sediments. Also Cu, Ni, Pt and Zn are correlated with several of the other heavy metals, Pb and Cr correlate

Table 3.5: **Rank correlation coefficients between heavy metal concentrations in sediments - class sand.**

P-Level	Ag	Cd	Cr	Cu	Ni	Pb	Pt	Zn
p<0.001			Zn (0.9)	Zn (0.9)	Cr (0.8)	Zn (0.7)	Cu (0.7)	Cu (0.9)
			Cu (0.9)	Cr (0.9)	Zn (0.8)	Cr (0.7)	Cr (0.7)	Cr (0.9)
			Ni (0.8)	Ni (0.8)	Cu (0.8)	Cu (0.6)	Ni (0.7)	Ni (0.8)
			Pt (0.7)	Pt (0.7)	Pt (0.7)	Ni (0.6)	Zn (0.6)	Pb (0.7)
			Pb(0.7)	Pb (0.6)	Pb (0.6)	Cd (0.5)		Pt (0.6)
								Cd (0.5)

Table 3.6: **Rank correlation coefficients between heavy metal concentrations in sediments - class silt/clay.**

P-Level	Ag	Cd	Cr	Cu	Ni	Pb	Pt	Zn
p<0.001		Cu (0.8)	Ni (0.6)	Zn (0.9)	Zn (0.8)	Cu (0.6)	Cu (0.6)	Cu (0.9)
		Zn (0.7)	Cd (0.4)	Cd (0.8)	Cd (0.7)	Cd (0.5)	Zn (0.5)	Ni (0.8)
		Ni (0.7)		Ni (0.7)	Cr (0.7)		Cd (0.5)	Cd (0.7)
		Pt (0.5)		Pb (0.6)	Cu (0.6)			Pt (0.5)
		Pb (0.5)		Pt (0.6)				

only with two of the other metals. Ag does not show highly significant correlations. In general, correlation coefficients in the silt/clay fraction are lower than in the sand fraction. Pt correlates with Cu>Zn>Cd.

Classification of heavy metal concentrations in sediment samples

With the introduction of the European Water Framework Directive in 2000, Environmental Quality Standards (EQS) are defined for metals in sediments. If the EQS are exceeded, the ecological status of a river can not be validated as "good". The EQS are given for Cu (160 µg/g), Cr (640 µg/g) and Zn (800 µg/g) in the silt/clay fraction. Accordingly, the respective concentration limits in sediments for Cu is exceeded at sampling points T-2/7, T-2/20 and T-3/2.

Prior to the introduction of the European Framework Directive, a sediment classification system

published by LAWA (1998) was used. Herein, the concentration of the metals Cd, Cr, Cu, Ni, Pb, and Zn are classified. To get an impression of the impact of the investigated inlet for runoff-water from the B10, the heavy metal concentration found in sediment samples were classified using this classification system (see Figure 3.10). The classification classes are changing for all heavy metals along all transects. Overall, lowest contamination loads are found for Cd, Ni, and Pb. Classes range between “very low contamination” (Class I-II) to “considerable contamination” (Class II-III). Cr and Cu already show a “considerable contamination” at the reference site which changes to an “increased contamination” (Class III) in Transect-2 and Transect-3. At T-2/7 the classification for Cu change into “high contamination” (Class III-IV). Highest loads are found for Zn. Here classes are inbetween “considerable contamination” (Class II-III) and “high contamination” (Class III-IV). In order to summarize the overall chemical state of the sediments at each sampling point, the highest impact class of all metals was chosen and is displayed as ”site classification” in Figure 3.10.

Even at the reference site sediments are “considerably contaminated”, due to Cr, Cu and Zn concentrations. Inlet-1 does not bring about a change in classification. After Inlet-2, however, the contamination situation worsen to “increased contamination” for about 7 m and then to “high contamination”, mostly due to the elements Cr, Cu and Zn. 50 m downstream from the second inlet, concentrations of heavy metals decrease again, and the classification is rated “considerable contamination”. Along Transect-3, especially Zn concentrations define the overall sediment classification. Consequently, 2 m downstream from Inlet-3, the sediment quality is rated as “high contamination”, the following sampling points between 15 m and 95 m downstream from Inlet-3 are still classified as “increased contamination”. The impact of road runoff on Transect-2 and -3 are thus clearly visible based on the changes in the sediment contamination classification. The contamination loads of the traffic related heavy metals change the initially contaminated sediment into increased or even highly polluted sediments.

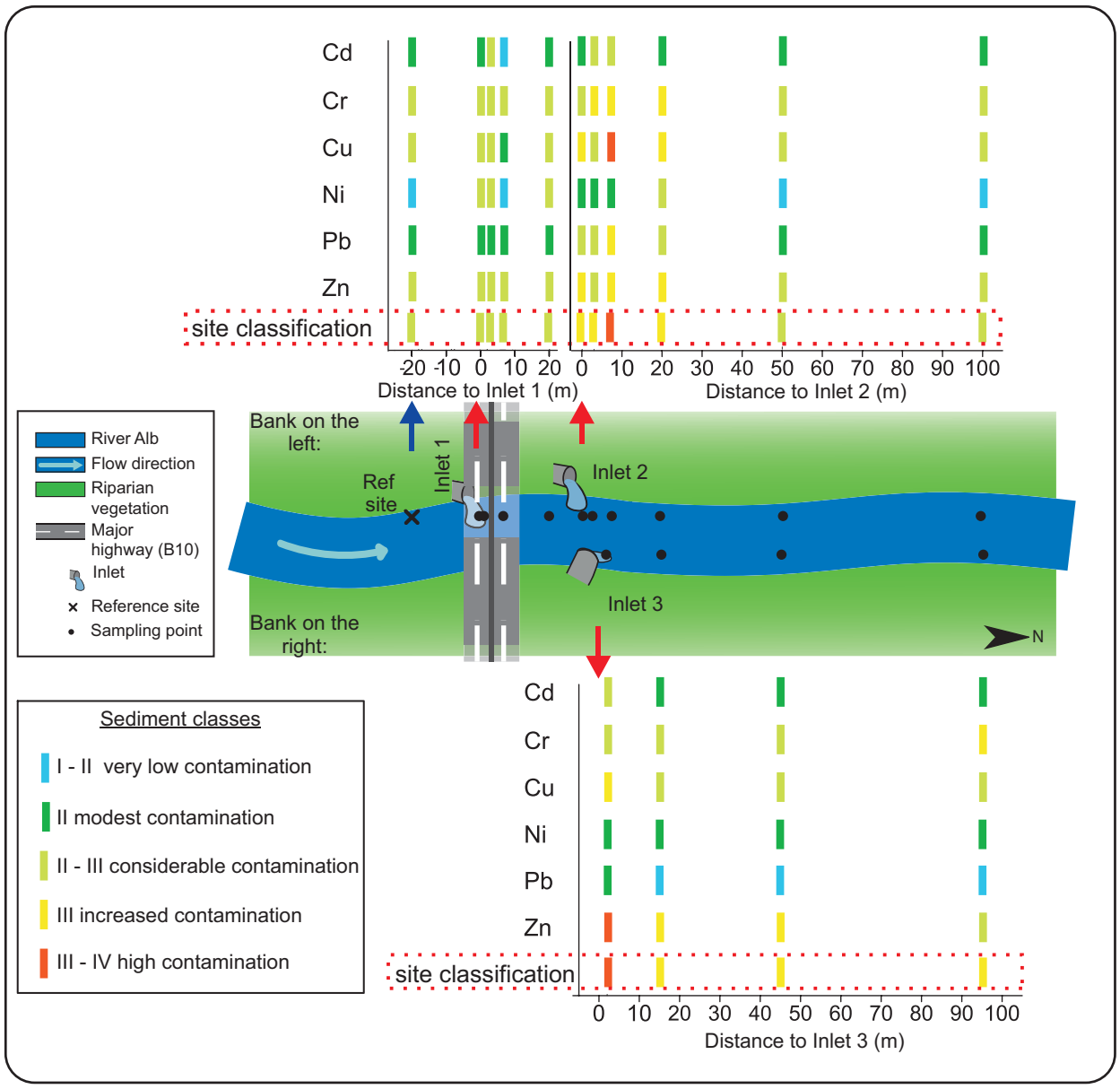

Figure 3.10: **Sediment classification in the transects of the river Alb. Analyzed metal concentration in the silt/clay fraction is classified due to the sediment classification system of LAWA (1998). The highest class of each sampling point was used to illustrate the sediment quality of the whole sampling site (i.e. site classification).**

3.3.3 Traffic related heavy metals in clam samples (*Corbicula* sp.) of the river Alb

Size and conditon factor of the clams at the different sampling points

At each sampling point approximately 30-45 clams were sampled and pooled to one sample. Figure 3.11 presents boxplots for the shell length and the condition factor of the clams in the pooled samples for all sampling points.

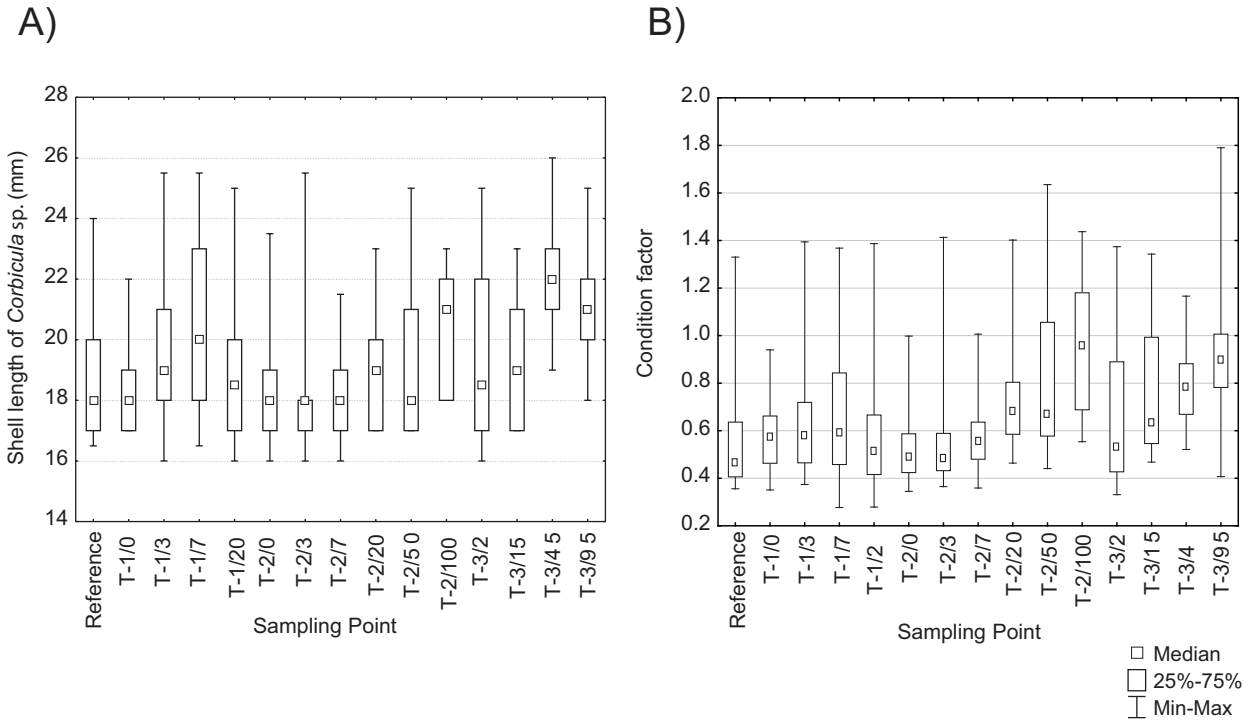

Figure 3.11: **Comparison of shell length and condition factor of *Corbicula* sp. at the reference site and the sampling points. A) shows boxplots of shell length, B) presents the conditon factor. The definition of the condition factor can be found in Equation 3.2**

It is obvious that there are some samples which differ significantly from others with respect to the shell length. The median of the sampling points T-1/7, T-3/45 and T-3/95 is greater in comparison to other sampling points (especially those with small medians, eg. T-1/0, T-2/0 to T-2/7). The differences were found to be statistically significant (U-Test, $p<0.05$). The same results can be found for the shell width and the shell height. Also the condition indeces vary among the different samples, as can be seen in part B of Figure 3.11. Statistically clams collected at the sample points T-2/7 to T-2/100 and T-3/15 and T-3/95 have a higher condition index than clams from other sampling points (e.g. reference site, T-1/0 to T-2/3), (U-Test, $p<0.05$). Shell measurements and the condition factor of all sampling sites can be found in Appendix A.11.

Heavy metal concentration in clam tissue collected at the reference site

In accordance with the treatment of the sediment samples, clam samples were collected and analyzed at the reference site to investigate the tissue concentration without the influence of the three road runoff inlets. Table 3.7 shows the mean metal concentration and the standard deviation of the analyzed clam samples.

Table 3.7: **Heavy metal concentrations in freeze dried clam tissue of the reference site (n=5).**

Metal		Mean concentration	Standard deviation
Ag	µg/g	0.02	0.01
Cd	µg/g	0.11	0.02
Cr	µg/g	2.1	0.43
Cu	µg/g	36	4.3
Ni	µg/g	0.77	0.03
Pb	µg/g	0.21	0.08
Pt	ng/g	0.09	0.06
Zn	µg/g	143	13

In the clam tissue, all metal concentrations are above the limit of detection. Furthermore, as has already been observed for sediment samples, concentrations vary depending on the analyzed metal. The lowest concentrations are found for Pt (pg/g range). Ag, Cd, Pb and Ni are found in a ng/g range, and higher concentrations are found for Cr<Cu<Zn in a µg/g range.

Platinum in clam samples

Pt was analyzed in all clam samples and compared to Pt concentration in clam tissue of the reference site. Figure 3.12 presents the detected concentration of five replicate analyses at every sample point.

At T-1/20, T-2/3, T-3/3 and T-3/95 Pt concentrations are below 0.05 ng/g and therefore below the limit of detection. Only at the sites T-1/3, T-1/7, T-2/20, T-2/100 and T-3/15, Pt concentrations are above the limit of quantification of 0.15 ng/g. Pt concentrations in *Corbicula* sp. are low in general. After Inlet-1, Pt concentrations show the tendency to slightly increase after 3 and 7 m in

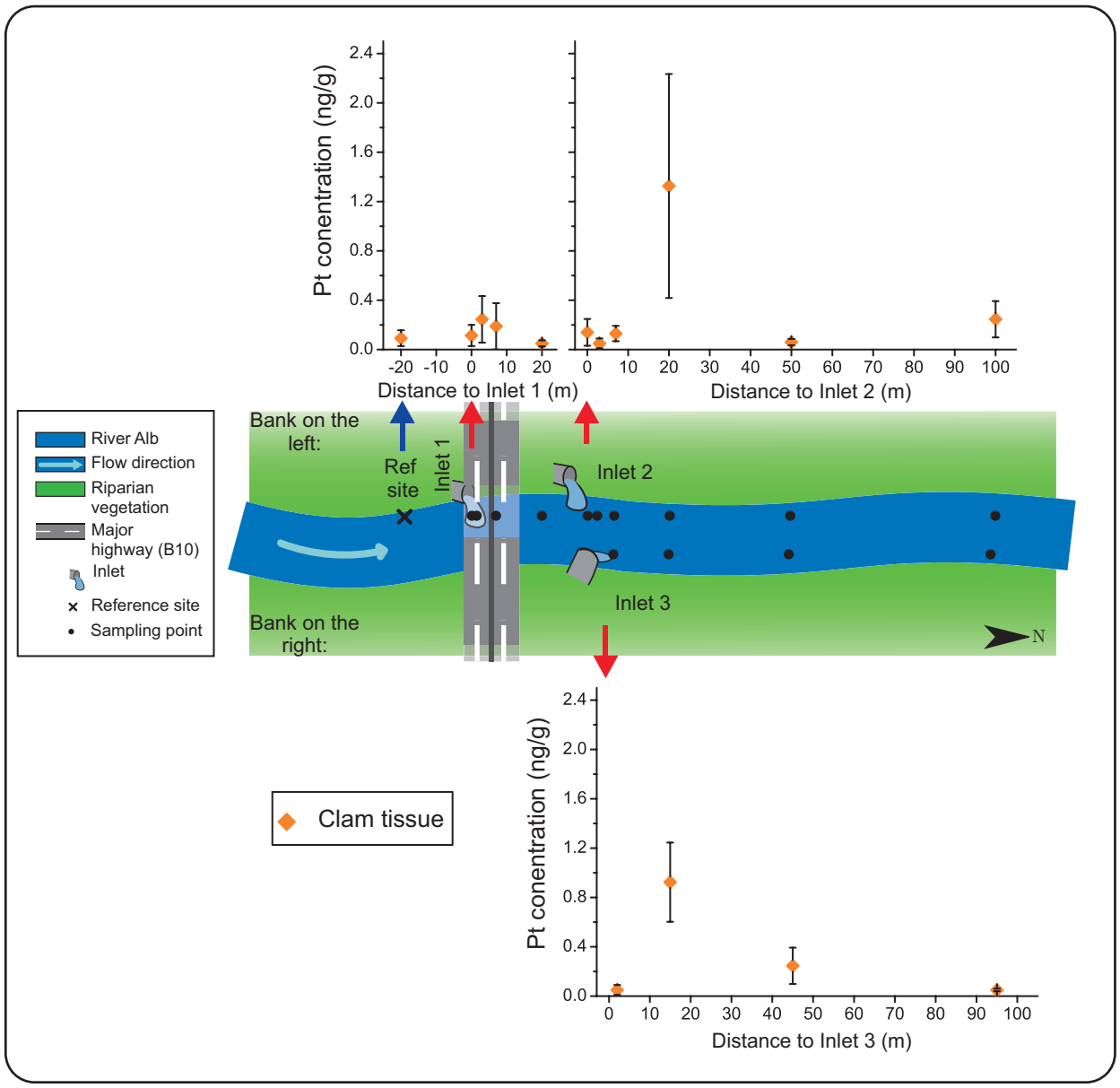

Figure 3.12: **Platinum concentrations in freeze dried clam tissues. Platinum concentrations are presented in ng/g (n=5). Error bars indicate the standard deviation.**

comparison to the reference site. In the Transects-2 and -3, Pt concentrations in clam tissue are more distinctly distributed. Shortly after the introduction of the runoff water, Pt concentrations are still similar to the concentrations at the reference site. This changes after 20 m in Transect-2 and 15 m in Transect-3, when Pt concentrations in *Corbicula* sp. increase considerably. The increase of Pt has been determined to be statistically significant for T-3/15. At T-2/20, however, the standard deviation of the analyses is too high to be tested as a statistically significant increase (U-Test, $p<0.05$). Further downstream from Inlet-2 and Inlet-3 Pt concentrations are decreasing, but are still higher than at the reference site, even 100 m downstream from Inlet-2. Thus, at two of three transects Pt concentrations in clam tissue increase following the introduction of runoff

water.

Platinum in clam samples: Comparison to other traffic related heavy metals

Also the elements Ag, Cd, Cr, Cu, Ni, Pb and Zn were analyzed in *Corbicula* sp. samples. The minimum and maximum concentrations for each metal are listed in Table 3.8. Means and standard deviations for all sampling points can be found in Appendix A.6. Furthermore, Appendix A.14 lists the results of Mann-Whitney U-Tests between means of metal concentrations at a specific sampling point to means of metal concentrations at the reference site.

Table 3.8: **Concentration ranges of all analyzed heavy metals in freeze dried clam tissue (n=5).**

Metal		Minimum concentration	Maximum concentration
Ag	µg/g	0.02	0.05
Cd	µg/g	0.08	0.2
Cr	µg/g	<1.48	6.7
Cu	µg/g	31	47
Ni	µg/g	0.61	1.4
Pb	µg/g	0.20	0.58
Pt	ng/g	<0.05	1.3
Zn	µg/g	95	154

As has been already observed for Pt, also Cr could not be detected in all samples. Cr concentration were below the limit of detection (1.40 µg/g) at several sampling points (i.e. T-1/0, T-1/3, T-1/7, T-2/3, T-2/20, and T-2/50). Only in Transect-3 Cr concentrations exceed the limit of quantification (i.e. 2.5 µg/g). All other heavy metals are detectable in all tissue samples. However, Ni concentrations are below the limit of quantification (i.e. 0.71 µg/g) at the sampling points T-1/3, T-1/7, and in nearly all sampling points downstream from Inlet-2 (with the exception of T-2/0). In Transect-3, Ni is detectable in all *Corbicula* sp. samples. With regard to their concentration in clam tissue the examined metals occur in the following order:

Zn>Cu>Cr>Ni>Pb>Cd>Ag>Pt

Zn and Cu concentration are found to be higher than Cd, Cr, Ni and, Pb by a factor of 10 to 100. They exceed the Ag concentrations by a factor of 1,000 and Pt by a factor of 100,000.

To verify that the road runoff of the inlets has any effect on the heavy metal content of the clam

tissue, concentrations for each heavy metal at each sampling point were plotted relative to the concentrations in the clam tissue of the reference site (see Figure 3.13 for Cr, Cu, Pb, and Pt and 3.14 for Ag, Cd, Ni, and Zn).

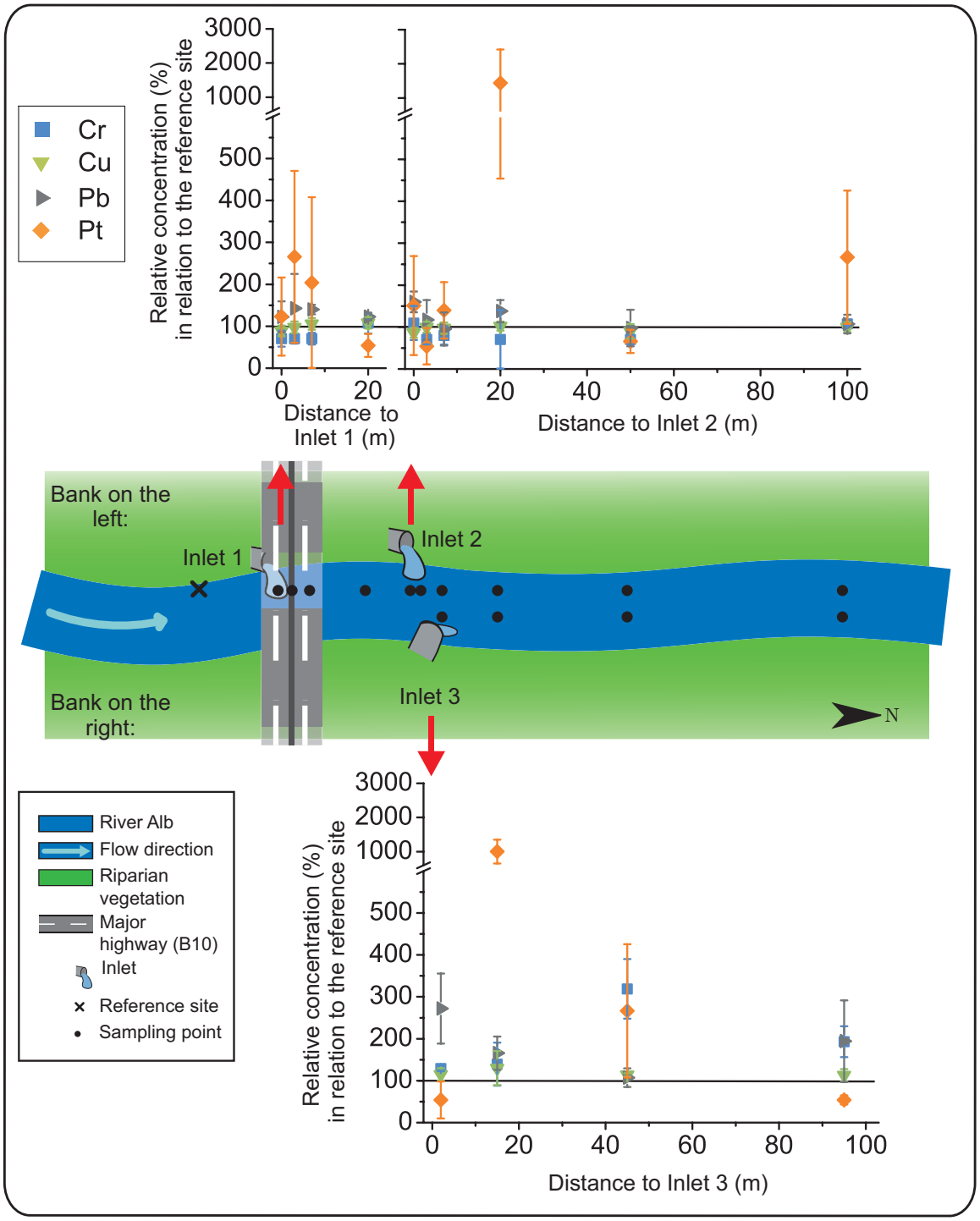

Figure 3.13: **Relative heavy metal concentrations in freeze dried clam tissue of *Corbicula* sp. in relation to heavy metal concentration in clam tissue of the reference site, Part 1. 100% match the concentration of Cr, Cu, Pb, Pt in clam tissue of the reference site (n=5).**

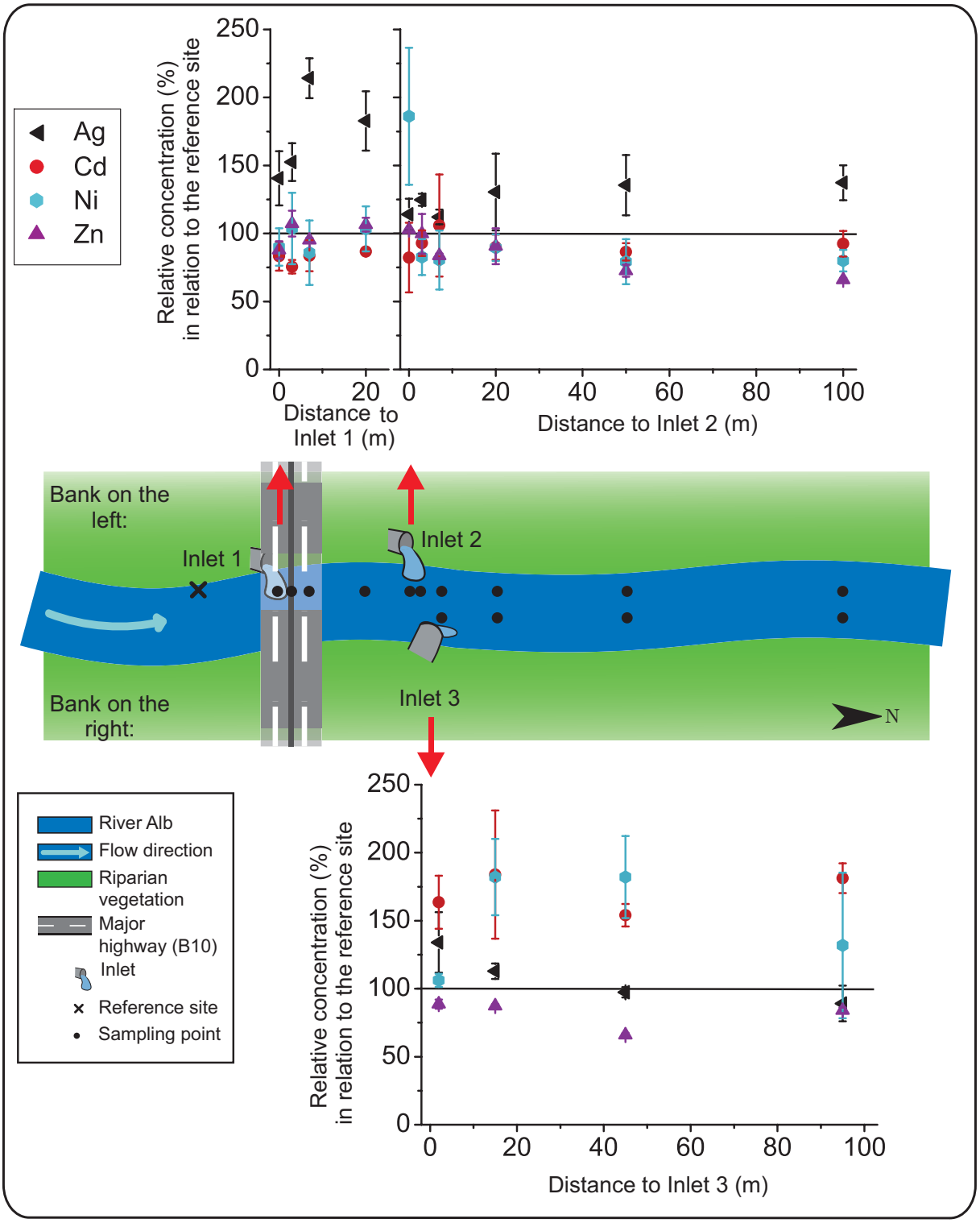

Figure 3.14: **Relative heavy metal concentration in freeze dried clam tissue of *Corbicula* sp. in relation to heavy metal concentration in clam tissue of the reference site, Part 2. 100% match the concentration of Ag, Cd, Ni, Zn in clam tissue of the reference site (n=5).**

Downstream from Inlet-1 only Ag and Pt show an increase in relation to the concentrations at the reference site. While the increase is statistically significant for Ag, at 7 m (U-Test, $p<0.01$) and at 20 m (U-Test, $p<0.05$) downstream from Inlet-1, it is not significant for Pt. All other heavy metals are still in the same concentration range as observed at the reference site. Downstream from Inlet-2 also no considerable increase of heavy metal in clam tissue can be observed for Cd, Cr, Cu, and Zn. At sampling point T-2/0 the amount of Ni, Pb and Pt is slightly increased. The increase to 185% for Ni is statistically different from the Ni concentration in clam tissue at the reference site (U-Test, $p<0.01$). Pt is the only metal which is increased to a considerable extend. 20 m after Inlet-2, Pt concentration in clam tissue is found to be higher than the reference site by a factor of 14 and 100 m after Inlet 2 it is 2.7 times higher than in clam tissues at the reference site. The highest differences of heavy metal concentrations in *Corbicula* sp. can be observed in Transect-3. With the exception of Ag and Zn, clam tissues of samples downstream from Inlet-3 indicate a higher accumulation of all heavy metals than for clams at the reference site (U-Test, $p<0.05$):

- Cd and Cu concentrations were tested to be higher than at the reference site at all sampling points in Transect-3
- Ni concentrations were tested to be higher at sampling points T-3/2, T-3/15 and T-3/45
- Pb concentrations were tested to be higher at sampling point T-3/2
- Cr concentrations were tested to be higher at sampling points T-3/45 and T-3/95
- Pt concentrations are higher than the reference values by factor 10 at sampling point T-3/15

Unlike for sediments, heavy metal concentrations in clam tissue do not show a clear spatial pattern correlated to the road runoff discharge. Still, some metals are increased after one or several inlets compared to the reference site. The highest accumulation of metals was observed downstream from Inlet-3. The highest relative concentrations are found to occur in the following order Pt>Cr>Pb>Ag>Ni>Cd>Cu. Zn was not observed to be above the reference values at all.

Correlations of heavy metals within clam tissue

To analyze correlations between metals within the clam tissue, Spearman Rank correlation analyses were carried out. All significant results ($p<0.001$) with an correlation coefficient higher than 0.3 are presented in Table 3.9, all results are listed in Appendix A.9.

It is evident that correlations between heavy metals in the clam tissue are relatively weak. Highest rank correlation coefficient can be found for Cr and Ni with R=0.64. All other correlation coefficients are lower than 0.5. There is one negative correlation for Ag with Cd, all other correlations

Table 3.9: **Significant correlations between heavy metal concentrations in clam tissues.**

P-Level	Ag	Cd	Cr	Cu	Ni	Pb	Pt	Zn
p<0.001	Cd (-0.5)	Ag (-0.5) Cr (0.4) Cu (0.4)	Ni (0.6) Cu (0.5) Cd (0.4)	Cr (0.5) Cd (0.4)	Cr (0.6)		no correlation	

are positive. The essential metal Zn does only correlate weakly with Ni. Pt does not show any correlations with other heavy metals in clam tissues.

Correlations of heavy metal concentration in clam and sediment samples

To test, if heavy metal concentrations in *Corbicula* sp. directly correspond to heavy metal concentration in sediments, additional correlation analyses (Spearman Rank correlation) were conducted. The following correlations were calculated for each metal:

- concentration in clam tissue vs. concentration in sand fractions
- concentration in clam tissue vs. concentration in silt/clay fractions
- concentration in clam tissue vs concentration in sediments <2 mm

Two statistically significant correlations were found (i.e. p<0.05). These include Ag in clam tissues and Ag in the sand fraction (R=0.4). Furthermore, for Cd in clam tissue and in the sediments <2 mm (R=0.54). Figure 3.15 illustrates the linear correlation plot for all significant correlations. All other results are listed in Appendix A.10.

Ag does show a clear linear correlation in clam tissue and the sand fraction. However, the y-axis indicates that the concentration changes in the clam tissues are very low.
For all other heavy metals, including Pt, concentrations in clam tissues do not directly depend on heavy metal concentration in sediments. Regardless of this finding, bioconcentration factors were calculated, since they often indicate in which matrix (clam or sediment) the metals accumulate most. Table 3.10 summarizes minimum, maximum and mean $BCF_{sediment}$ which were calculated for all sampling points. All results are presented in Appendix A.15.

As the mean concentration in clam tissue is divided by mean concentrations in sediment samples (<2 mm), $BCF_{sediment}$ >1 indicate a higher accumulation of the metal in the tissue than in the sedi-

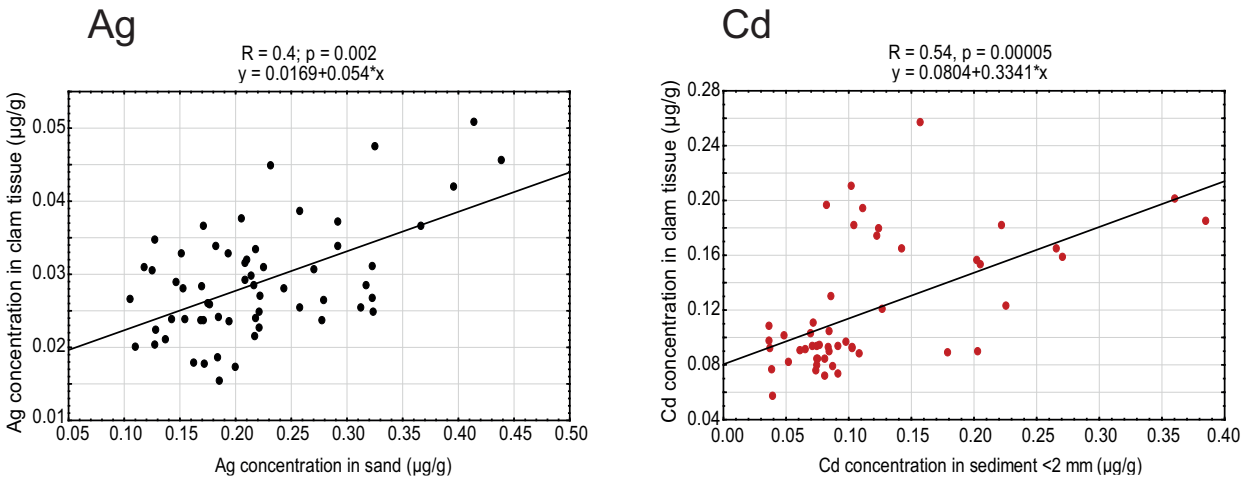

Figure 3.15: **Spearman Rank correlations between metals in sediments and clam tissue. Presented are all correlations with p<0.05.**

Table 3.10: **Bioconcentration factors ($BCF_{sediment}$) for heavy metals in clam tissue and sediment <2 mm.**

	Ag	Cd	Cr	Cu	Ni	Pb	Pt	Zn
Min	0.1	0.37	0.01	0.18	0.02	0.002	0.002	0.62
Max	0.2	**1.5**	0.37	**4.4**	0.13	0.02	**1.3**	**2.9**
Mean	0.14	0.81	0.08	**2.0**	0.06	0.01	0.33	**1.5**

ment. Table 3.10 demonstrates, that Cr, Ni and Pb are accumulated to a larger extend in sediments than in clam tissues at all sampling points. Some metals, however, are accumulated to a higher degree in clam tissues than in sediments at some sampling points. This holds true for Pt at sampling point T-3/15; for Cd at all of the sampling points in Transect-3; for Cu and Zn in all sampling points of Transect-1 and 3 (without T-3/2) and some of the sampling points in Transect-2 (T-2/20, T-2/50 and T-2/100). $BCF_{sediment}$ decreases in the following order:
Cu>Zn>Cd>Ag>Pt>Cr>Ni>Pb.

3.4 Discussion

3.4.1 Platinum and other traffic related heavy metals in sediments

To study the introduction of Pt in river systems through traffic discharges, Pt was analyzed in different grain sizes of sediments samples . The results clearly reveal, that Pt concentrations increase dramatically after an introduction of runoff water and decreases again to initial concentration levels within 20 to 50 m downstream from the respective inlet. Therefore, the traffic on the B10 road is obviously a source for Pt in the river Alb. Pt could further be detected in all fractions at all sampling points. In the silt/clay fraction concentrations are higher than in the sand fraction. However, taking into account that for the total sediment sample the percentage of sand is much higher than the percentage of silt/clay at all investigated sampling points, it can be stated that most of the Pt mass is present in the sand fraction. This is reflected by the comparison of the Pt concentration of the sediment fraction <2 mm to the concentrations in the sand and silt/clay fraction. The Pt concentration curve of the sediment fraction <2 mm resembles the curve of the sand fraction. This results can partly be explained by the Pt division in road dust. Pt particles deriving from automobile catalyst converters were found to have particle diameters in between <0.39 and 65.3 µm (Gómez et al., 2002), and should therefore, predominately enrich in the fraction <63 µm. Still, in road dust, particles are agglomerating and Pt can be found predominately in the fraction 250-63 µm (Jarvis et al., 2001). Also in road dust the fraction of the 250-63 µm is larger. Jarvis et al. (2001) found that typical proportions of Pt in sieved road dust were 13% (250-63 µm) and <1% (<63 µm) of the total sample. Therefore, the main load of Pt is already greater in the larger road dust fraction than in the small road dust fraction, which is then reflected also in the sediment samples. Similar results were found by Pratt & Lottermoser (2006) for other traffic related heavy metals. However, it can be assumed that in this study part of the introduced Pt particles did not precipitate at the sampling points but is transported in the particulate matter of the water column. This hypothesis is supported by the results of Flemming et al. (2004) who found that most of Pt in river systems is transported in the suspended particulate matter. These findings would explain, why concentrations at the reference site are above the geological background concentrations for Pt. As can be seen in the results, Pt concentrations in the silt/clay fraction are higher than the geological background values. It is assumed that this is the result of regional transport of Pt in the suspended particulate matter.

To analyze if the Pt concentrations in sediments of the river Alb are typical, high or low, it is further compared to other studies. Pt concentrations in sediments have already been studied by several other authors. In the recent literature, PGE data for approximately 100 different sampling sites (most of them in European urban regions) can be found. Unfortunately, PGE were analyzed in varying grain size fractions of the sediment samples and the studies are therefore not comparable in all aspects. Nevertheless, some of the authors provide a detailed description of the conditions at

the sampling sites, allowing for a classification into sampling sites which are highly polluted, like those which are in short distance to inlets of sewer systems or mine drainage systems, and lightly polluted sites (e.g. in urban surroundings, but not directly affected by discharge of Pt containing waters, or upstream/downstream from such discharges). In highly polluted sediments, concentrations are above 20 ng/g for Pt (Moldovan et al., 2001; Whiteley & Murray, 2005; Prichard & Jackson, 2008). In lightly polluted sediments Pt concentrations are usually below 10 ng/g (de Vos, 2002; Haus et al., 2007b; Prichard & Jackson, 2008; Pratt & Lottermoser, 2006). In this study Pt can be found in concentration ranges in between 15 and 45 ng/g at sampling points up to 20 m downstream from the inlet in all transects, in the silt/clay fraction, and commonly also in the sand fraction. At the reference site and the sampling points further downstream, Pt concentrations are approximately 10 ng/g or below. The only exception is sampling point T-3/45, where Pt concentration is 17 ng/g. Relatively high concentrations of Pt in sediments at the studied sampling site are therefore limited to a section of 20 m downstrem from the inlets in Transect-1 and Transect-2 and to 45 m downstream from the inlet for Transect-3. A similar result was found for the other traffic related heavy metals. Here the degree of pollution could be classified by classification classes developed by LAWA (1998). In case of most metals the introduction of road runoff water results in a downgrading of their respective contamination class. Commonly, this effect disappears again after 20 m downstream from the source of contamination. Pt can therefore be rated as a good tracer metal in sediments for impacts of traffic related heavy metals.

In this and already in other studies (Haus et al., 2007b, 2009a), it was shown that Pt concentration in sediments are nearly negligible in comparison to other traffic related heavy metals. This can be explained by the rare natural occurence of Pt. Furthermore, it is already well-known that also in the source of road runoff (i.e. road dust), Pt concentrations are among the ng/g range, while other heavy metals are found in the µg/g range (Zimmermann et al., 2002; Gómez et al., 2001). Nonetheless, the results of this study demonstrate that the relative impact of Pt in comparison to the relative impact of the other traffic related heavy metals is high, as Pt concentrations increase for up to 36 fold as the result of the introduction of road runoff. It should be noted so, that this drastic effect is a result of the very small amount of Pt in the sediment at the reference site. Cd however, which was also found in a ng/g range at the reference site, does not show an effect that is as pronounced as for Pt. The results of the concentrations range for other heavy metal than Pt, can also be compared to concentration ranges found in other studies. There are only a few and not really recent studies which directly characterize traffic related heavy metals near a potential sources (most studies focus rather on heavy metals in road dust or directly in runoff water). Those were performed by Mudre & Ney (1986); Watts & Smith (1994); Maltby et al. (1995) and Karouna-Renier & Sparling (2001). In the study published by Mudre & Ney (1986), Cd, Pb, and Zn contaminations were analyzed in six streams near the same highway in the sand fraction. Compared to the present study, concentrations for Pb and Zn were lower (2 - 25 µg/g for Pb and 2 - 70 µg/g for Zn), whereby Cd levels were nearly the same in both studies.

Watts & Smith (1994) analyzed sediments in the River Lagan crossing the city of Belfast, Ireland, at 25 sites. In the silt/clay fraction they found concentrations of Cu (65 - 230 µg/g), Pb (65 - 350 µg/g) and Zn (185 - 1025 µg/g), which are comparable to the here discussed study. However, concentrations of Cr, were higher documented (Watts & Smith, 1994) (80 - 960 µg/g). But Cr in river sediments of the cited study also exceeded Cr concentrations in road dust samples of the same study, which implies that there probably was another Cr source. Comparable to this study at the river Alb, also Watts & Smith (1994) found highest sediment concentrations in the proximity of road runoff discharge site.

Karouna-Renier & Sparling (2001) analyzed metal concentrations of Cu, Pb and Zn in stormwater treatment ponds. One category of the investigated ponds were ponds receiving stormwater from highways, state and county roads. Median concentrations in sediments were 7.55 µg/g for Cu, 11.85 µg/g for Pb and 21.25 µg/g for Zn. While Pb levels in the stormwater treatment ponds are comparable to Pb concentrations analyzed in the river Alb, Cu and Zn concentrations in the Alb exceed the concentrations of the stormwater treatment pond, especially at sampling points near Inlet-2 and Inlet-3.

Maltby et al. (1995) used a similar study design as was applied in this study. The authors sampled sediments of three different rivers, which received runoff water from a pipe. Each river was sampled twice. The first sample point was approximately 400 m upstream of the inlet, the second sample point was less than 100 m downstream from the inlet. Unfortunately, Maltby et al. did not sieve the sediment samples and did not provide any details on the vicinity of the sampling points downstream from the inlets. Maltby et al. (1995) found significantly elevated concentrations of Cd and Pb in two of three rivers and significantly elevated concentrations of Zn and Cr in one of the investigated rivers.

In general, the analyzed metal concentrations found in this study are comparable to metal concentrations found in other traffic affected sediments.

It can be stated that in river systems concentrations of traffic related heavy metals in sediments are highest in direct proximity to the source. The distribution of the traffic related heavy metals are similar to the distribution of Pt, which was also demonstrated by the correlation analysis performed in this study. Clear correlations ($R > 0.5$, $p < 0.001$) of Pt were found for Cr, Ni, Zn, Pb and Cu in the sandfraction and only for Zn and Cd in the silt/clay fraction. No correlation was found with Ag. This lack of correlation is not surprising as Ag is not a traffic related heavy metal. It was only included in this study to proof that metal concentration analyzed are due to the discharge of road runoff and can not be associated with other metal sources. Cd was only detectable in the silt/clay fraction. This is interesting, as Cd derives from the corrosion of cars as well as from tyre abrasion (see also Table 2.1). But also Harrison et al. (2003) analyzed traffic related metals in airborne particulate matter and found Cd mostly in the finest fractions 0.1 - 2 µm and only to a very small proportion in the coarser fractions. In general, it is evident that there exist

more and stronger correlations between metals in the sand than in the silt/clay fraction. This is clearly visible for Pb. While Pb correlates with all heavy metals in the sand fraction to a very high degree it does not clearly correlate with any of the other heavy metals in the sand/silt fraction. This could be taken as another indication for the fact that mostly the greater particles immediately settle into the sediment, while smaller particles are transported further within the water column.

Even though a good correlation and the same distribution could be found for nearly all metals, a different distance range of all metals could be observed at each of the three transects. Transect-1 shows the lowest sediment concentrations, which do not differ greatly in comparison to the reference site. Sediments in Transect-2 do have high concentration loads and the effect of road runoff discharge can be detected up to 50 m downstream from Inlet-2 for most metals and at least up to 100 m for Pt. In Transect-3 sediment concentrations 2 m downstream from Inlet-3 are very similar for all metals than observed in Transect-2 3 m downstream from Inlet-2, but already 15 m downstream from Inlet-3, the effect has nearly disappeared. It has to be concluded that each Transect does underlie different factors which influence the sedimentation of heavy metals. There are already several factors described by different authors, which are influencing the distribution and concentration of traffic related heavy metals in river sediments. There are the number of cars passing the street, the amount of precipitation and the size or the hydrologic characteristics of the receiving water system (van Hassel et al., 1980; Maltby et al., 1995; Mudre & Ney, 1986). The design of this study tries to control for these factors since all three transects are impacted by runoff water of the same street, into the same river. Therefore, the above mentioned factors can be ruled out as reasons for the observed differences in the heavy metal concentration ranges in the three transects. The high concentrations after Inlet-2, compared to the relatively low concentrations after Inlet-1 can likely by attributed to the size of the drained section of the street. The dimensions of the respective drained road section is largest for Inlet-2 and shortest for Inlet-1. Furthermore, next to the metals discharge through the inlets an additional metal input could derive from road dust which is transported by the wind into the river. As the main wind direction in Karlsruhe is from South-West (LUBW, 2007), road dust could be transported by wind directly from the road on the bridge into the river. According, to several authors (e.g. Jarvis et al., 2001; Ely et al., 2001; Wichmann et al., 2007; Singer, 2008; Zereini et al., 2007) transport of the main load of traffic related metals into soils next to a highway takes place up to 10 m distance from the source, with very high concentration in the first cm next to the road. Nearest sampling point downstream from the bridge is sampling point T-1/20. All other sampling points after Inlet-1 are below the bridge. Concentrations in sampling point T-1/20, however, are not elevated in the sand fraction and for almost none of the metals in the silt/clay fraction (with the exceptions of Cu and Pb). As T-1/20 is located close to the bridge, it can be assumed that particles transported by wind are not detectable at this sampling point, since they are further transported with the water current.

Another factor, which obviously can effect the heavy metal distribution in sediments, is the current velocity of the water. Mudre & Ney (1986) stated that a rapid water flow prevents fine particles

from sedimentation to the ground. The current velocity was not directly analyzed in this study, however, a qualitative difference of the current velocity between sampling points can be estimated based on the relationship between the masses of the sand and the silt/clay fraction in the whole sample at the respective sampling points. It can be seen that the velocity at the sampling point T-3/2 is very low compared to all other sampling points. This is indicated by the high proportion of silt/clay in the sample, which is more than twice as high as in all other samples. This could explain, why in Transect-3 concentrations are very high at sampling point T-3/2 and immediately drop to the level of the reference site, just a few meters further downstream. Here, the current velocity is so low that nearly all particles are precipitated, while after Inlet-2 particles are carried along by the current and precipitate further downstream.

The main factors that influence the traffic related heavy metal concentration in river sediments identified in this study are therefore: the length of the drained street section, the distance to the source of runoff (i.e. inlets and bridge) and the current velocity.

In summary this study shows, that the introduction of heavy metals from traffic into aquatic water systems can be detected using Pt as a marker in sediment samples. Furthermore, it has been observed, that the behavior of traffic related heavy metals with regard to transport and concentration in the short distance range is comparable between aquatic sediments and terrestrial soils. Like in soils, traffic related heavy metals tend to show a high heavy metal load near the source, followed by an exponential decrease. Due to the water properties, high concentration loads in water can be transported 10 to 30 m further away from the source than in the air, especially when the velocity of the current is high. In both systems a regional or long-range transport cannot be ruled out. When looking at the overall load of heavy metals in sediments, it has been found, that most of the traffic related heavy metal load is accumulated in the sand fraction. The accumulation of traffic related heavy metals in sediments is at least within the first 20 m is high, as reflected by the Pt concentrations as well as the sediment classification system used in sediment monitoring. Especially near the inlets, sediment quality is shown to be increased or even highly polluted for Cr, Cu and Zn.

3.4.2 Uptake of Platinum and other traffic related heavy metals by *Corbicula* sp.

Pt concentrations in *Corbicula* sp. are rather low or do not reflect the high concentrations of Pt in some of the sediment samples. This is also true for most of the other traffic related heavy metals. A statistically significant increase of the metal burden is only recognizable in Transect-3 for Cr, Cu, Ni, Pb, Pt, in Transect-2 for Ni at the sampling point T-2/0, and in Transect-1 for Ag. These low Pt concentration found in the clam tissues are not supported by findings in other studies. Even though, literature on field studies investigating the Pt uptake of aquatic organism is scarce, there are some studies analyzing the Pt uptake of asselids and gammarids (Rauch & Morrison, 1999;

Moldovan et al., 2001; Haus et al., 2007b).

Rauch & Morrison (1999) analyzed samples of *Asellus aquaticus* of a stormwater detention pond, which receives rainwater from a swedish inner city highway and from two different rivers, both receiving discharges of mixed stormwater overflow systems. Asselids of the detention pond had a Pt concentration of 1.0 µg/g Pt, while *Asellus aquaticus* of the two rivers had concentrations of 0.16 to 4.5 µg/g Pt. Moldovan et al. (2001) sampled the same rivers a couple of years later at sampling sites near the highway and near a parking lot. Both sampling sites received surface runoff and combined sewer overflow. They found relatively high concentrations of Pt in the sediments (i.e. 53-54 ng/g) which indicate a high pollution due to traffic. Also high concentrations were found in *Asellus aquatic* with 5.1 to 118 ng/g at site 1 and 33 to 83.4 ng/g at site 2. Both studies, however, showed that Pt levels in *Asellus aquaticus* are highly variable. Even if sampled at the same sampling site and the same day. This could be due to physiological statuses of the individual organism or due to analytical problems as the analysis of Pt in animal tissue is often challenging. Nonetheless, concentrations documented for asselids are much higher than those found in the clam tissues at the traffic influenced sampling site of the river Alb.

Haus et al. (2007b) studied Pt and other traffic related heavy metals in sediment and animal samples of different rivers and ponds. The difference to the above mentioned studies is, that these sampling sites were only slightly polluted as there was no direct discharge of runoff water near the sampling sites. This was reflected in the relative low concentration of Pt in sediment samples (i.e. <0.3 to 5.01 ng/g). However, Pt concentrations up to 0.5 ng/g were found in in *Asellus aquaticus* and up to 1.3 ng/g Pt in different species of gammarids. Those Pt concentrations are in the same concentration range than found in *Corbicula* sp. samples of the river Alb.

It is striking, that the biomonitoring results for *Corbicula* sp. do not match the findings of the sediment analysis, which showed that traffic related heavy metals are introduced by the three inlets. One potential explanation for this finding could be, that *Corbicula* sp. is not suitable for detecting the introduction of road runoff.

Even though no other studies exist, analyzing Pt in bivalves, there are studies investigating the accumulation of other traffic related heavy metals in bivalves. Karouna-Renier & Sparling (2001) analyzed sediment and faunal samples in ponds receiving street runoff. In order to get enough material for the analytical procedure, they had to pool different species as they were not able to find enough individuals of one species for a sample. Therefore they differentiate between Molluscs, Odonata and a composite sample. Fortunately, this study is comparable with this study of the river Alb, as the sediment concentrations for the analyzed elements Cu and Pb are similar. Only the Zn concentrations were higher in sediments of the river Alb. Like *Corbicula* sp. in the river Alb, the molluscs in the study of Karouna-Renier & Sparling (2001) did accumulate Cu and Zn to a higher degree than concentrations found in the sediment. They found median concentrations of 37 µg/g Cu and 52 µg/g Zn in the mollusc sample. Pb concentrations, however, were not higher in the

mollusc tissue than in the sediments and median concentration of Pb were found to be 1.37 µg/g. In comparison to *Corbicula* sp. tissues of the river Alb, concentrations of Cu and Pb are similar (median in *Corbicula* sp.: 32 µg/g Cu and 0.3 µg/g Pb). Zn concentrations, however, with 127 µg/g are higher in the clam tissue of the river Alb. Furthermore, Cu and Zn concentrations in Molluscs collected in (highway)- stormwater treatment ponds were higher than in Molluscs of ponds with only little anthropogenic influence. It can therefore be concluded, that the uptake of traffic related heavy metals by *Corbicula* sp. seems to be low in comparison to asellids and gammarids, but similar to that of other mollusc species.

The question remains, why asellids and gammarids do accumulate traffic related heavy metals to a higher extend than the clams studied here. To explain these findings, it is helpful to analyze the possible uptake routes of *Corbicula* sp. In the context of this study the following scenarios are possible and will be discussed:

- no uptake of metals through direct avoidance by the clam
- uptake/ no uptake of specific soluble metals through filter feeding
- uptake/ no uptake of specific non soluble metals through filter feeding or pedal feeding

Metals in runoff water derive mainly from street dust, which is mobilized during a precipitation event. In water, metals partition into soluble and particulate bound fractions (Sansalone & Buchberger, 1997). This partitioning is mainly influenced by the time the metal already rested on the pavement and the pH of the rainwater (Sansalone & Buchberger, 1997). During a precipitation event, the soluble fraction of metals is the first fraction to enter the road runoff (and therefore the river) (Harrison & Wilson, 1985). This causes the often observed first flush of metals during a storm event. Later on mostly suspended particles discharge through the runoff into a river system. During a precipitation event, the particles distribution shifts from fine material to coarser particles sizes. The main load of metals is found in the particle sizes of >125 µm (Wilber & Hunter, 1979).

When this discharge reaches the clam population there are several possibilities for the clam respond: It could open the valves and when it starts to rain and the metal peak is floating to the clam it just behaves as usual and filters the water including soluble and particle bound metals. The other possibility is, that the clam stops filtrating and the valves are closed. Studies of Ortmann & Grieshaber (2003) confirm that part of the natural behavior of *Corbicula* sp. includes a diurnal cycle of valve motions with extended periods of valve closure, frequently up to 10 to 12 h per day. The clams tend to open their valves during daytime when the chance of available nutrition is highest and consequently valves are mostly open during the afternoon hours. Even though a precipitation event might carry nutrients into the river, there are studies suggesting that *Corbicula* sp. notices heavy metals in the water and closes the valves as a response to elevated metal concentration. Legeay et al. (2005) could demonstrate that *Corbicula* sp. exposed to Cd,

reduces valve opening times, as an effect to the exposure. None the less, it takes some time, until *Corbicula* sp. recognizes a flush of metals and responds with a valve closure. Moroishi et al. (2009) recognized that a reaction of *Corbicula* sp. to low Cd or Cu concentrations changes can be detected after 15 min to 2 h after exposure start, depending on the metal concentration. Also Doherty et al. (1987) could demonstrate that the mean valve parting time was inversely related to Cd and Zn concentrations in the water. It can therefore be suggested, that the low concentrations of traffic related heavy metals in clam tissues, could be the result of an active avoidance of the accumulation of heavy metals by the clams in the river Alb. As the introduction of heavy metals via street runoff is not continuous it is possible that a discharge can occur when the clams are generally not active or that the clams can detect the discharge and consequently stop to filtrate until the discharge event ends.

However, some metals were accumulated by *Corbicula* sp. Cd and Cu were significantly increased in all clam tissue samples downstream from Inlet-3, Pt and Ni were increased at some sampling points in Transect-2 and 3, compared to the concentrations of clam tissues at the reference site. Zn was not increased at all. Furthermore, some heavy metals in the clam tissues did correlate. This gives evidence for the suggestion that metals are accumulated on different uptake paths or in different chemical speciations. As already mentioned, metals are discharged in soluble and particulate forms. This difference in form does have an influence on the bioavailability of the metal. A number of studies investigating the partitioning of traffic related metals have shown that Cd is mainly discharged in a soluble form, while Cu, Ni and Zn are partly (approximately 50%) discharged in a soluble form (Harrison & Wilson, 1985; Sansalone & Buchberger, 1997; Tuccillo, 2006). The discharge and the speciation of Pt into water systems is still under discussion, but there are some authors concluding from dissolution exposures and analysis from sediments and water samples of a gully pot that up to 40% of Pt is discharged in a soluble form (Fliegel et al., 2004; Wei & Morrison, 1994). Others report, that the solubility of Pt is far lower (Jarvis et al., 2001).

Corbicula sp. is able to accumulate Cd in a soluble form. The uptake of Cd was shown to be concentration and time dependent (Baudrimont et al., 1997a; Lee & Lee, 2005). This relationship in between water concentration and clam tissue concentration is non-linear, following a Michaelis-Menten type of saturation (Qiu et al., 2005). The depuration of Cd takes a long time. After an exposure experiment with 5 µg/L for 14 days, Inza et al. (1998) could still analyze the same amount of Cd in the clam tissue even after 30 days of depuration. They elongated the depuration experiment and found a decontamination rate of 25% after 120 days. Also Qiu et al. (2005) estimated a half-life for Cd of approximately 100 days for *Corbicula* sp. In a field study, where certainly a mixture of soluble and particle bound Cd was available, Baudrimont et al. (2003) even estimated a half life of 500 days for Cd in clam tissues.

Only a few studies investigated the uptake of Cu by *Corbicula* sp., like Harrison et al. (1984). They exposed *Corbicula* sp. to Cu concentration from 1 µg $CuCl_2$/L to 12 mg/L. They observed a clear dose response accumulation of Cu by the clam and a high level of sensitivity with regard to

Cu based on the fact, that mortality was high. They also noticed that the clams closed their valves in the early stages of the experiments, especially in treatment groups with high Cu concentrations.

Other than Cd and Cu, Zn is an essential element for bivalves. It is a key component of many enzymes including carbonic anhydrase (Rainbow, 2002). The organism needs a certain quantity of the metal to meet its essential metabolic requirements. Still, any further accumulation of Zn beyond the essential requirements would potentially be toxic. Therefore, many bivalves are able to regulate the accumulation and excretion/detoxification rates of Zn. Belanger et al. (1986) showed that for short time exposures of less than five days, there is no concentration dependent accumulation of Zn in a concentration range of 0.025 to 1.0 mg/L. *Corbicula* sp. just stopped filtration when exposed to high concentrations of Zn. However, for longer exposure periods instead (i.e. more than five days) they found a clear dose-response accumulation pattern. In contrast to Cd, depuration times for Zn are short. Belanger et al. (1986) documented a complete depuration of Zn within 17 days for all exposure concentrations. Also other authors (Baudrimont et al., 2003; Qiu et al., 2005) confirmed that the depuration rate for Zn is higher than for Cd.

No laboratory exposure studies investigating the uptake of soluble Pt and Ni by *Corbicula* sp. could be identified in the literature and it is thus assumed that it has not yet been investigated systematically.

In accordance to the above mentioned studies it can be hypothesized that in Transect-3 of the river Alb, Cd and Cu were available in a soluble form and accumulated by *Corbicula* sp. This transect differs from the others due to its lower current velocity and soluble metal fraction may be easier available for the clams than in other transects. Furthermore, clams in this transect were larger, which would imply that those clams are older and have been exposed longer to the metals than clams at other sampling points. The similar uptake of Cd and Cu is also reflected in the relatively high correlation coefficients found for both metals in the clam tissues. Zn, however, was not accumulated by *Corbicula* sp., because of the high regulation of Zn concentrations by the clam.

While Cd, Cu, Ni, Pt and Zn are thought to be discharged in a mainly soluble form, it is assumed that Cr and Pb are being discharged mainly in a particulate form (Harrison & Wilson, 1985; Sansalone & Buchberger, 1997; Tuccillo, 2006). In some of the sampling points of Transect-3, also Cr and Pb concentrations were higher in the clam tissues than compared to clam concentrations at the reference site. As Cr and Pb are assumed to be discharged mainly in a particulate form, there are two routes for their uptake by *Corbicula* sp.: Via filter feeding or pedal feeding.

Corbicula sp. is usually assumed to be a non-selective feeder (Way et al., 1990; Vaughn & Hakenkamp, 2001). Boltovskoy et al. (1995) found 61 different algae species in the gut content of *Corbicula* sp., sampled in the field, and estimate that those algae are covering 2 to 50% of the organic matter needed for respiration only. They suggest that most of the organic matter needed is supplied by particulate organic matter. Therefore, the clam has to filtrate particles others than algae and bring them into their digestive system. Way et al. (1990) found that *Corbicula* sp. does

not change filtration rates when algae or latex microspheres in the same size range were being fed. It can thus be assumed that *Corbicula* sp. is capable of also filtrating suspended particles from road runoff. It is still under discussion if filter-feeding on suspended particles is size dependent. While Way et al. (1990) found an upper size limit of ingested particles at 20 to 25 µm, Boltovskoy et al. (1995) could not find any size preferences. If a size restriction of 20 µm for ingested particles is assumed, it can be concluded that *Corbicula* sp. is not able to ingest a great portion of the metals bound to suspended particles offered via road runoff, as the main load is bound to particles larger than 20 µm (Sansalone & Buchberger, 1997; Tuccillo, 2006). Especially for Cr and Pb this would imply that the main part of the discharged metal load is not available for the clam, since only a very small percentage (2-5%) is discharged in a dissolved state (Harrison & Wilson, 1985; Sansalone & Buchberger, 1997; Hallberg et al., 2006; Tuccillo, 2006).

The uptake of particles by *Corbicula* sp. is not restricted to its filter feeding behaviour. Some authors assume that *Corbicula* sp. ingests large sediment particles via pedal feeding (Way et al., 1990; Reid et al., 1992; Hakenkamp & Palmer, 1999; Sousa et al., 2008). Pedal feeding is a process in which sediment particles are transported with ciliary currents of the foot into the mantle cavity and from there into the digestive trakt (Reid et al., 1992).

In this study however, there is no evidence for a metal uptake via pedal feeding, as only one correlation between traffic related heavy metals in sediments and the clam could be observed. The correlation was only significant between the calculated sediment fraction <2 mm and the clam tissues. No correlation was observed for other grain fractions. Also in other studies it was demonstrated that an uptake from sediments is small or neglectable (summarized in Doherty, 1990). Elder & Mattraw (1984) could not find a correlation between sediment concentrations and clam tissue concentration at five different sampling points in the same river. It is assumed that pedal feeding only occurs if the nutrition concentration in the water is not high enough to match the metabolic demands of *Corbicula* sp. (Sousa et al., 2008). Even if particles are ingested by pedal feeding, the assimilation of the ingested metals is very low. Lee & Lee (2005) could demonstrate that only 2% of the ingested Cr was eventually assimilated.

In general, it can be concluded that the uptake of metals is restricted by the form in which metals are discharged into the river. Further, accumulation is controlled by the feeding of *Corbicula* sp. with regard to this offered metal speciations. The accumulation of Cd, Cu, Ni and Pt can mainly be explained by uptake of soluble metal forms. Zn, however, does not show any enrichment in *Corbicula* sp. This can most likely be explained by the fact that *Corbicula* sp. can regulate Zn uptake and excretion. The uptake of Cr and Pb can mainly be accounted for by filter feeding of very small suspended particles. As *Corbicula* sp. does not accumulate metals which are bound to larger particles in the suspended particulate matter or the sediment, it is likely that the uptake of traffic related heavy metals observed in the river Alb was lower than observed for other aquatic animals, like asselids or gammarids in other studies. Those organism mainly feed from detritus and may therefore also ingest larger particle on which more traffic related heavy metals are bound.

Even though the uptake of traffic related heavy metals and especially of Pt is small, the question remains if this uptake is toxicological relevant or not.

In toxicological studies three different levels of toxicological effects are distinguished: Effects on the population level, effects on the individual level and effects on suborganism level (molecular, biochemical, physiological changes) (Adams & Greeley, 2000).

But what is the contribution of Pt to the toxicity of road runoff for aquatic organism?

The acute toxicity of PGE on aquatic organisms was mainly proven by using soluble PGE species in exposure and toxicity studies with different organism groups (crustaceans and fish). They reveal that Pt is the most toxic of the three PGE. Borgmann et al. (2005) demonstrated in a exposure study with *Hyallela azteca* that the toxicity of Pt, was far behind the toxicity of e.g. Cd, Cr, Hg and Pb with LC_{50} values ranging between 0.57-8.4 µg Pt/L in soft water and 1.05 to 159 µg Pt/L in tap water. It was assumed to be as toxic as Se, Ce and Lu. Besides the acute toxicity, also sublethal effects of Pt were investigated. In several studies the effect of different Pt concentrations were tested on *Danio rerio*. While Jouhaud et al. (1999a,b) found reversible intestinal changes after an exposure with relatively high Pt concentrations (16 µg/L Pt (IV)), Osterauer et al. (2010a) found reactions of *Danio rerio* at lower concentrations. Beginning with 1 µg/L $PtCl_2$, they found different histopathological effects of Pt. At concentrations of 100 µg/L, the livers of the exposed animals showed strong reactions (i.e. vacuolisation of the cytoplasm, beginning of cloudy swelling of the hepatocytes, and caryopycnosis). Also the embryonic developement of *Danio rerio* was tested. Osterauer et al. (2009) exposed embryos of *Danio rerio* to Pt concentrations between 38 and 74 ng/L and found a diminishing of the heart rate even at the lowest tested concentration. Furthermore, at high Pt concentrations (50 and 100 µg/L) the hatching success of the fish was significantly reduced.

Also the paradise snail *Marisa cornualis* was used for histophathological effect studies and further the embryo test were conducted. Osterauer et al. (2010a) found slight effects on gills and epidermis at Pt concentrations between 10 to 100 µg/L. More severe effects were observed at the hepatopancreas already at low concentrations of 0.1 µg/L. In the embryo tests *Marisa cornualis* was exposed to Pt concentrations between 200 ng/L and 100 µg/L (Osterauer et al., 2009). Like the zebra fish, *Marisa cornualis* showed an decreased hatching success. Furthermore, snail embryos exposed to 100 µg/L Pt did not develop an external shell (Osterauer et al., 2010b).

On the suborganism level an EC_{50} value of 62 µg/L was conducted for *Daphnia magna* after exposure with Pt (IV) (Biesinger & Christensen, 1972) . Besides a significant reduction of weight and protein content of the animals they found a reduction in glutamic oxaloacetic transaminase (GOT) activity. Singer et al. (2005) conducted a test with a bivalve. They investigated the effect of PGE on stress biomarkers in *Dreissena polymorpha*. Their studies lasted about 10 weeks. They demonstrated that Pt can induce the production of heat shock proteins (hsp70). These proteins have important functions in terms of protein folding, protein transportation and cell stabilisation.

They were already used as biomarkers of adverse effects of other heavy metals eg. Pb and Cd, also for *Corbicula* sp. (Baudrimont et al., 2003, 1999, 1997b,a). In the study of Singer et al. (2005) the threshold levels for hsp70 induction decreased in the order: Cd >Pt >Pb >Pd >Rh after exposure of *Dreissena polymorpha* to diluted standard metal solutions (500 µg/L). The increase of hsp70 was time dependent.

It is therefore obvious that Pt has effects at the organism and suborganism level. However, all effects observed, occured at Pt tissue concentrations which are far above concentrations found in the clam tissue of *Corbicula* sp. in the river Alb. As the EC_{50} values for Pt are relatively high it can be concluded that lethal effects of Pt to *Corbicula* sp. due to Pt in road runoff can be excluded. It could be possible that some effects on the suborganism level could occur due to the exposure to Pt. Those are mostly involved in detoxifying processes and should not severely harm the clam. But there is one group of effects which is sever for organism even if in low exposure concentrations. These are substances with genotoxic potential. Furthermore, carcinogenic and mutagenic compounds are extremely dangerous as their effects may exert a damage beyond that of individuals and may be active through several generations (Bolognesi & Hayashi, 2011). Therefore, the genotoxic potential of Pt for aquatic organism is studied in the following chapters of this thesis.

Chapter 4

Accumulation of different Platinum concentrations by *Corbicula* sp. and the genotoxic effects of Platinum on gill cells and hemocytes

4.1 Introduction

In Chapter 3 of this thesis it was shown, that Pt is introduced into river systems via stormwater runoff. Also an uptake of Pt by *Corbicula* sp. could be demonstrated . However, Pt concentrations in the clams were low and could not be correlated to sediment concentrations. This led to the question if *Corbicula* sp. is a suitable sentinel and whether there are adverse effects of Pt on the clams.

Corbicula sp. was already described as a promising sentinel for heavy metal pollution and is also often used to detect heavy metals. It started to become a famous sentinel in the USA after its introduction into freshwater systems. Up to now several reviews have been written about the sentinel characteristics of this clam (Doherty & Cherry, 1988; Doherty, 1990). Phillips (1977); Beeby (2001) and Sures & Siddall (2001) defined most of the commonly used criteria for sentinels, from which the following are well documented for *Corbicula* sp.:

- Its life span is sufficient to allow a sampling of more than one-year class. Due to Sousa et al. (2008) and Mouthon (2001) *Corbicula* sp. was observed to have a life span of 3 to 5 years.

- Sampling of the clams is easy, because they can be taken with sediment samples (they are burrowed in sediments) and the density of the clams in the sediment is often very high. In sediments which are inhabited by *Corbicula* sp. densities of several hundred to several thousand individuals per square meter are found (Mouthon, 2001; Elliott, 2008; Sousa et al., 2008).
- The size of the clam allows adequate tissue samples for analyses as they grow rapidly. Some authors already analyzed heavy metals in individual clams (Graney et al., 1983; Peltier et al., 2008) as well as in different organs of *Corbicula* sp. (Fraysse, 2000; Legeay et al., 2005).
- *Corbicula* sp. is tolerant to different maintaining conditions in the laboratory, which was shown in several laboratory studies (Inza et al., 1998; Fraysse, 2000; Ortmann & Grieshaber, 2003; Lee & Lee, 2005; Legeay et al., 2005; Champeau et al., 2007).

However, some characteristics of good sentinels have not been thoroughly investigated yet, especially with regard to Pt. Those are the following:

- The organism should accumulate the pollutant without showing increasing mortality.
- A high metal concentration factor should be exhibited by the organism.
- A simple correlation should exist between the metal content of the organism and the average metal concentration in the ambient environment (e.g. water or sediments).

These characteristics were investigated in a long term exposure study. *Corbicula* sp. was exposed to different concentrations of Pt for 70 days. The different concentrations used in this study should be high enough to still detect an uptake, but low enough to reflect environmental conditions. Only a few studies are available to get information about environmental concentrations of Pt in surface water bodies. Concentrations analyzed in the water column were mostly found to be below the detection limit. The IWW (2004) analyzed several sources of PGE for river systems and also analyzed Pt in stormwater and stormwater retention ponds. The latter can be suggested to be an extreme site for organisms exposed to Pt. Their study illustrated that Pt concentrations in the stormwater retention ponds have a high variability when analyzed at different time points. They found concentrations which were below the limit of detection (1 ng/L) and concentrations up to 100 ng/L.

Pt concentrations used in this study were therefore adapted to these results and the range of exposure levels were chosen to be between 10 to 100 ng/L. For a better comparison to other studies a further concentration of 100 µg/L was also tested.

Next to the suitability of *Corbicula* sp. as a sentinel, the question of effects of Pt in environmentally relevant concentrations is raised in this chapter. Genotoxic effects could to be relevant for

serious consequences of individual clams or even the clam population. Alterations in the DNA structure can easily have effects on individuals by reducing the fecundity or increasing tumor risks (Tucker & Preston, 1996; Theodorakis, 2001). Even more, they can also have effects on population structures, like decreasing genetic variability (Theodorakis, 2001).

There are some studies confirming that Pt is able to induce changes on the DNA. Pt is used as an anticancer drugs in Pt coordination complexes like cisplatin or carboplatin complexes. In those complexes they are well known to be mutagenic and carcinogenic (Slavutsky et al., 1995; Gebel et al., 1997; Hoppstock & Sures, 2004).

But also Pt salts and other inorganic Pt complexes have been reported to show positive results in different genotoxicity tests. Those tests all differ in the cell lines and/or organisms used, the chemical species of Pt tested and the test itself. Most of the effects were studied in cell lines. The Ames Test showed that $PtCl_4$ induces genotoxic effects in bacteria (*Salmonella typhimunium*) (Bünger et al., 1996). These results were confirmed by Lantzsch & Gebel (1997) who found positive results in the SOS chromotest which is used in the tester strain PQ37 of *Escherichia coli*. The genotoxic effects were induced by cisplatin, transplatin, $K_2[PtCl_4]$ and $PtCl_4$, no effects were found for $K_2[PtCl_6]$. In human lymphocytes, Gebel et al. (1997) and Migliore et al. (2002) could demonstrate that next to cisplatin, transplatin and carboplatin complexes also $K_2[PtCl_6]$, $PtCl_4$, and $PtCl_2$ induced micronuclei (MN).

Next to *in vitro* studies, the genotoxicity of Pt was also tested in a few *in vivo* studies. Gangnon et al. (2006) investigated the genotoxic effect of Pt (H_2PtCl_6) in animals and plants. The plant model they used was *Sphagnum magellanicum* and it was treated for four weeks with medium containing 0.1 to 10 mg/L. In a second experiment they fed six week old female Sprague-Dawley rats with different Pt solutions (0.1 to 10 mg/L) for four weeks. After the exposure period the capitula of the mosses and liver cells of the rats were investigated for DNA damage using the Comet Assay. In the mosses genotoxic effects increased with increasing Pt concentrations. Also in rats DNA damage was observed for all Pt concentrations, however, no linear dosis-effect relationship could be detected.

Osterauer et al. (2011) did expose aquatic organisms to Pt and tested genotoxicity. Embryos of the paradise snail *Marisa cornuarietis* and the zebra fish *Danio rerio* were exposed to different Pt concentrations (100 ng/L to 200 µg/L) and the Comet Assay was used to detect DNA damage. After 4 days of exposure no DNA damage could be found for *D. rerio*. *M. cornuarietis*, however, did suffer DNA damage after an exposure of 8 days beginning with concentrations of 1 µg/L.

However, the Comet Assay used in all *in vivo* studies is a very sensitive test. It discovers single and double strand breaks, cross links between DNA and DNA-proteins and alkali lable sites (Fairbairn et al., 1995; Tice et al., 2000). Some of this damage can still be repaired by the cells and positive test results do not always implicate that the substances tested do alter the DNA structure

irreversible (Klobucar et al., 2003) and thus have severe effects for the individuals or even the population.

As the aim of this study is to examine if Pt can induce DNA damage which potentially could harm the individual clam or even more the population structure, another test was used in this study: The micronuleus test (in the following abbreviated as MN test). The MN test aims to find chromosomes or parts of chromosomes which are not included in the regular cell dividing. They are not attached to the spindle apparatus during the metaphase of the cell cycle and often not included to one of the new cell nuclei. A micronucleus indicates DNA damage which is non repairable (Klobucar et al., 2003). No data could be found for MN test performed in *in vivo* studies after Pt exposure.

4.2 Material and Methods

4.2.1 Origin of the clams

The clams for this experiment were sampled in a pond called "Niederwiesenweiher". This pond is part of the landscape protection area "Pfälzische Rheinauen". It is a former gravel pit which was in operation until 1976. Now the pond serves as public swimming pond during the summer. It has an extend of approximately 10 ha and a depth off up to 6 m. It receives ground water, rain and spring water only and discharges into a creek (called Steinbach) (Ministerium für Umwelt, 2010).

Sediments of the "Niederwiesenweiher" were analyzed for Pt as described in Chapter 2 with 2.9 ng/g Pt in the sand fraction and 3.5 ng/g Pt in the silt/clay fraction. According to Chapter 3 this can be assumed to be a low Pt contamination. *Corbicula* sp. can easily be found in the sediment of the pond due to its relative high abundance. For the study, 3000 adult clams were sampled in June of 2005 and transported to the laboratory. During the transport clams were kept in a tank filled with pond water. The water was aerated during the trip to the laboratory (approx. 1 h). For acclimatization clams were kept in flow through tanks for three month. For tests analyzing genetic damage, an acclimatization of 30 days was recommended by Rigonato et al. (2005) in order to exclude the detection of DNA effects of substances which were present in the field. During acclimatization, clams were fed twice a week with the green microalgae *Chlorella vulgaris* (air dried algae powder, Drak Aquaristik, Dr. Andreas Kremser, Stuttgart, Germany) .

4.2.2 Exposure study

The exposure study had a duration of 70 days. The clams were divided into five groups with 715 clams each. Every group was placed in a plastic tank filled with 20 L non chlorinated tap water. The tanks had a ground platform with the size of 48 x 41 cm, in order to allow the clams to lie solitary on one spot and to avoid spacial stress. The water was well aerated with two aeration stones for each tank. The tanks were placed a in climate chamber in order to control the temperature of the experiment. The climate chamber was regulated to 18 °C. One group was not exposed to Pt and served as the control group. All other groups were exposed to different Pt concentrations (10, 50, 100 ng/L and 100 µg/L). As Pt source a $H_2[PtCl_6]$ standard solution was used (1000 mg Pt/L, Ultra Scientific, Wesel, Germany)and added in respective quantities to the water of the tank (see also 4.1).

Water and Pt were renewed three times in 10 days. Prior to and 60 min after the change of water and Pt addition, physical and chemical values of the water were analyzed. The pH-value, the temperature and the redox potential were analyzed with pH-325, WTW, Weinheim, Germany and

Table 4.1: **Design of the exposure study with *Corbicula* sp.**

Treatment-group	Nominal exposure concentration	Sample collected for Pt analysis [days after exposure start]	Sample collected for micronucleus test [days after exposure start]
Control	0 ng/L	12, 20, 30, 40, 60, 70	10, 49, 58
10 ng/L	10 ng/L	12, 20, 30, 40, 50, 60, 70	10, 49, 58
50 ng/L	50 ng/L	12, 20, 30, 40, 50, 60, 70	10, 49, 58
100 ng/L	100 ng/L	12, 20, 30, 40, 50, 60, 70	10, 49, 58
100 µg/L	100 µg/L	12, 20, 30, 40, 50, 60, 70	10, 49, 58

the conductivity was analyzed with the set LF-318 also manufactured by WTW.

Samples were taken every 10 days. As the mortality of the clams was especially high in the control group, no sample of the control group was taken at day 50 after the start of the exposure study. For the Pt analysis 45 clams of each treatment group were taken on each sampling day. Clams were kept in clean water for 24 h for a depuration phase. Angelo et al. (2007) were able to show that *Corbicula* sp. eliminates a considerable amount of metals due to depuration. Lee & Lee (2005) showed that 16 hours of depuration led to an egestion of 70 to 90 % of ingested metals. Afterwards clams were killed by freezing. Until sample preparation they were stored at -20°C. For the micronucleus test 7 clams of each group were taken and slides for the micronucleus test were prepared immediately after taking the samples.

4.2.3 Platinum Analysis

Clams were defrozen at room temperature. Measurements (i.e. mass, length, width, height, tissue mass), homogenization and freeze-drying were performed as described in Chapter 3.2.2. Each sample was divided into three to five subsamples. All subsamples were digested with the high pressure asher and analyzed by adsorptive cathodic stripping voltammetry (ACSV) as described in Chapter 2.2.

4.2.4 Micronucleus test

There are several tests which are used for testing the mutagenicity or genotoxicity of substances in surface waters. One of the most frequently used test is the micronucleus test (Ohe et al., 2004).

With this test it is possible to point out chromatin breakage or dysfunctions of the spindle apparatus induced by clastogens or spindle poisons (Mersch & Beauvais, 1997) (see Figure 4.1).

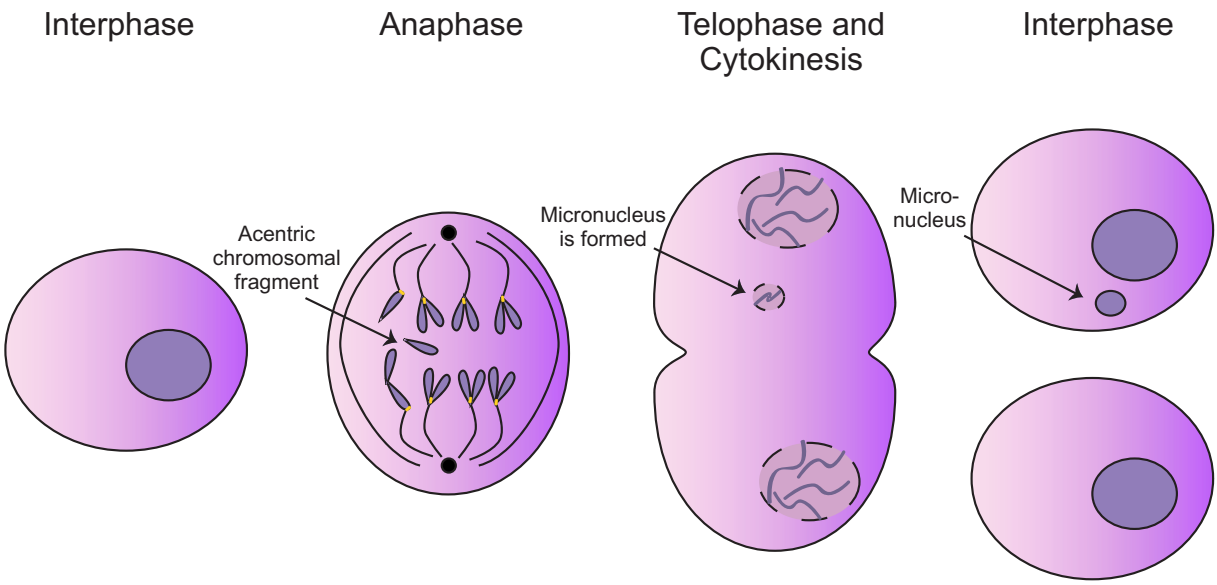

Figure 4.1: **Formation of micronuclei after chromosomal abberation. After a genotoxic effect which causes an acentric chromosomal fragment, this fragment can not be attached to the spindle in the Metaphase of the Mitosis. In the Anaphase this fragment is not transported to the spindle pole and forms a micronucleus in the Telophase and the Cytokinesis of the cell cycle.**

Micronuclei derive from malfunctions in the mitosis of the cell cycle. After an exposure with clastogenic substances chromosomes or chromatid fragments that lack a centromere (due to chromatin breaks or chromosome aberrations) are not attached to the mitotic spindle during the metaphase of the mitosis. In the anaphase of mitosis, when chromosomes move towards the spindle poles, those acentric chromosomes or fragments lag behind. They are, however, often included into the cytoplasm of one of the daughter cells during the telophase and form a secondary nucleus which is much smaller than the main nucleus of the new daughter cell (Al-Sabti & Metcalfe, 1995), see also Figure 4.1. Furthermore, micronuclei can be formed if the spindle apparatus is malfunctioning (e.g. due to spindle poisons) and chromosomes with centromere are not guided to the spindle poles. Micronuclei are formed naturally at a low spontaneous frequency. Due to clastogenic substances and spindle poisons this frequency can be increased. These micronuclei can be stained and detected by light microscopic analysis (Al-Sabti & Metcalfe, 1995).

Preparing of the slides for a micronucleus test

For the micronucleus test seven clams of each treatment group were taken for each sample point (see Table 4.1). These clams were not incorporated in the metal analysis. They were taken independently of the clams for the metal analysis. The animals were layed on ice for five minutes before carefully opening the shell for a few mm. In accordance to Mersch et al. (1996) and Mersch & Beauvais (1997) a needle of a hypodermic syringe was introduced near the posterior adductor muscle and 0.5 to 1 ml hemolymph was drawn up. The hemolymph was spread out on an prior prepared slide (before starting the procedure, slides were washed with 95% alcohol, cooled on ice and already labelled) and the cells were allowed to settle on the glass for 10 min at room temperature. Afterwards cells were fixed with 0.5 ml fixative consisting of one part acetic acid (100%, p.a., Carl Roth GmbH and CoKG, Karlsruhe, Germany) and three parts methanol (Rotipuran, 99.9%, p.a., Carl Roth GmbH and CoKG, Karlsruhe, Germany). The slides were then air dried and stained with undiluted GIEMSA solution (Azur-Eosin-Methylenblue solution in methanol, Merck KGAA, Darmstadt, Germany) for 1 min.

Next to the hemocytes also gill cells were prepared for the micronuleus test. After the hemolymph sample was taken, the adductor muscle of the clam was cut through and the clam was opened entirely. With forceps the gills were removed and layed on a cooled microscope slide and covered with a washing buffer (consisting of 190 mg/L ethylene glycol tetraacetic acid (EGTA) diluted in phosphate buffered saline (PBS) (Dulbecco's Phosphate-buffered-saline, Gibco Products, New York, USA). Isolation of the gill cells resulted from a combination of mechanical and enzymatic isolation. For the mechanical isolation gills were screwed with a drigalski spattel. The resulting cell suspension was rinsed (with 0.5 ml of the above described wash buffer) into a 10 ml polyethylene container and digested in 2 ml Trypsin (Gibco Products, New York, USA) for 5 min at room temperature. The suspension was filtered through a gaze (70 µm) into 5 ml of minimum essential medium (MEM with Earl's salts, Applichem GmbH, Darmstadt, Germany) to stop the enzymatic digestion and centrifuged with an Hettich centrifuge (Hettich Universal 16 R, Hettich rotor 1629, Hettich lab, Tuttlingen, Germany) for 15 min at 4°C and a speed of 200 x g. For a second washing step, the resulting pellet was resuspended into 50 µl of MEM and again centrifuged for 15 min at 4°C and a speed of 200 x g (Eppendorf centrifuge 5415D, Eppendorf rotor F45-24-11, Eppendorf, Hamburg, Germany). Resulting pellets were then resuspended in fixativ consisting of three parts of methanol (Rotipuran, 99.9%, p.a., Carl Roth GmbH and CoKG, Karlsruhe, Germany) and one part of ethanol (99.8%, with 1% of MEK, Carl Roth GmbH and CoKG, Karlsruhe, Germany) and spread on a prepared objectiv slide. After air drying, cells were also stained with GIEMSA. This time GIEMSA was dilutet (3% in buffer after Weise, MERCK, Darmstadt, Germany) for 4 min.

Cell and micronuclei counting

Before starting the examination of cells, slides were coded for a blind scoring. Of each treatment group and sampling time four, out of seven slides were chosen for cell counting. Those slides were screened at a magnification 250x for regions with a suitable quality (many well spread cells, good staining contrast, intact cell membranes). Those could mostly be found at the end of a smear. Cells were then counted. It was carefully monitored that cells were not counted twice.

At a magnification of 1000x, 500 hemocytes or gill cells were counted for each slide (2000 cells per sample) and the number of micronuclei (MN) in those cells were noticed. The MN frequency is expressed as the number of micronucleated cells per 1000 cells scored (i.e. ‰).

Cells which were counted had to meet specific criteria which were already defined by Majone et al. (1987); Venier et al. (1997) and Mersch & Beauvais (1997):

- Cells belong to the dominant cell type.
- Cells inhabit an intact cell membrane. The borders of the cells are clearly distinguishable, indicating that the cell membrane is intact.
- Cells are separated. The counted cell is clearly visible as a single cell and does not rest on another cell. If cells lie on another micronuclei could be hidden under the nucleus of the other cell.

Counted micronuclei also had to fulfil already described criteria (Majone et al., 1987; Venier et al., 1997; Mersch & Beauvais, 1997) to distinguish them from other fragments or staining crystals:

- Micronuclei are within in the same cytoplasm than the main nucleus.
- Micronuclei are clearly separated from the main nucleus.
- Micronuclei are roundish and have clear borders, suggesting a nuclear membrane.
- Their chromatin structure and the color of the staining was similar to or weaker than that of the main nucleus.
- The size of the micronucleus is between 1/3 to approx. 1/10 of the main nucleus.

4.2.5 Data analysis

Replicate handling Three to four aliquots of the clam tissues were digested and analyzed for the Pt concentrations. Outliers, mean values and standard deviation were obtained as already

described in Chapter 3.2.3. Mean values and standard deviation were also generated for chemical and physical water parameters, condition factors and MN frequencies.

Boxplots Data regarding the chemical and physical water parameter are presented as boxplots. In boxes percentiles, the mean, and median of the data are shown as explained in Figure 4.2.

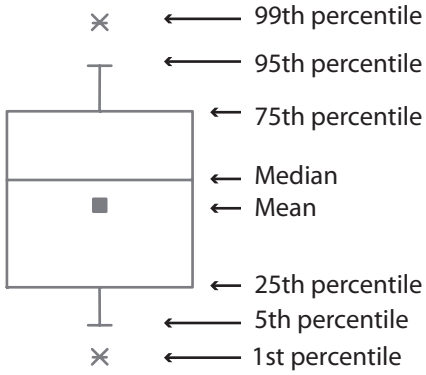

Figure 4.2: **Design of boxplots used to illustrate chemical and physical water parameters.**

Condition factor The condition factor was calculated as defined in Equation 3.2 (Chapter 3.2).

Mortality The mortality was calculated for the specific sample events (see Equation 4.1). The aim is to evaluate which fraction of the clams died between two sampling events.

$$mortality_n = \frac{Dead\ clams_n}{clams_{n0} - clams(\sum_{n-1}^{n0})} * 100 \tag{4.1}$$

$n = sampling\ event$
$n_0 = starting\ time\ of\ exposure$

Maximum possible Platinum uptake (MPU) To compare how much of the offered Platinum was found in the clam tissues, the maximum possible Pt uptake was calculated (see equation 4.2).

$$max\ uptake_n = \frac{M_{Pt\ n}}{M_{Clam\ n}} + max\ uptake_{n-1} \tag{4.2}$$

$n = sampling\ event$
$M_{Pt\ n} = Platinum\ mass\ of fered\ between\ the\ last\ sampling\ event\ and\ the\ current\ sampling\ event$
$M_{Clam\ n} = Clam\ mass\ in\ the\ treatment\ group\ as\ dry\ weight\ at\ the\ current\ sampling\ event$

Bioconcentration factor The bioconcentration factor is used to analyze a chemical potential to bioaccumulate through exposure to water (Luoma & Rainbow, 2008). With the help of this measure the bioaccumulation capacity of different species for the same substance or the accumulation of different chemicals in the same species can be compared (4.3).

$$BCF = \frac{C_{Clam\ tissue}}{C_{Water}} \tag{4.3}$$

$C_{Clam\ tissue} = Metal\ concentration\ in\ freeze\ dried\ clam\ tissue$
$C_{Water} = Metal\ concentration\ in\ the\ water$

Statistical tests Several statistic tests were used to find differences between mean values of analyzed parameters. The Kruskal-Wallis-Test (H-Test) was used for chemical and physical water parameters, as well as for the condition factor. This non parametric test was used to compare more than two treatment groups at different sampling events. It implied the following two hypothesis:

H0:
Mean values of the tested chemical or physical parameter in all treatment groups are equal.
H1:
Mean values of the tested chemical or physical parameter in all treatment groups are not equal.

If the test result indicated that there are differences of the mean values, another test was performed: The multiple Mann-Whitney Test (U-Test).

The U-Test was also performed to analyze differences of micronuclei inductions. In this case the test was used one-sided under the following hypothesis:

H0:
Mean frequency of the micronuclei induction in the control group equals the mean frequency of micronuclei induction of a specific treatment group.
H1:
Mean frequency of the micronuclei induction in the control group is smaller than the mean frequency of micronuclei induction of a specific treatment group.

4.3 Results

4.3.1 Comparison of physical and chemical water parameters during the exposure study

The physical and chemical water parameters temperature, pH-value, conductivity, and redox potential were monitored in all treatment groups throughout the exposure study. The results are plotted in Figure 4.3.

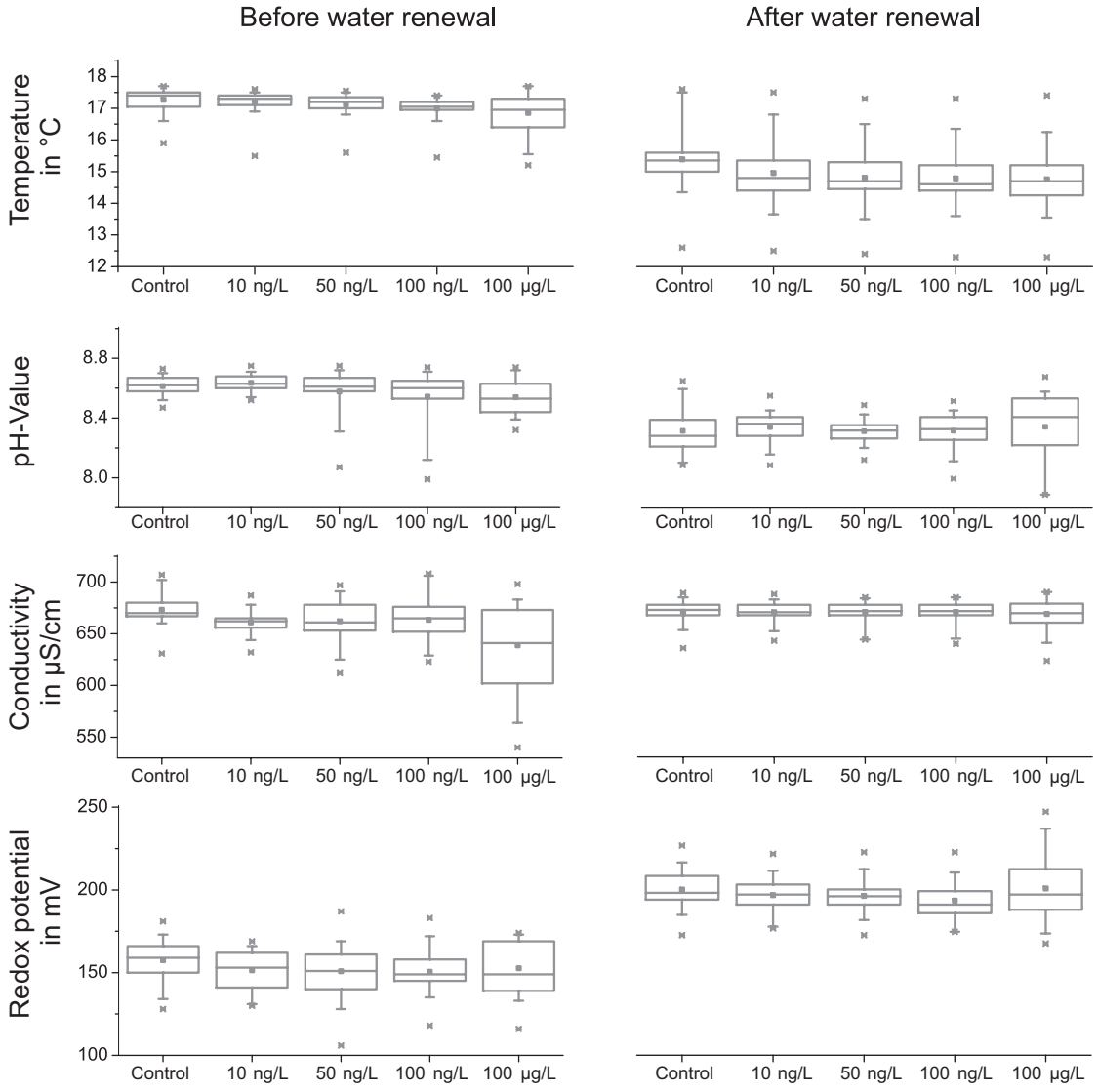

Figure 4.3: **Physical and chemical water parameters during the exposure study with clams. Boxplot analyses of temperature, pH-value, conductivity and redox potential values during the exposure study.**

Multiple U-tests reveal, that none of the parameters differs significantly from another within the treatment groups before the water renewal. Also after the water renewal the means of the chemical and physical parameters do not differ significantly for all treatment groups (Kruskal-Wallis test, $p>0.05$). However, temperature, pH-values and the redox potential do differ when comparing groups before and after the water renewal (U-Test, $p<0.05$). The temperature and the pH-value decrease after the water renewal and the redox potential increases. The values of the conductivity do not significantly change due to the renewal of water and Pt (U-Test, $p>0.05$).

4.3.2 Performance of clams during the exposure study (mortality and condition factor)

Clams which were used for the Pt analysis were further investigated for differences in size and mass, as those factors could lead to different accumulation capacities (e.g., Shoults-Wilson et al. (2009)). As can be seen in Figure 4.4 no differences could be observed between the treatment groups. Overall shell length was 19 mm (with a standard deviation of 2.2 mm) and the mean mass of the tissues was 329 mg (with a standard deviation of 126 mg). Data for the height, width and total mass can be found in Appendix A.16.

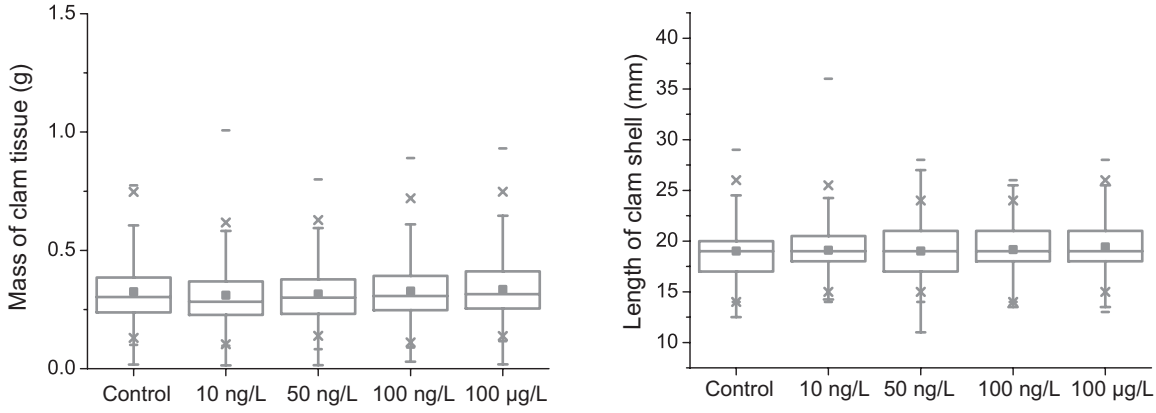

Figure 4.4: **Boxplot analysis for shell length and tissue mass of clams. A) Mass of clams for the different treatment groups. B) Length of clams for the different treatment groups.**

To get an overview about the performance of the clams the condition factor was calculated (see Figure 4.5).

For all treatment groups it can be observed, that the condition factor is higher before the exposure study started. With the start of the experiment the condition factor decreases (H-Test; $p<0.05$; U-Tests for all groups $p<0.05$). Between day 12 and day 60 after the exposure start the condition factor remains stable in most of the treatment groups (exceptions is treatment group 50 ng/g, here the condition factor of day 60 is lower than on day 50 and 100 ng/L, and the clams of day 60 do

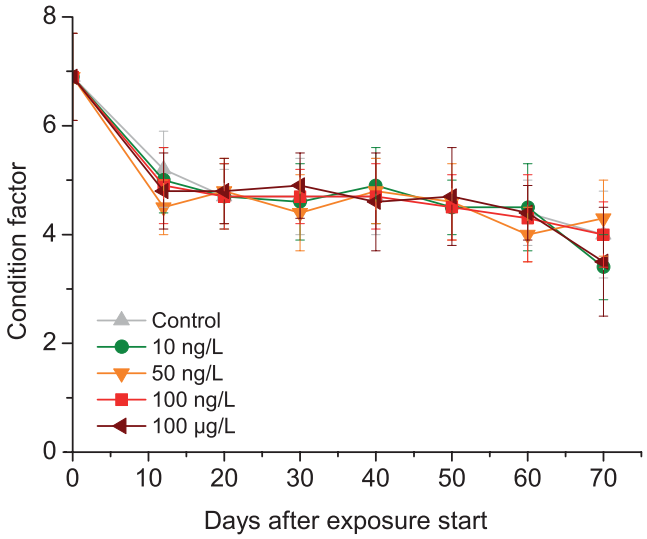

Figure 4.5: **Condition factor of the clams during the exposure study. The condition factor gives an indication of the nutritional condition of the clams.**

show a significant lower condition factor than on day 12). However, at day 70 again a significant lower condition factor was found in the treatment groups control, 10 ng/L, 100 ng/L and 100 µg/L compared to all other sampling points.

Another measure of the performance of the clams is the mortality rate. The mortality rate in all treatment groups was high (see Figure 4.6).

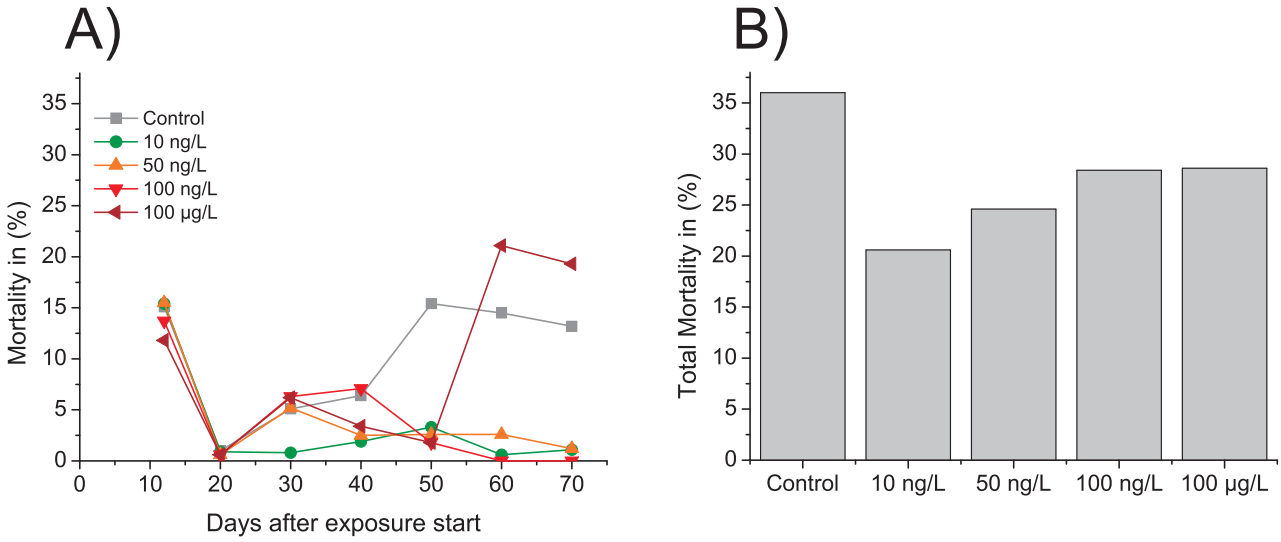

Figure 4.6: **Mortality of clams during the exposure study. A) Mortality rate in the different treatment groups during the exposure study. B) The percentage of total dead clams in comparison to all clams for the respective treatment group.**

Figure 4.6A presents the percentage of clams which died during the exposure study in the respective treatment groups between the sampling events, as described by equation 4.1. As can be seen for all treatment groups, mortality was high in the first 12 days of the exposure study. In the middle of the study (day 12 to day 40) mortality was relatively low and then it was rising in the Control group and in the group exposed to 100 µg Pt/L . Figure 4.6B furthermore shows, that the overall mortality was highest in the control group, followed by the treatmentgroups 100 ng/L and 100 µg/L. Lowest mortality rates were observed for the clams treated with 10 ng Pt/L. However, results of the Kruskal-Wallis test reveal that the mean mortality between the treatment groups does not differ significantly.

4.3.3 Accumulation of Platinum by *Corbicula* sp.

The accumulation of Pt by *Corbicula* sp. was investigated by taking samples at eight different times during the exposure study. Clams of each sample were pooled and Pt analysis was repeated for three to four times for each sample. Figure 4.7 shows a plot of the results of the Pt analyses.

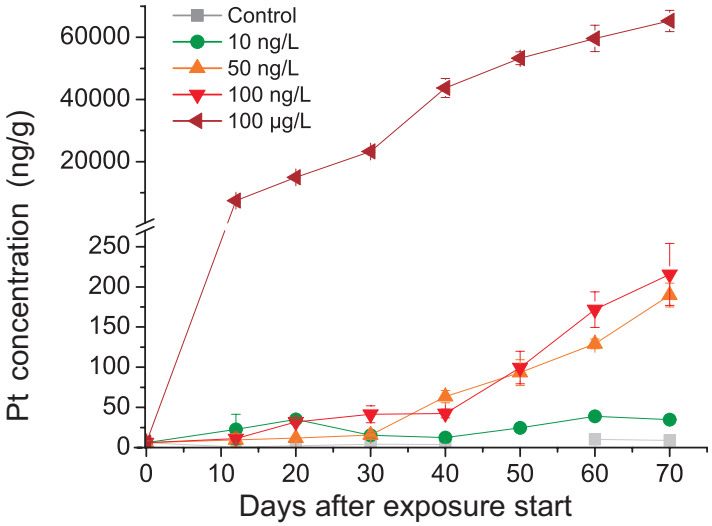

Figure 4.7: **Platinum concentrations of clams at different times of the exposure study. Each datapoint represents the mean of three or four repeated analyses. Standard deviation is given within the error bars.**

As can be seen in Figure 4.7, Pt concentrations in the control group were the lowest (0.6 to 10 ng/g) and remained stable over time. At exposure day 50 no Pt concentration is plotted for the control group. At this day no sample was taken, because the number of clams in this group was already diminished due to the high mortality rate in this treatment group. All other treatment groups differ from the control group. As the number of analysis is low (n=3 or n=4), no statistical test

was conducted, but standard deviations are clearly separated from each other, indicating that Pt concentration ranges do not overlap between the control group and the different exposed groups. The highest Pt accumulation could be found in clams which were exposed to the highest exposure concentration (100 µg Pt/L). In this treatment group concentrations lie between 7 to 65 µg/g, depending on the exposure day. Furthermore, concentrations are rising constantly during the exposure study and the highest accumulation rate occurs between day 0 and day 12 after the exposure study.

The treatment groups exposed to 50 and 100 ng Pt/L do show low accumulation rates up to day 40. From day 50 onwards, higher Pt concentrations can be found in the clam tissues, with the highest concentration at day 70. As concentrations are still rising it can be concluded that a steady state situation is not reached even at the end of the study for these two treatment groups.

Also for the treatment group exposed to 10 ng Pt/L, an accumulation is already visible at day 12 after the exposure starts. Up to day 30, Pt concentrations in the clams of the treatment groups with 10, 50 and 100 ng Pt/L are in a comparable concentration range.

The plots in Figure 4.8 compare the uptake of Pt with the maximum possible uptake of Pt for the clams. The maximum possible uptake was calculated as described in Equation 4.2.

It can clearly be seen that the maximum possible uptake for the clams is rising exponational. With every sampling event clams were taken out of the experiment. Therefore, the same metal mass within the aquarium was available for less clams after each sampling event. The observed uptake of Pt only includes a very tiny part of the maximum possible uptake. Furthermore, with increasing exposure concentrations in the water, the percentage of the uptake decreases. Therefore, highest uptake rates can be found for the treatment group 10 ng/L. Especially in the beginning of the exposure study, half of the offered Pt can be found in the clams. With the exposure day 30, the relation between observed and possible uptake gets smaller and only 6-12% of the offered Pt can be found in the tissues of the clams. A different picture can be observed for treatment group 50 ng/L. Here, the analyzed uptake only covers 3-4% of the theoretically possible uptake in the beginning of the study (i.e. up to day 30) and increases then to 6-7% for the following exposure days. In the treatment groups 100 ng/L and 100 µg/L the rate between observed and possible uptake remains stable throughout the whole exposure study. While in the clams of the treatment group 100 ng/L 2-5% of the possible uptake can be found, it is only 1-2 % for clams of the treatment group 100 µg/L.

The results indicate that the uptake of Pt is dependent on the Pt concentration in the ambient water as higher exposure concentrations result in higher tissue concentrations. However, the percentage of the uptake of Pt is decreasing with increasing exposure concentrations. Figure 4.9 includes two plots.

Plot A shows the relation between the exposure concentrations and the Pt concentrations in the

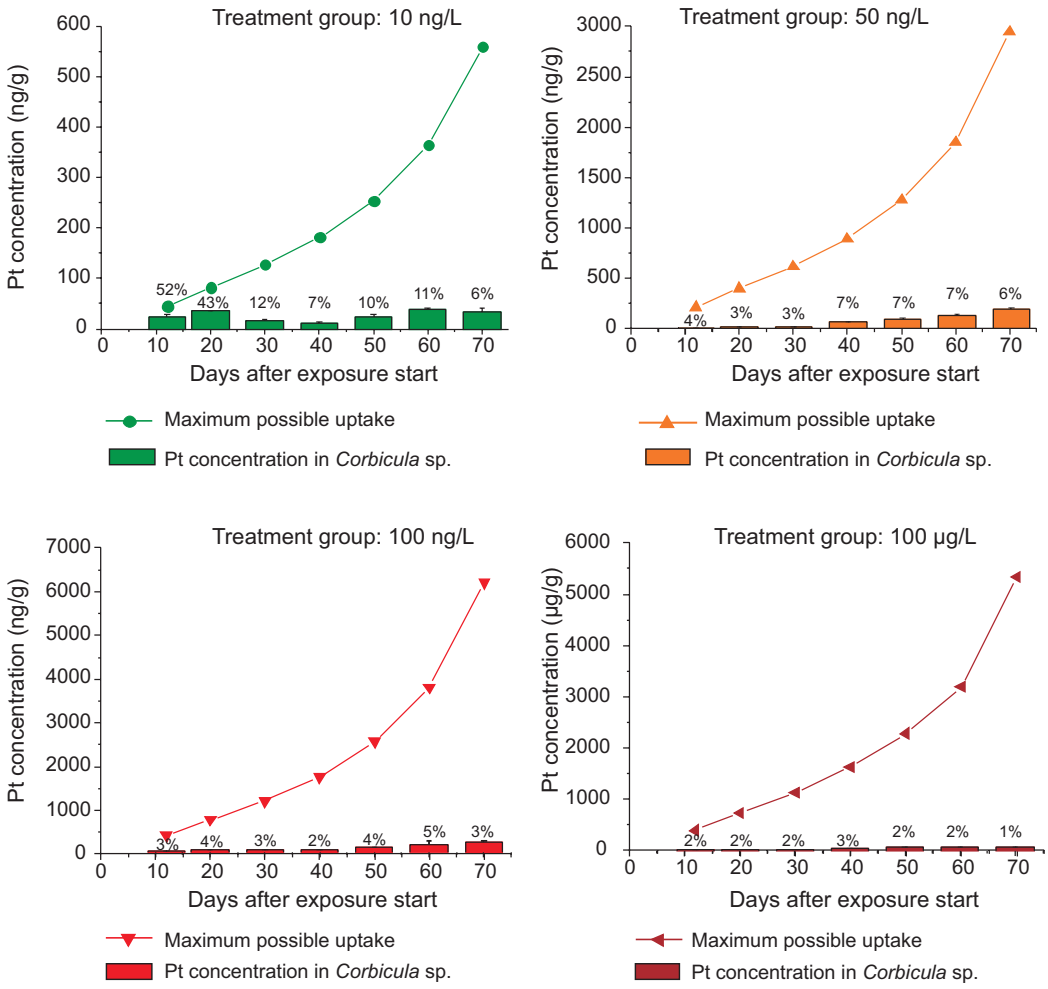

Figure 4.8: **Platinum concentrations of clams in comparison to the maximum possible uptake. The plots describe the maximum possible uptake of Pt per gram dry clam tissue, while the bar plots show the actual analyzed Pt concentration for each sampling time. The percentage numbers indicate the rate between observed uptake and maximum possible uptake.**

clam tissue of the last exposure day (i.e. day 70) for all treatment groups. As can be seen, the curve resembles a saturation curve. However, it should be noted that the treatment groups 50 and 100 ng/L (see Figure 4.7) do not indicate that the steady state of the uptake process has been reached yet. Plot B instead directly compares the uptake of the treatment groups 10 ng/L, 100 ng/L and 100 µg/L. In this plot the factors of 10 and 1000 are levelled to the same intervals on the y-axis. The plot shows that the uptake of the clams exposed to lower Pt concentrations are always higher than the uptake observed in the clams of treatment groups with higher exposure levels. Both plots therefore underline that the relation between exposure concentrations and Pt uptake decreases with increasing exposure concentrations. This unequal accumulation is also visible in the bioconcentration factors (BCF) calculated for all groups at day 70 after the exposure start. In

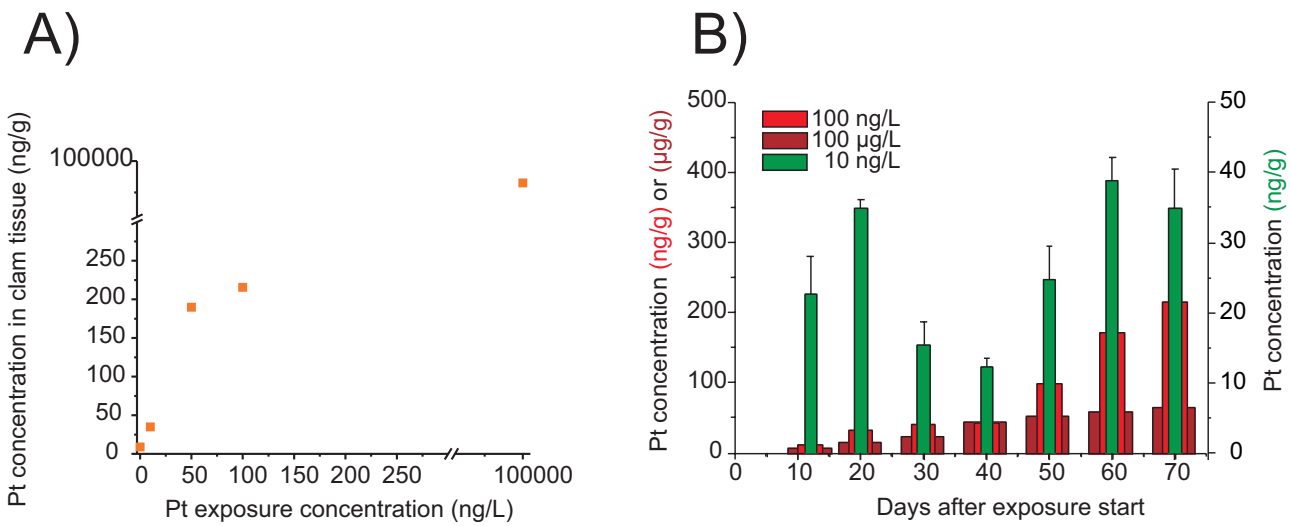

Figure 4.9: **Correlation between exposure concentrations and concentrations in clam tissue. A) shows the correlation between exposure concentrations and clam tissue concentrations at exposure day 70 for all treatment groups. B) Direct comparison between clam tissue concentrations of the treatment groups 10 ng/L, 100 ng/L and 100 µg/L.**

the groups 10, 50 and 100 ng/L bioconcentration factors are 3500, 3800 and 2200, respectively. For the group exposed to 100 µg/L the bioconcentration factor is only 650. However, also for the calculation of bioconcentration factor a steady state condition should be established. Therefore, BCF values are only indicators and have to be interpreted with care.

4.3.4 Genotoxic effects of Platinum to *Corbicula* sp.

Genotoxic effects of Pt to *Corbicula* sp. were analyzed by counting micronuclei (MN) in hemocytes and gill cells of the clams at three different days during the exposure study. Results are plotted in Figure 4.10.

The average frequency of micronucleated cells in the control ranged between 2.5 to 14.7 ‰ in the gill cells and between 8 to 13 ‰ in the hemocytes.

10 and 49 days after the exposure start no differences could be seen between the means of micronucleated cells in the exposed groups compared to the control goups. Therefore, no effect of Pt on the chromosomes is detected. This is different at day 58 after exposure start. Here the number of micronucleated cells in the control group is lower and in the gill cells of the treatment group 100 µg/L the frequency of micronucleated cells is significantly higher than in the control group (one sided U-test, $p < 0.05$). In the hemocytes no differences between the exposed and the control groups was observed.

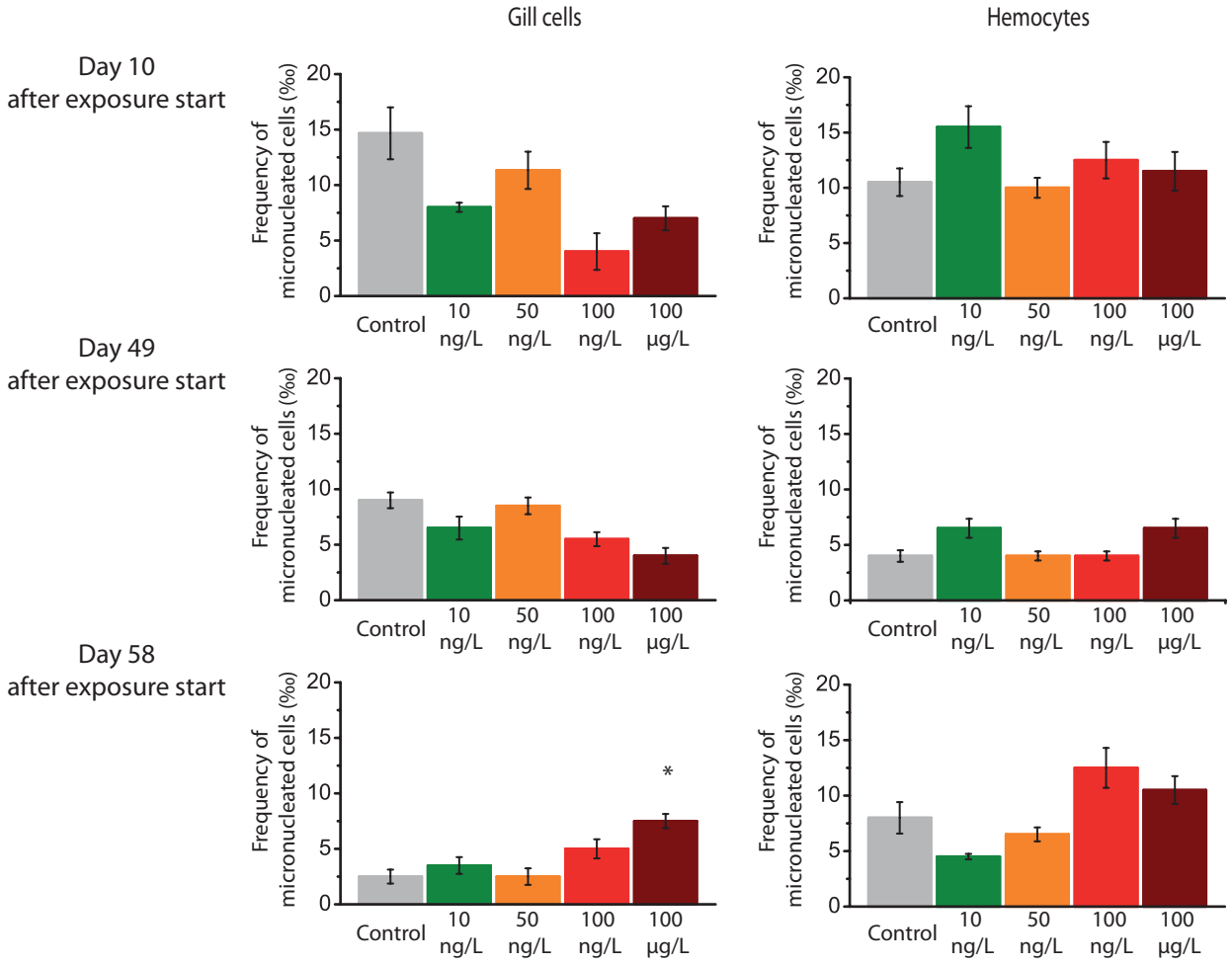

Figure 4.10: **Comparison of micronuclei frequencies in gill cells and hemocytes. For each column four clams were analyzed and 500 cells per clam were counted. Standard deviations are indicated by the error bars. Significant differences (U-test, p<0.05) between the mean of Pt exposed groups compared to the control group are indicated by an asterisk.**

4.4 Discussion

In the introduction of this chapter two main questions were raised, which should be answered through the investigated experiments. The first question of the study was if *Corbicula* sp. is a suitable sentinel for Pt in an exposure scenario where Pt concentrations in the water are considerably low and thus comparable to worst case scenarios in the field. The ability of the clam to cope without suffering in the concentration range in question, the capacity of metal accumulation and the relationship of ambient Pt concentrations and tissue concentrations were in the focus of the study. The second question raised in this study was, if a Pt exposure does have any severe effect on the chromosomes in hemocytes and gill cells of the clams.

***Corbicula* sp. as a sentinel for Pt** A precondition for an exposure study with different treatment groups is that the variables, with the exception of the variable under observation, are stable and comparable within all treatment groups. Variables which can significantly influence the Pt uptake of *Corbicula* sp. are:

- physical and chemical water parameters,
- size and age of the analyzed clams,
- performance of the clams during the exposure study,
- time of exposure,
- exposure concentrations.

The results of the study imply that despite the exposure concentrations no differences in the physical and chemical water parameters occurred. Also the size and the condition factors of the analyzed clams were the same or at least comparable for all treatment groups.

It can therefore be assumed that differences in metal accumulation can be explained by the variables time and exposure concentrations.

To characterize an organism as a good or suitable sentinel for Pt, it has to be able to tolerate Pt within the typical concentration range found in the field. A high mortality was observed within all groups of the exposure study. As the highest mortality was observed in the control group, an acute toxicity of Pt can be excluded as a reason for the clams death. However, it should be discussed what else did cause the high mortality within the study. Temperature range and pH-value were optimal for clam growth and reproduction (see also McMahon (1983); Sousa et al. (2008)) and the tanks were well ventilated. Next to the mortality, also the condition factor is

decreasing after the exposure start. This can be explained, as the feeding of the clams stopped with the exposure start. However, *Corbicula* sp. was already used in several exposure studies without feeding (Harrison et al., 1984; Champeau et al., 2007) and without a mortal effect. It should be noted, that already during the acclimatization phase several clams already died. Thus the limitation of nutrients in itself can not serve as a sufficient explanation for the observed mortality rate. Unpublished results of the research group of Prof. Dr. Bernd Sures (University of Duisburg-Essen, Department of Aquatic Ecology) show that other cultivation conditions, like offering sediment to burrow or offering food during the exposure study, do not alter the mortality rates between different treatment groups. As several other studies with *Corbicula* sp. were already conducted in tap water (Inza et al., 1998; Labrot et al., 1999; Baudrimont et al., 2003; Legeay et al., 2005; Champeau et al., 2007) also the higher conductivity of tap water compared to river water can be excluded as a reason for the mortality rate. The only possible reasons for a high mortality rate could further be an illness or a substance in the tap water, which stressed the clams.

As highest mortality rates were found in the control group, no additive Pt effect on the mortality rate were observed and the question if *Corbicula* sp. can accumulate Pt without suffering lethal effects has to be approved.

The second attribute of sentinel, which was analyzed in this study is the accumulation capacity of the clam in respect to Pt. According to Phillips (1977); Beeby (2001); Sures & Siddall (2001) a sentinel is characterized by the fact that it does accumulate a specific substance to a high degree compared to the ambient concentrations. Ideally, the sentinel increases the analytical sensitivity of the contaminant (Beeby, 2001). Prelimary BCF factors were calculated in 4.3.3.

It can be seen that in environmental relevant concentrations (i.e. ng/L range), BCFs are between 2200 and 3800. As no steady state conditions in the accumulation curves of the groups 50 and 100 ng/L has been established at the end of the exposure study, BCF values are probably even underestimated for these two groups.

Compared to other studies, BCF values calculated for Pt are lower than reported for *Corbicula* sp. by Graney et al. (1983) for Cu (17000 and 22000). They are in the same range than BCFs for Cd (1800 to 3800) and higher than for Zn (360 to 630). BCFs for Pt were also calculated for *Dreissena polymorpha*. Singer et al. (2005) calculated a BCF of 300 after an exposure with 500 µg/L Pt_4^+ for 70 days. BCFs calculated by Sures & Zimmermann (2007) are better comparable to this study as they exposed *D. polymorpha* to 100 µg/L Pt. With 43 the BCF value in the study of Sures & Zimmermann (2007) is lower than observed for *Corbicula* sp. in this study with 650.

These results clearly indicate that *Corbicula* sp. does accumulate soluble Pt species to a high degree. Analysis of Pt in clam tissues can increase the analytical sensitivity of Pt in the field, as Pt concentrations in water are far lower than Pt concentrations in the clam tissues and often below the limit of detection. Therefore, it can be concluded that *Corbicula* sp. is a good sentinel for Pt with regard to the attribute ”accumulation capacity”.

The third requirement for a good sentinel questioned in this study is a clear and simple correlation between the metal uptake of an organism and the ambient metal concentrations (Phillips, 1977; Beeby, 2001; Sures & Siddall, 2001). A simple linear correlation between Pt in the clams and the different exposure concentrations in the water could not be found in this study. The clams tend to accumulate a higher rate of Pt, when they are exposed to a lower concentration. Therefore, the correlation resembles more a saturation curve. A similar observation was already done for Cu, Cd and Zn for *Corbicula* sp. (Graney et al., 1983; Qiu et al., 2005). Also for other bivalves this kind of correlation was documented, e.g. for Pd in *D. polymorpha* (Frank et al., 2008).

This behavior can be considered as difficult for monitoring studies using *Corbicula* sp. as a sentinel in cases were concentration differences in the environment should be compared and are only slightly different at the sampling sites. The result of an higher accumulation rate at lower exposure concentrations could lead to similar tissue concentrations for clams, even if they were exposed to different ambient concentrations. This phenomenon can be seen in the accumulation kinetics of the treatment groups 50 ng/L and 100 ng/L (see Figure 4.7). Even after 70 days of exposure, a difference in the tissue concentrations of these two treatment groups is not apparent due to the higher accumulation rate of Pt in the treatment group 50 ng/L. For other metals than Pt (or also PGE) a difference of 50 ng/L in the exposure concentration would not be interesting as field concentrations for other metals are normally higher. In the case of Pt, however, the differences in the exposure concentrations are very low in the environment. This is already indicated in Chapter 3. Here, sediments with Pt concentrations above 40 ng/g were considered as highly influenced, while concentrations below 10 ng/g were considered as slightly influenced.

It can therefore be assumed, that different Pt concentrations in the field can only be distinguished from each other by analyzing samples of *Corbicula* sp. if soluble Pt concentrations in the ambient water are very different from each other (are not in the low ng/g range).

Besides the question if *Corbicula* sp. is a suitable sentinel or not also other interesting results were found. Even if those results were not in the main focus of the study they will be discussed here.

The results indicate, that the uptake rate appear to increase between specific exposure days: Day 30-40 for treatment group 50 ng/L, and day 40-50 for the treatment groups 10 ng/L, 100 ng/L and 100 µg/L. This is apparent in the accumulation curves as a bend in the curve and also in the rising Pt proportion of the maximum possible uptake found in the clam tissues.

Also in other studies a similar increase in metal uptake or shifts in molecular biomarkers were observed in the same time frame of different exposure studies. Frank et al. (2008) observed a similar shift in the metal accumulation rate for *Dreissena polymorpha* after 7 weeks (42 days) of exposure to different concentrations of soluble Pd (Frank et al., 2008). At the same time they observed in most of the treatment groups that the proportion of Pd bound to metallothioneins decreased. They suggest that the demand of metallothioneins exceeds the physiological capacity to produce those proteins. Also Singer et al. (2005) found a change in the protein metabolism 40

days after the exposure start with different soluble PGE. While they could not observe an increase of the metal accumulation during this state of the exposure study for Pt and Pd, a steady state was already arrived, they could demonstrate that the production of hsp70 is more or less ceased after 45 days of exposure.

Those results lead to the suggestion that *Dreissena polymorpha* and *Corbicula* sp. do change the metal metabolism strategies of PGE after approximately 7 weeks of exposure. The main pathways of metal compensation in invertebrates are the induction of metal binding proteins within the cytosol and the inclusion of the metals into structures like granules, lysosomes and vesicles (Newman & McIntosh, 1991). Despite of the compensation due to metal binding proteins (e.g. metallothioneins), it now can be suggested that *Dreissena polymorpha* and *Corbicula* sp. increase the storage process into compartmentalized structures, which (partly) replace the binding of Pt (or Pd) on metal binding proteins. Consequently, the demand for protein repair mechanisms would decrease (hsp70), the Pt (or Pd) concentrations in the cytosol would also decrease. The latter would imply that the concentration gradient between cell interior and exterior would increase and the passive transport of Pt into the cells would be enhanced. This could explain the increased accumulation rate observed in the accumulation kinetic curve of this study. However, this assumption should be subject to further investigations.

In conclusion: *Corbicula* sp. is an ideal sentinel regarding the attributes "Accumulation without suffering mortality" and ”high accumulation capacity”. Furthermore, by analyzing Pt concentrations in clam tissues, different Pt exposure conditions between different sampling sites can be estimated as there is a simple relation between the soluble Pt concentrations of ambient water and the tissue concentrations. However, small quantitative differences (in the low ng/g range) are difficult to detect as the relationship between the ambient Pt concentrations and the Pt concentrations in the clam tissue is not linear.

Genotoxic effects of Platinum to *Corbicula sp.* Another question raised in this study was, if Pt does induce DNA damage in the clams.

Corbicula sp. is frequently used as a bioindicator also with respect to the testing of genotoxicity of different substances. Different tissues have been used to proof the genotoxicity of organic and anorganic substances in the alkaline filter elution (Kramer & Hubner, 2000), electrophoresic tests for DNA strand breaks (Barfield et al., 2001), Comet Assay (Rigonato et al., 2005; Caffetti et al., 2008; Fedato et al., 2010; Rigonato et al., 2010) and also in the MN test (Fedato et al., 2010).

Most of the studies cited above, used gill cells or hemocytes. An exception is the study of Barfield et al. (2001), who used tissue of the foot. The reason for choosing these tissues is based on the fact that both tissues are considered to be parts of the target tissues in metal contamination (Bolognesi & Hayashi, 2011). Hemocytes are part of the hemolymph. They are the circulating

cells in the clam body and constantly exposed to water soluble metals. Moreover, they are also involved in metal excretion processes (Bolognesi & Hayashi, 2011). Gill cells are the first cells to get in contact with heavy metals taken up by clams. They suffer the highest accumulation loads (Bolognesi & Hayashi, 2011).

Fedato et al. (2010) could proof that the micronucleus test is suitable for both cell types. In this study the MN induction of substances with unknown effects were tested as well as the induction of MN with methyl methanesulfonate (MMS), a well known MN inductor. Compared to the results of Fedato et al. (2010) the induction of MN in the present study is relatively high. While they found a natural induction level of approximately 0.5 ‰ in the control groups in this study 2.5 to 15 micronucleated cells per thousand gill cells or hemocytes were found. These findings are rather comparable to the induction of MN in the cell lines of the positive control exposed to MMS in the above mentioned study of Fedato et al. (2010). Also in studies with other bivalves (like *Dreissena polymorpha*) control levels in both analyzed cell lines were lower than in the study presented here.

Especially combined with the relatively high mortality rate in all groups this indicates again that the clams used in this study had severe problems. This could be due to an illness the clams were suffering from or other substances within the used tab water. Taking the high frequency of micronucleated cells in both cell types into account, it has to be concluded that this illness or the substance also have an effect on the MN induction. Despite the fact, that micronuclei were obviously induced, no differences could be found between the control and the treatment groups at the sampling events 10 and 49 days after the exposure start. Indicating, that no additional MN induction through the exposure of Pt occured.

However, at day 58 after the exposure start a statistically significant difference was found for the frequency of micronucleated cells in the gill cells of the control group and the group exposed to 100 µg/L Pt. This effect was not supported by the findings in the hemocytes, which are in general known to be more sensitive to the MN test than gill cells (Mersch et al., 1996; Bolognesi et al., 1999). In hemocytes no differences were found between the frequency of micronucleated cells of these two groups on day 58 after the exposure start. Furthermore, it has to be pointed out that a comparison of the frequencies between the sampling events on day 10,49 and 58 does not indicate, that the frequency of micronucleated cells in the exposed groups did increase during the exposure study. On the contrary, the frequency of micronucleated cells in the control group decreased, while the frequency of micronucleated gill cells in the clams exposed to 100 µg/L was already at 7‰ at day 10 after the exposure start. This indicates that the observed differences in the frequency of micronucleated cells are not induced by an effect of Pt on the DNA in gill cells. Furthermore, it indicates that the control group is starting to recover from the DNA damage induced by other substances than Pt.

As the clams were obviously influenced by other genotoxic substances all statements regarding the

genotoxicity of Pt in this study have to be treated with great caution and further tests are needed. Thus there was no conclusive evidence for the hypothesis that Pt can increase MN induction in *Corbicula* sp. Since there was also no indication that Pt exposure effects MN induction, it is assumed that Pt does not cause genotoxic effects on *Corbicula* sp.

Chapter 5

The accumulation of Platinum by *Squalius cephalus* and *Pomphorhynchus* sp. and the genotoxic effect of Platinum on fish erythrocytes

5.1 Introduction

Besides invertebrates also vertebrates are known to be effected by heavy metals in river systems (Luoma & Rainbow, 2008). It was already demonstrated that Pt is accumulated by different fish species in the field. Essumang et al. (2008) found Pt concentrations in between 5 to 110 ng/g in five different fish species in an estuarine stretch of the river Pra, Ghana. In laboratory studies Pt was also found in control groups prior to the exposure experiment. Concentrations were below 1 ng/g, but could be found in all analyzed organs (muscle, liver, kidney, gills, intestine) and also in the parasite *P. laevis*. Those fish were caught in the Danube River (Sures et al., 2005). It is therefore clear that fish in the field take up Pt. Several laboratory studies have further demonstrated, that different chemical speciations of Pt are accumulated by fish. Soluble Pt salts were taken up by *Anguilla anguilla* (Zimmermann et al., 2001, 2004a) and *Danio rerio* (Jouhaud et al., 1999a,b; Osterauer et al., 2009), ground catalytical material was taken up by *Barbus barbus* and *A. anguilla* (Sures et al., 2005; Zimmermann et al., 2005a) and also Pt from road dust samples was accumulated by *A. anguilla* (Zimmermann et al., 2002).

Effects of Pt on fishes, however were only analyzed in very few studies. *Danio rerio* showed reversible intestinal changes after exposure to 16 µg/L of Pt (IV) (Jouhaud et al., 1999a,b). Since

Borgmann et al. (2005) could demonstrate that the toxicity of Pt is far lower than toxicity of e.g. Cd, Cr, Hg and Pb, some studies already focused on the genotoxic effect of Pt. Studies regarding the effects of Pt on the DNA of fish, however are not satisfactory. Osterauer et al. (2011) investigated the genotoxicity of Pt using the Comet Assay protocol for zebra fish embryos. While Pt was already found to be genotoxic for mammals (Gangnon et al., 2006), Osterauer et al. (2011) could not find any effects. However, the authors concluded, that the embryo was only exposed for 96 h and it was protected by the chorion. Therefore, the question of genotoxic effects of Pt on fish in aquatic systems still remains to be answered.

Therefore, genotoxic effects on fish DNA is one of the aims addressed in this study. To overcome the problem of short term exposures, an exposure duration of 35 days was chosen and samples were taken on a weekly basis to see if hypothesized effects depends on the time of exposure.

Therefore the first hypothesis of the current study is the following:

- Pt does induce genotoxic effects in fish DNA during a long term study.

Heavy metal effect studies with fish do include some difficulties in terms of interpretation of the results, as it was shown that infection with parasites of the phylum Acanthocephala does influence metal uptake of fish (reviewed by Sures, 2008). Acanthocephalans are known to accumulate heavy metals to a very high degree. Concentrations found in the parasites exceed tissue concentrations of the hosts by far. This could be shown for Pb and Cd in laboratory studies (reviewed by Sures, 2004) and also in field studies for As, Cd, Cu, Co, Mn, Pb, V and Zn (Nachev et al., 2010). Also Pt, Pd and Rh were shown to be taken up by acanthocephalans to a high degree, even if PGE concentrations were not detectable in fish tissues (Sures et al., 2003b; Zimmermann et al., 2004b; Sures et al., 2005; Zimmermann et al., 2005a).

But according to the literature, acanthocephalans do not only accumulate heavy metals to a high degree, they also change the uptake rates of heavy metals by their fish hosts. Sures & Siddall (1999) as well as Sures et al. (2003a) did show that infected chub, *Squalius cephalus*, did incorporate less Pb in the intestinal tissues than uninfected chub.

As Pt is taken up by fish and acanthocephalans, it can further be hypothesized that

- Pt concentrations are lower in tissues of fish infected by acanthocephalans compared to the tissues of uninfected fish.

This hypothesis does further raise the question if possible Pt induced effects are also altered by a parasitic infection. As effects are often directly correlated to the metal concentrations in the test organism it was already stated in several studies that acanthocephalans could possibly mitigate the

effects of heavy metals in their hosts (Sures & Siddall, 1999; Sures et al., 2003a; Sures, 2008). Still, this hypothesis was not yet investigated. Therefore, it is also raised in this study:

- Fish infected by acanthocephalans do show a reduced genotoxic effect, compared to uninfected fish

To proof the three hypothesis mentioned above, *Squalius cephalus* and acanthocephalans of the genus *Pomphorhynchus* were chosen as a suitable host-parasite test system.

This system is a well studied parasite-host systems and was already used in numerous studies in respect to heavy metal accumulation, especially in Pb and Cd exposure studies (e.g. Sures & Siddall, 1999, 2001; Sures et al., 2003a), but also in field studies (Sures et al., 1994).

Squalius cephalus, formely named *Leuciscus cephalus* is not only an already often used indicator species in field studies (Thielen et al., 2004; Agtas et al., 2007; Podrug & Raspor, 2009; Akbulut & Akbulut, 2010) and laboratory studies (Sures et al., 1994; Sures & Siddall, 1999) as well as for genotoxicity tests using the comet assay (Devaux et al., 1998; Winter et al., 2004; Frenzilli et al., 2009) or the micronucleus test (Kolak et al., 1999; Frenzilli et al., 2008). The most common reasons for using *S. cephalus* as a sentinel mentioned by these authors, are characteristics which were defined as the classical characteristics for sentinel species(see Beeby, 2001; Luoma & Rainbow, 2008). It is widely distributed at least in European streams, long living, easily available, tolerable to different pollution levels.

The fish was infected with acanthocephalans of the genus *Pomphorhynchus*, subsequently metal accumulation and micronucli formation was observed during an exposure study of 35 days. In summary, the aim of the study is ,

- to analyze the kinetics of Pt accumulation in different organs of *S. cephalus*,
- to compare these kinetics between infected and uninfected *S. cephalus*,
- to evaluate the genotoxic effects of Pt in erythrocytes of *S. cephalus* and
- to analyze if *Pomphorhynchus* is capable to reduce metal induced genotoxic effects in its host *S. cephalus*.

5.2 Material and Methods

5.2.1 Origin of the test animals

The chub used in this experiment were bought in the Aquaculture Research Centre, of the Research Institute for Nature and Forest (INBO), a scientific institute of the Flemish Government in Belgium. They were bought as stocking fish and held in the laboratory for 2 years before the exposure started. During this time the fish were maintained in laboratory tanks with a supply of tap water and fed on a regular basis with flaked food. For the experiment 112 chub were chosen.

For the infection of chub with acanthocephalans, two species of the genus *Pomphorhynchus* were used. The cystacanths were obtained from naturally infected gammarids. The gammarid *Gammarus roeseli* was sampled with a hand net in the creek Bruchbach at the village Steinfeld (Pallatine, Germany). Approximately 3% of the gammarids were infected with cystacanths of the species *Pomphorhynchus laevis*. The gammarid *Gammarus balcanicus* was sampled in a creek in Kovice (Slovakia) and infected gammarids were transported within 20 h to the laboratory in Karlsruhe. During the transport gammarids were kept in water and cooled. All gammarids arrived alive. *Gammarus balcanicus* was infected with *Pomphorhynchus tereticollis*. Both cystacanth species were isolated from the gammarids and stored in minimum essential medium for one day until the infection of the fish took place. 100 specimens of *Pomphorhynchus laevis* and 215 specimens of *Pomphorhynchus tereticollis* could be isolated. Figure 5.1 shows an infected gammarid as well as the isolated cystacanths. Under the binocular cystacanths were checked for their developmental status. If the cystacanth already everted its proboscis, it was sorted out and not used for the subsequent experimental infection.

5.2.2 Infection of chub with cystacanths

In total 36 fish were infected with *Pomphorhynchus tereticollis* and 20 fish were infected with *Pomphorhynchus laevis*. For each infection, five cystacanths were introduced into the stomach of a chub. For the infection a 2 ml syringe fitted with a 12 cm by 1 mm plastic tubing was used. The plastic tube was inserted through the mouth of the fish into the esophagus and cystacanths were injected. Fish were not anesthetized as this procedure just lasted a few seconds. After the infection each fish was kept in a bucket with fresh tap water to observe if cystacanths were thrown up. This was the case for two fish and the infection had to be repeated.

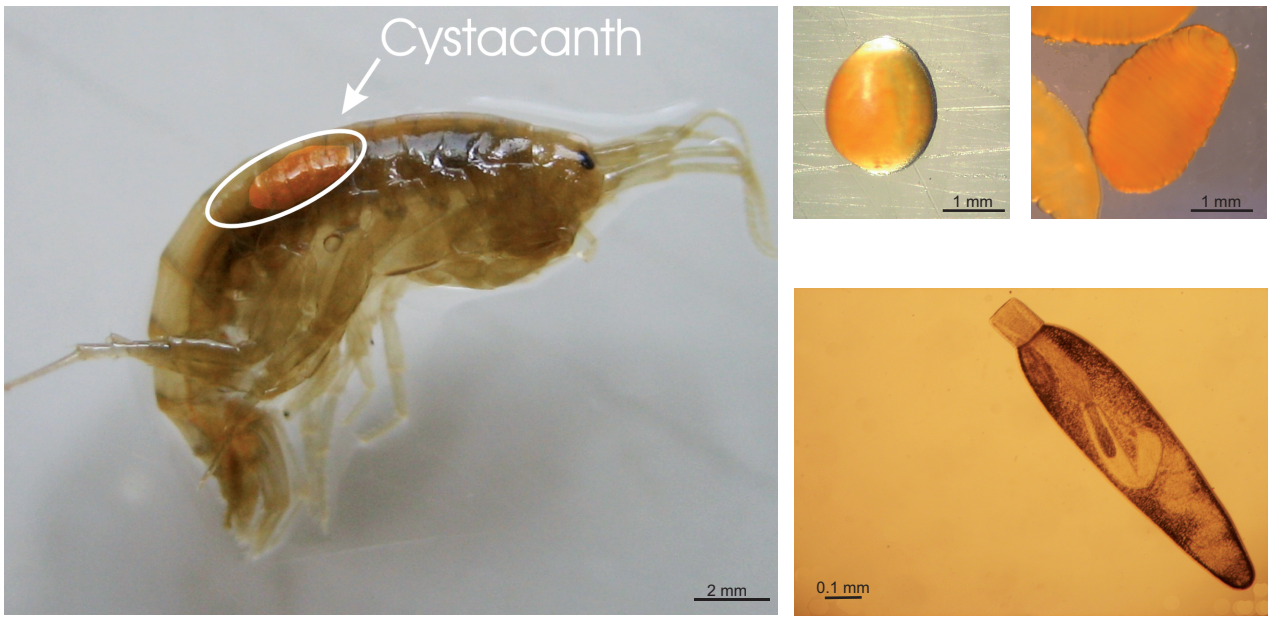

Figure 5.1: **Cystacanths used for infection. Big picture (left): *Gammarus balcanicus* infected with *Pomphorhynchus tereticollis*. Top Pictures: Left - Isolated cystacanths (*P. laevis*) under the binocular. Right side - Isolated cystacanths *P. tereticollis*, under the binocular. Below: Pressed cystacanth (*P. tereticollis* under the microscope).**

5.2.3 Exposure study

After the infection, tanks were prepared for the exposure study. The exposure study took place in glass tanks containing 80 L of non chlorinated tap water and a filter system each (Fluval interior filter system 40 plus, 1000 L/h, Hagen Deutschland GmbH, Holm, Deutschland). Four tanks were prepared: Two tanks held infected, the other two uninfected fish. To keep track which fish was infected with which acanthocephalan species, all tanks were divided into two unequal parts. For the compartmentalization a gaze in a wooden frame was used. In the smaller part (1/3 of the tank) 10 fish were kept (in tanks with infected fish, the fish were infected with *P. laevis*), in the bigger part (2/3 of the tank) 18 fish were kept (if fish were infected, they were infected with *P. tereticollis*). In one of the tanks six extra fish were kept, those were sampled shortly before the exposure started, to sample fish material and fish blood for exposure day 0 and to make sure, that fish were not infected with other parasites. Fish were acclimatized for 50 days in those tanks before the exposure started. In this time span, the acanthocephalans had enough time to settle and develop in the gut of the fish.

At the exposure start, two of the four tanks were exposed to a $H_2[PtCl_6]$ standard solution (1000 mg Pt/L, Ultra Scientific, Wesel, Germany). Figure 5.2 shows the scheme of the exposure study.

The nominal exposure concentration was 100 µg/L. Pt was added in the respective quantities to

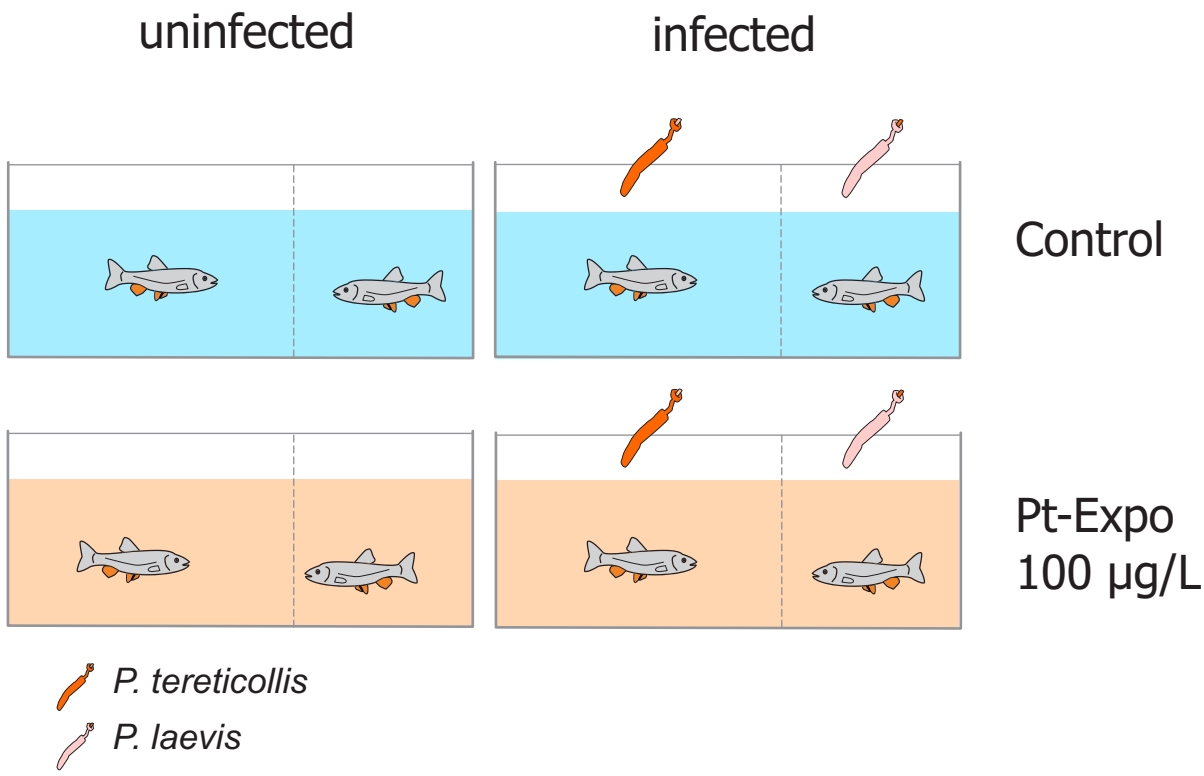

Figure 5.2: **Experimental design. In total there were six treatment groups. Three treatment groups were exposed to Pt: a non infected group (n=28), a group infected with P. laevis (n=10) and a group infected with P. tereticollis (n=18). Further three treatment groups are not exposed to Pt (control groups).**

the aquarium water and was stirred. Water and Pt were renewed weekly, whereby 2/3 of the water was removed of the tank and renewed. Two days before the water change, fish were fed with commercial flaked food (TetraMin, Tetra GmbH, Melle, Deutschland). This is necessary in exposure studies with fish as hunger would lower the excretion of heavy metals through the bile and the accumulation of heavy metals would be higher than in a well fed condition of the fish (Hofer & Lackner, 1995). Before and 30 min after the change of water and Pt addition, physical and chemical values of the water were analyzed. The pH-value, the temperature, and the redox potential were analyzed with pH-325 (WTW, Weinheim, Germany) and the conductivity was analyzed with the set LF-318 (also manufactured by WTW).

5.2.4 Sample preparation

Fish and water samples were taken weekly on the same day when water change took place. As fish were infected with different parasite species, fish sampling was adapted in the following way. One week fish infected with *P. laevis* and the other week fish infected with *P. tereticollis* were sampled.

In treatment groups without infections, fish were taken from the small or the big compartment, respectively. The design of the exposure study can be found in Table 5.1.

Table 5.1: **Design of the exposure study with *Squalius cephalus*.**

Treatment-group	Nominal exposure concentration	Sample collection (days after exposure start)
Control uninfected	0 µg/L	7, 13, 21, 28, 35
Control *P. laevis*	0 µg/L	13, 28
Control *P. tereticollis*	0 µg/L	7, 21, 35
Pt uninfected	100 µg/L	7, 13, 21, 28, 35
Pt *P. laevis*	100 µg/L	13, 28
Pt *P. tereticollis*	100 µg/L	7, 21, 35

For the water samples a 10 ml sample of tank water was taken from each tank and filtered (cellulose nitrate filter, pore size 0.45 mm, Sartorius AG, Göttingen, Germany), acidified with 10 µl H_2SO_4 (96% suprapure quality; Merck, Darmstadt, Germany) and stored at -21 °C until metal analysis.

Chub were killed immediately after the removal of the tank. Blood was taken from a cut into the neck of the fish and smeared on a cleaned and cooled glass objective for the micronucleus test. Before dissection the total length (i.e. head to end of the tail fin) and the total mass were recorded. The fish were dissected using stainless steel scissors and forceps. Those instruments were cleaned thoroughly with 1% ammonium- EDTA-solution and double-distilled water, prior to the dissection. The following tissues samples were taken, cleaned with double distilled water and weighed: gills, liver, intestine, gonads, muscle. The sex was determined after the dissection of gonads. Muscle tissue samples consisted of approximately 1 square cm sample taken from the right site of the fish, behind the pectoral fin. It was carefully cleaned from scales, skin and spines and checked under the binocular. For gill, liver and gonad samples the entire organ was removed. The intestine was also taken as a whole sample, but it was examined carefully for parasites (Figure 5.3). All parasites were counted for each fish and detached from the intestine. Then the intestine was intensely scraped to remove all mucus. After the isolation of the parasites, the intestines itself was rinsed with double distilled water and dried on a clean laboratory paper. Parasites of each fish were pooled to one sample and also weighed. As the mass of the tissues was relatively low, samples could be analyzed completely and a homogenization of the tissues was not necessary. All samples were frozen at -20°C prior to Pt analysis.

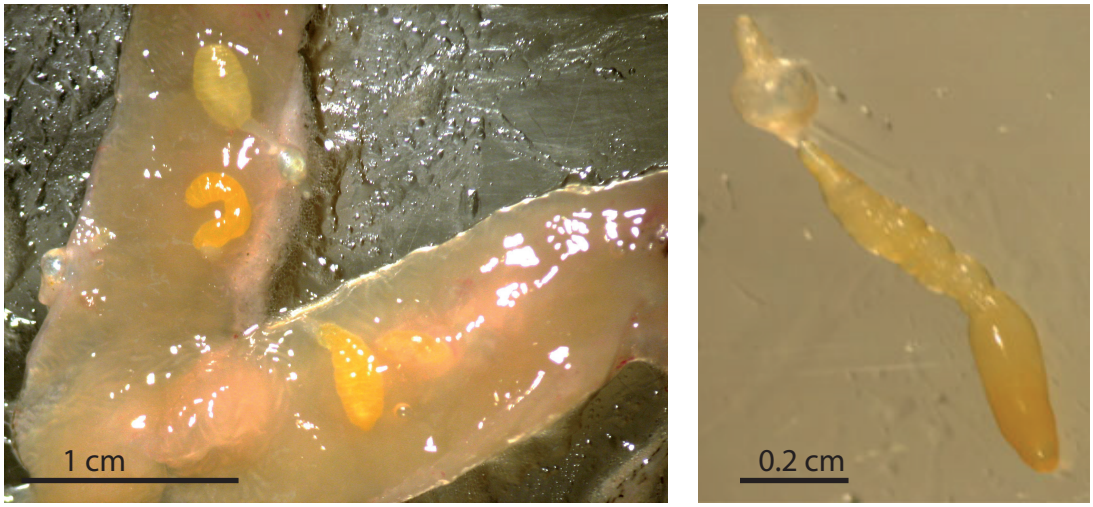

Figure 5.3: **Pictures of sampled parasites after the fish dissection. On the left site: Parasites are still attached to the open intestine. On the right site: *P. laevis* isolated from the intestine after the exposure experiment.**

5.2.5 Platinum Analysis

All water and tissue samples were thawed at room temperature. Water samples were directly analyzed via AAS as described in Chapter 2. Three aliquotes were analyzed for each sample. Fish samples were digested using the high pressure asher and Pt concentrations were detected with the ACSV analysis also described in Chapter 2. Muscle, liver and samples were analyzed for each fish, without dividing the samples into aliquots. This resulted in n=5 for weeks in which fish infected with *P. laevis* were sampled and in n=6 for weeks in which fish infected with *P. tereticollis* were sampled. As the chemical analysis was very time consuming it was decided to limit the number of fish analyzed for all control groups to n=3.

5.2.6 Micronucleus test

Preparing of the slides for a micronuclei test

As fish is one of the rare vertebrate groups containing a cell nucleus in erythrocytes, they can easily be used for the micronucleus test. Immediately after a fish was killed some blood drops of the fish were transfered to a cleaned microscope slide and smeared. For each fish one slide was prepared. The blood was air dried and then placed in methanol (100%) for three minutes to fix it on the slide. Again the blood smear was air dried. Nuclei and micronuclei were stained with GIEMSA (3% in dilution buffer, Weisepuffer) for 30 min. Slides were washed with double

destilled water, dried overnight and stored in darkness until counting of the micronuclei.

Cell and micronuclei counting

For each sample point and treatment group four of the slides were chosen and counted by a single observer. As the formation of micronucleus appear to be variable (Frenzilli et al., 2008; Viganò et al., 2002), on each slide 2500 erythrocytes were counted, resulting in 10000 cells for each sample point and treatment group. The number of 2500 counted erythrocytes per individual and 10000 cells per sample point were chosen as it had already been proofed by other studies to be suitable for MN tests for chub (Frenzilli et al., 2008) and for gobius and tilapia (Arslan et al., 2010; Manna et al., 1985). Criteria for counting MN in fish erythrocytes were defined by Al-Sabti & Metcalfe (1995) who reviewed several studies including teleost fish and MN test in laboratory and field studies. These criteria, listed below, were also used in this study:

- Blind scoring (counting coded slides without knowledge of the treatment group or the time of sampling)
- Overlapping and damaged cells were excluded from scoring
- Cells were carefully checked for debris and precipitates of the staining process which could be mistaken for micronuclei

The appearance of erythrocytes and micronuclei are presented in Figure 5.4. Micronuclei are roundish, similar stained as the main nucleus, separated from the main nucleus and smaller than the main nucleus.

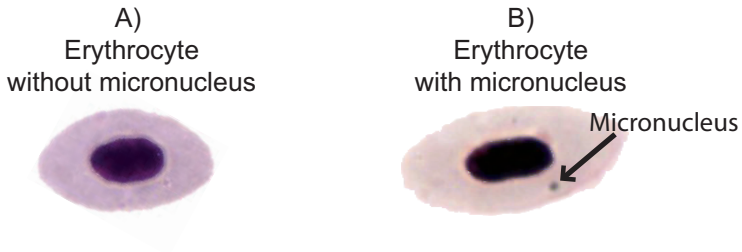

Figure 5.4: **Erythrocytes in fishblood. The blood smears of chub were analyzed for micronuclei in erythrocytes, the figure shows A) an erythrocyte without and B) one with an micronucleus.**

5.2.7 Data analysis

Boxplots Data regarding the chemical and physical water parameter are presented as boxplots. In those boxes different percentiles, the mean, and median of the data are shown as explained in Figure 4.2, Chapter 4.

Fulton condition factor The Fulton condition factor is a standard method to describe the condition of a fish. It is a measure taking into account the length and the weight of a fish (Nash et al., 2006). It is calculated using the following Equation 5.1:

$$CF = \frac{M * 100}{L^3} \tag{5.1}$$

$CF = condition\ factor$
$M = Mass\ of\ fish\ (g)$
$L = total\ length\ of\ fish\ (cm)$

Hepatosomatic index A common and easily available stress indicator is the hepatosomatic index (HSI) as defined in Equation 5.2.

$$HSI = \frac{M_{liver} * 100}{M_{fish}} \tag{5.2}$$

$HSI = hepatosomatic\ index$
$M_{liver} = Mass\ of\ liver\ (g)$
$M_{fish} = Mass\ of\ fish\ (g)$

Statistical tests Statistical tests were performed to analyze if mean values or median values of different data groups differ from each other significantly. For samples which are independently from each other (e.g. the Pt concentrations of different treatment groups) the Kruskal-Wallis Test was used, as already described in section 3.2.3. If the test was conducted to support one of the hypothesis described in this chapter, the test was performed one-sided. All other Kruskal-Wallis tests were performed two-sided. Dependent samples (e.g. the Pt concentrations of different organs of the same fishgroup) were tested with the Wilcoxon-Test. It tests the following hypothesis for two dependent groups.

H0:
Metal concentrations for Pt in a specific organ of a certain chub group are equal to Pt concentrations in another organ for the same chub group.

H1 (one sided):

Metal concentrations for Pt in a specific organ of a certain chub group exceed Pt concentrations in another organ for the same chub group..

5.3 Results

5.3.1 Physical and chemical water parameters during the exposure study

As some of the treatment groups were held in the same water, the data of only four water tanks are shown in this subsection (see also Figure 5.5).

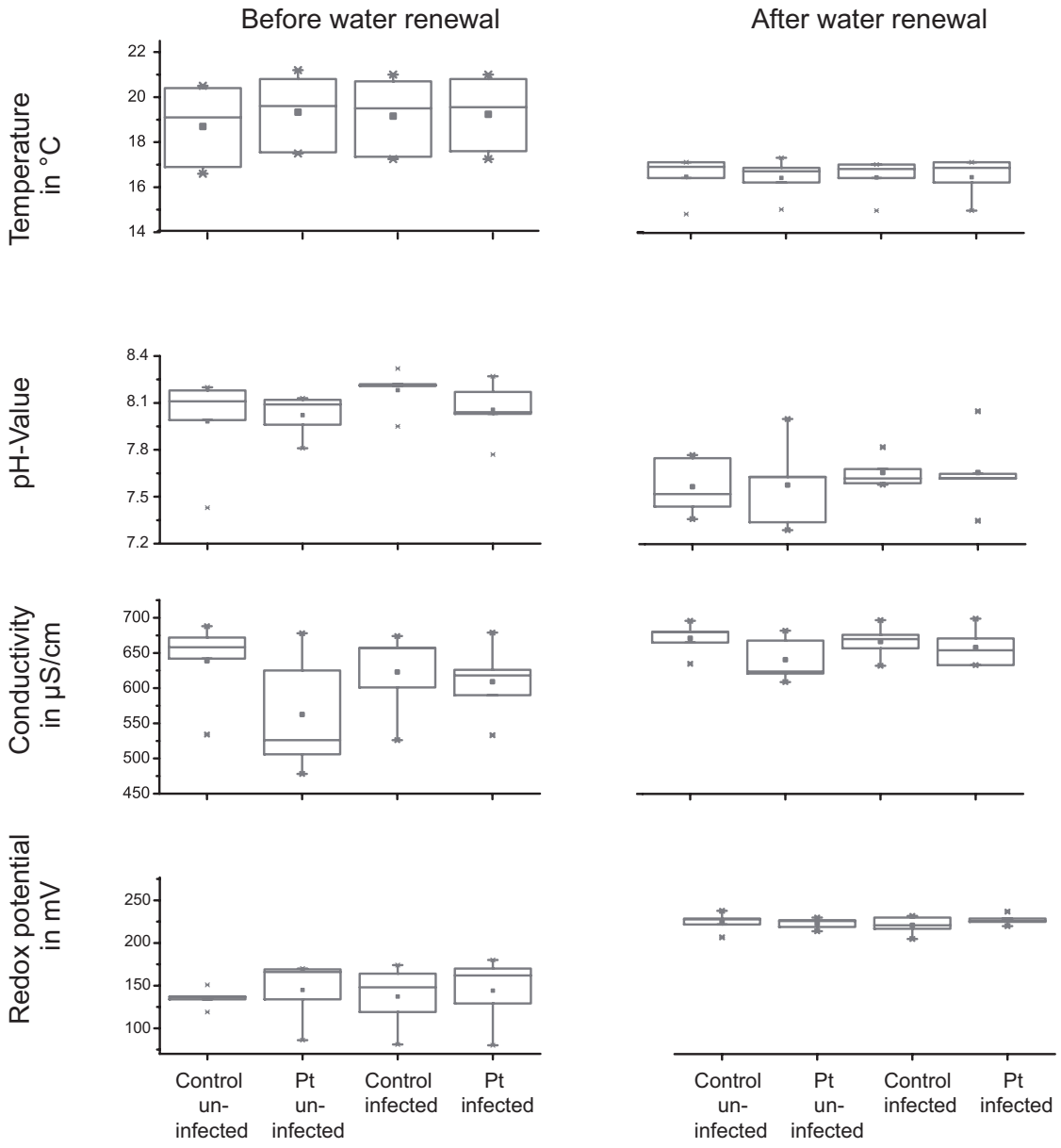

Figure 5.5: **Physical and chemical water parameters during the exposure study with chub. Boxplot are divided in values before and 30 min after the weekly water change.**

All parameters were statistically tested for differences between means (Kruskal-Wallis-Test). It can be seen, that values for the treatment groups did not vary, within the groups before the water

renewal and within the groups after the water renewal for all analyzed chemical and physical parameters. However, as already observed in the exposure study with clams (see Chapter 4) also in this exposure study there are differences between the values before and after the water renewal. The temperature decreases by 2 K ($p<0.01$) from 19 to 17°C, also the pH-value decreases by 0.5 ($p<0.5$) from pH 8.1 to pH 7.6 and the redox potential increases by 70 mV ($p<0.01$) from 150 mV to 220 mV after the water renewal. An exception is the conductivity. Mean values in all treatment groups do not differ due to the water change and are approximately 630 µS/cm.

Mean Pt concentrations for the exposed treatment groups within the water are shown in Figure 5.6. Platinum concentrations in both control groups were also analyzed, but were below the detection limit and were therefore not included in the plot.

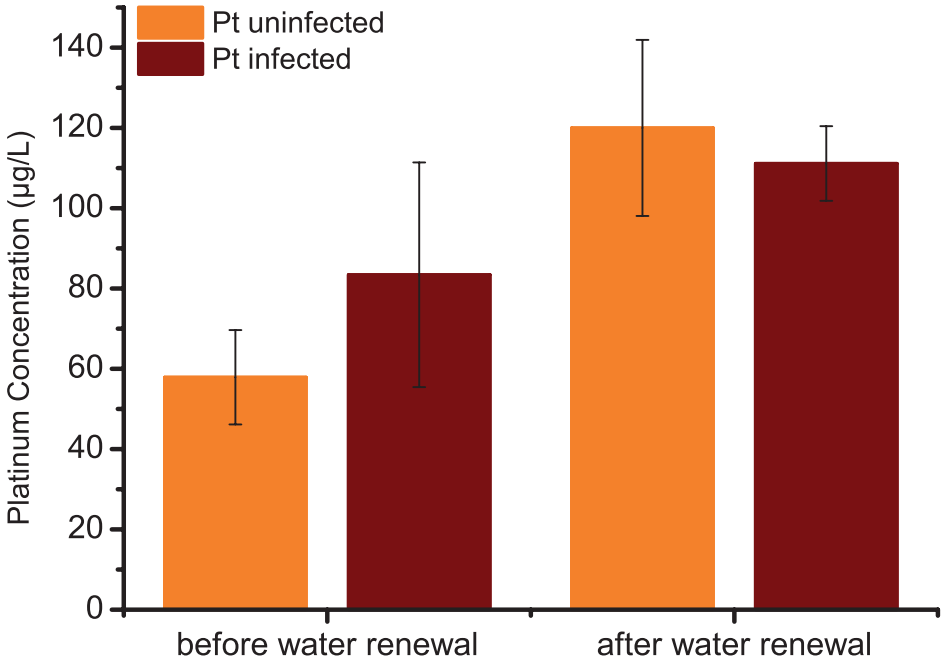

Figure 5.6: **Mean Platinum concentrations in the water of the exposed treatment groups. Mean values of Pt concentrations in water samples before and after the water renewal are plotted. Error bars indicate the standard deviation of all analyses.**

As can be seen in Figure 5.6, Pt concentrations were higher after the water renewal.

5.3.2 Performance of chub during the exposure study (Mortality, sex rates, Fultons condition factor and hepatosomatic index)

While one fish died prior to the exposure start, during the exposure period all of the fish survived. The majority of the fish was female (97 fish were female, 14 male). The male fish were equaly

distributed over all treatment groups (four males in treatment groups "Control uninfected" and "Pt uninfected", 3 males in the treatment group "Control *P. tereticollis*", 2 males in the treatment group "Pt *P. tereticollis*" and one and none male fish in the treatment groups "Pt *P. laevis*" and "Control *P. laevis*", respectively). The mean Fulton condition factor was 0.7 (standard deviation of 0.07) over all treatment groups and sampling times. Due to the results of the Kruskal-Wallis Test the Fulton condition factor does not significally differ in between the treatment groups or during the exposure study for all treatment groups ($p>0.05$) (see also 5.7). It can therefore be concluded that the overall condition of the fish were the same for all treatment groups and did not change during the experiment.

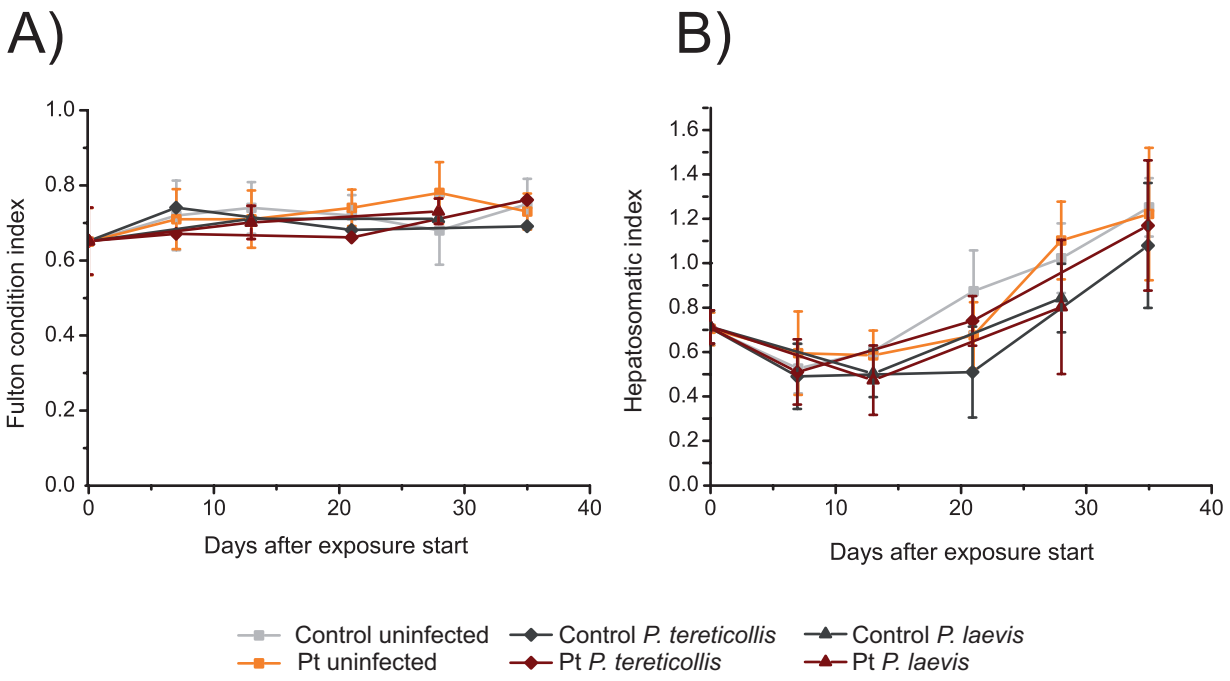

Figure 5.7: **Fulton condition factor and Hepatosomatic index for chub of all treatment groups. A) is presenting the Fulton condition factor as described in Equation 5.1 and B) is presenting the hepatosomatic index as described in Equation 5.2.**

Furthermore, the Hepatosomatic index (HSI) was analyzed as described in Equation 5.2. As can be seen in Figure 5.7 the variance in the HSI is high. Therefore, no statistical differences were found in between HSI of the different treatment groups (U-Tests, $p<0.05$). However, the HSI is rising for all treatment groups during the exposure study, although only for the group "Control uninfected" a statistical difference can be found in HSI values for day 7 and day 35 after the start of the exposure (U-Test, $p<0.05$). For the other groups the same tendency is visible, but no significant difference could be identified.

5.3.3 Infection of chub with *Pomphorhynchus* sp.

The opening of the intestine of the fish allowed to determine the success of the infection of the chub. In total 280 cystacanths were injected into the digestive tract of the chub, 180 *P. tereticollis* and 100 *P. laevis*. In this experiment, 177 adult acanthocephalans were found, eleven of them outside the intestine in the body cavity of the fish. This results in a recovery rate of 56% for acanthocephalans found inside the intestines. However, the two species do not show an equal infection success as can be seen in Figure 5.8. The infection success in individual fish can be found in Appendix A.17.

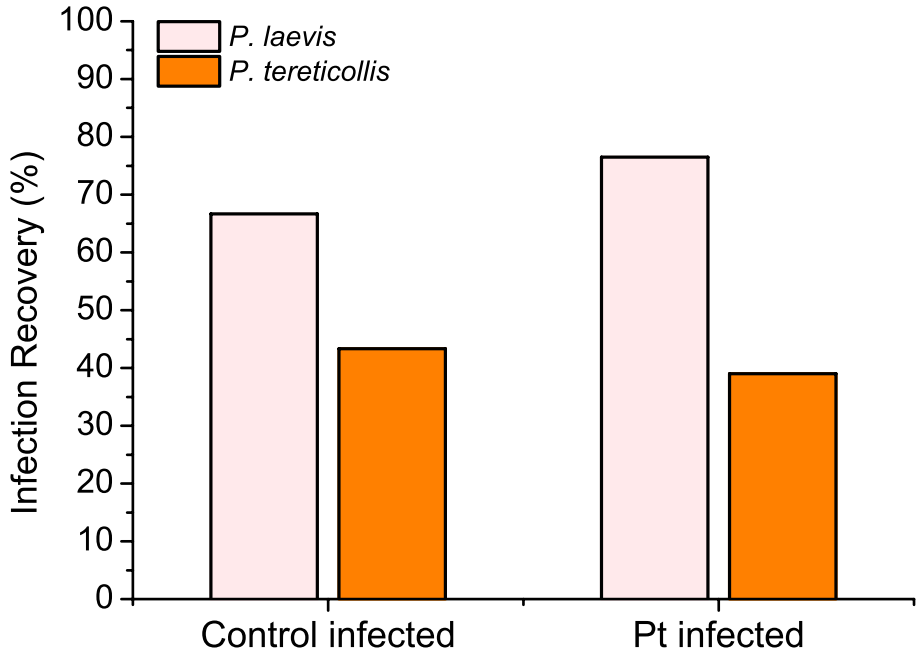

Figure 5.8: **Recovery of parasites in fish intestines after infection. Percentage of adult acanthocephalans found in the intestine of chub compared to the number of initially incorporated cystacanths.**

While 80% of the injected *P. laevis* larvae could be found as adults in the intestines of their hosts, only 46% of the injected *P. tereticollis* were found. Therefore, the experimental infection of chub with *P. laevis* was more successful than the infection with *P. tereticollis*. This difference was proved to be statistically highly significant (U-Test, $p<0.001$).

5.3.4 Accumulation of Platinum by *Squalius cephalus* and acanthocephalans of the genus *Pomphorhynchus*

Pt was analyzed in the organs muscle, intestine and liver of all individual fish. Furthermore, it was analyzed in the pooled parasite sample of each fish. Results are presented in Figure 5.9.

Pt concentrations in fish organs and acanthocephalans of control groups (Control uninfected, Control *P. tereticollis*, Control *P. laevis*) are all low. In the muscle tissue most of the samples do not show detectable Pt concentrations. Exceptions are Control uninfected at day 7, Control *P. laevis* at day 13 and Control *P. tereticollis* at day 35, with concentrations just above the detection limit. In the intestine Pt could be found in all samples with concentrations in between 0.05 to 7.74 ng/g. In the liver of the control groups, Pt concentrations are mostly below the detection limit, but can rise to 7.46 ng/g in some samples. Also in the acanthocephalan samples Pt is detectable, but concentrations are low (*P. tereticollis*: 5.5 to 10.6 ng/g and *P. laevis*: 0.6 to 4.3 ng/g).

Pt accumulation can be observed in the fish organs of Pt exposed fish groups. Pt concentrations in muscle samples are still low. Mean values for the different samples are in between 1.7 and 7 ng/g. However, in some individual chub no Pt above the detection limit could be found, especially after one week of exposure. Higher concentrations can be found in the organs intestine and liver. The Pt concentrations in both organs are comparable to each other for each treatment group. They can not be distinguished with the Wilcoxon Test ($p<0.05$). The accumulation of Pt in all organs of exposed chub rises until day 13 after the exposure start. Pt concentrations remain stable afterwards. An exception are eventually Pt concentrations in the liver of chub infected with *P. laevis*. Here, Pt concentrations seem still to rise after 28 days of exposure.

Highest Pt concentrations can be found in acanthocephalans. In the case of *P. tereticollis* Pt accumulation follows an expected accumulation curve, with high Pt uptake in the beginning of the exposure study and stable Pt concentrations from day 21 of the experiment on. The accumulation of Pt in *P. tereticollis* is 3 times higher than in the liver, 5 times higher than in the intestine and 40 times higher than in the muscle tissue.

For *P. laevis* uptake is very high 13 days after the exposure start, but it decreases on day 21 and is then equally high to the concentrations of the organs liver and intestine. Compared to the muscle tissues, *P. laevis* accumulated 25 times more Pt.

In a further step, differences in the Pt accumulation of infected and non infected chub were analyzed (see Figure 5.10).

It can be seen, that the intensity of Pt accumulation is differently according to the parasite with which the chub is infected. For all three organs, Pt concentrations are comparable for uninfected and infected chub, when chub are infected with *P. laevis*. The picture differs a bit, when chub

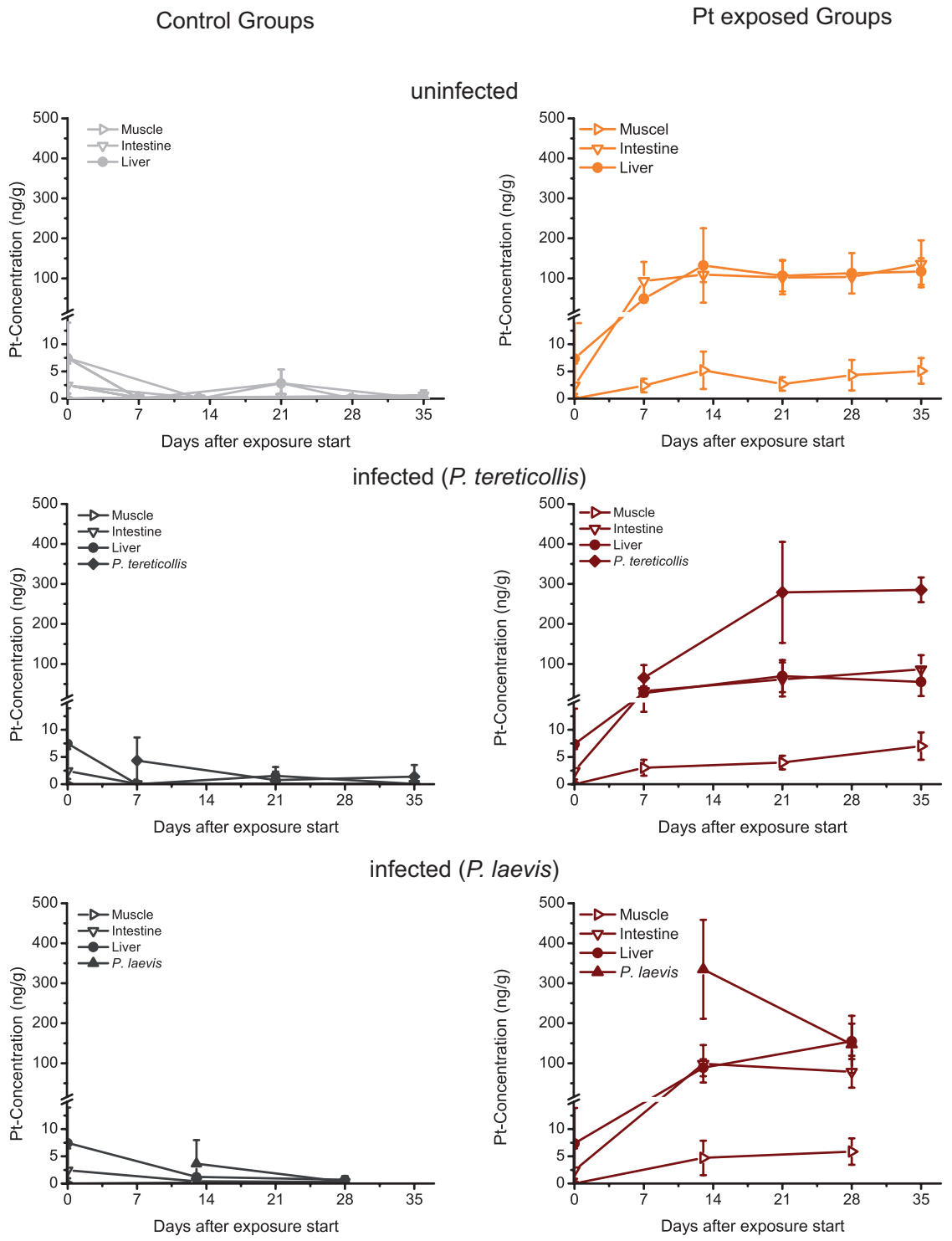

Figure 5.9: **Accumulation of Platinum in different organs of *Squalius cephalus* and its parasites *P. tereticollis* and *P. laevis*. Errors bars represent the standard deviation of n=3 for controls or n=5 or n=6 for infected chub.**

are infected with *P. tereticollis*. In muscle tissues Pt concentrations of infected chub seem to be a bit higher, than Pt concentrations in uninfected chub beginning at day 21 after the exposure

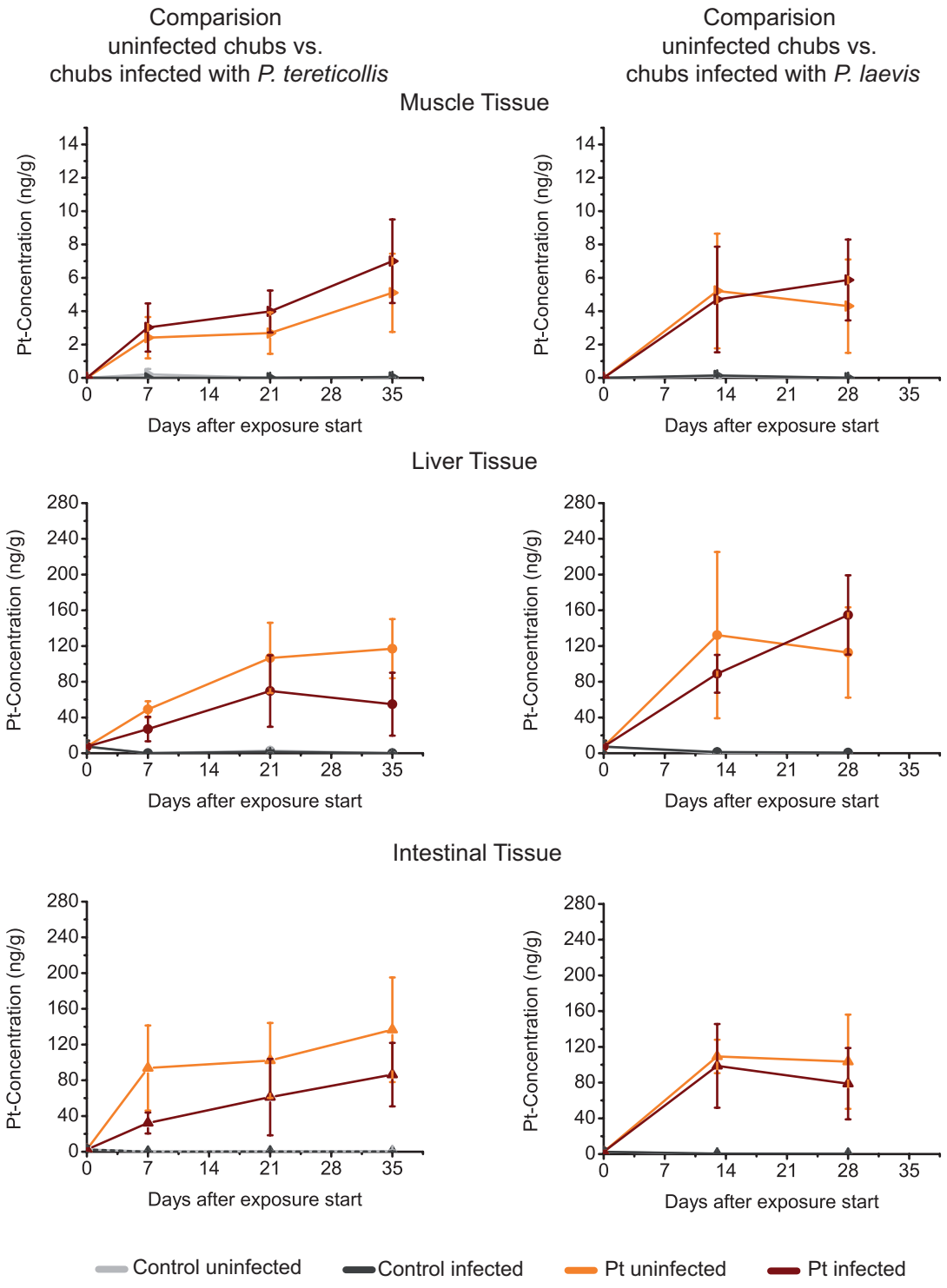

Figure 5.10: **Comparison of Platinum accumulation in different organs of infected and uninfected *Squalius cephalus*. Errors bars represent the standard deviation of n=3 for controls or n=5 or n=6 for infected chub organs.**

start. In liver and intestinal tissues however, Pt concentrations are higher in uninfected chub, than in infected chub for all analyzed exposure days. Furthermore, at day 7 after exposure start this difference can be proven to be statistically significant for both tissues (Kruskal-Wallis Test, $p < 0.05$). It is further be statistically different at day 35 after exposure start for Pt concentrations in the liver tissues. For intestinal tissues differences after day 7 of the experiment can not be proven statistically significant, but the trend is still visible.

5.3.5 Genotoxic effects of Platinum on *Squalius cephalus*

Accordingly to the clams exposure study, also the fish were analyzed for genotoxic effects. In this case, the micronuclei (MN) frequency was analyzed in the erythrocytes of the fish blood.

Overall, the spontaneous MN induction was in between 0.3 and 1.8‰ in the blood samples of chub. The mean of the spontaneous MN frequency was 0.83‰. The rate of binucleated cells was overall negligible. Figure 5.11 shows the frequencies of micronucleated cells at the different exposure days.

In part A the uninfected Control group is compared to the uninfected group exposed to Pt. As can be seen no effects occur in the beginning of the exposure. At Day 13 after the exposure start, frequency of micronucleated cells in the Pt exposed group exceed the frequency of micronucleated cells in the Control group. On day 21 after the exposure start this difference can be found to be statistically significant (one-sided U-test, $p<0.5$). Afterwards, the level of micronucleated cells is again comparable to the Control group. Also for the other two treatment groups (fish infected with parasites as well as infected and exposed to Pt) a similar trend is observable. Also here, frequency of micronucleated cells is highest at day 21 after the exposure start. However, the effect is not tested to be statistically significant (two-sided and one-sided U-Test, respectively, $p>0.05$). A higher frequency of micronucleated cells is also visible on day 28 for the treatment group infected and Pt exposed fish. In Graph 5.11, D it is clearly visible, that in fish exposed to Pt the induction of MN was highest, followed by infected fish and then infected and exposed fish.

Therefore, a clear effect of Pt on the induction of MN could be found. For the induction of MN in erythrocytes of Pt exposed fish there was no difference found between infected and uninfected fish.

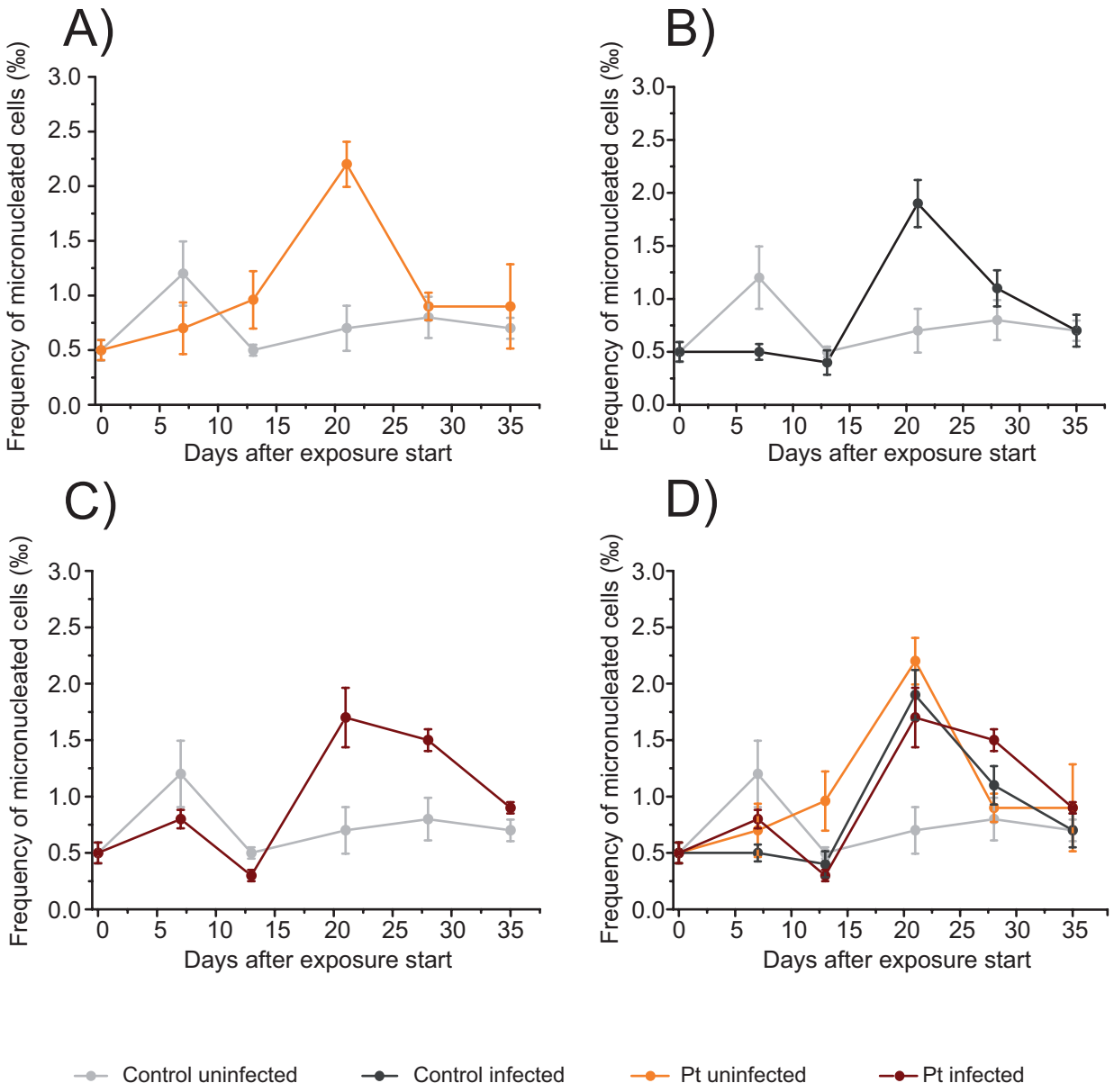

Figure 5.11: **Comparison of micronuclei frequencies in erythrocytes of all treatment groups. Graphs compare the mean value for micronucleated cells in 1000 erythrocytes for all treatment groups. A), B) and C) compare the treated groups against the uninfected Control Group. D) shows all treatment groups in one graph. For each fish 2500 erythrocytes were checked, resulting in 10000 erythrocytes for each treatment group and sampling point. Standard deviations of n=4 are indicated by the error bars.**

5.4 Discussion

Platinum accumulation The results clearly indicate that chub do accumulate Pt. Most of the Pt was found in liver and intestine, both known as target organs for metals as both organs are involved in the uptake and metabolism of metals (Dallinger et al., 1987). Also in muscle tissues Pt was accumulated, but at a much lower concentration rate. In all tissues a steady state was found after 13 days after the exposure start. Pt concentrations in liver and intestinal tissues are comparable to each other. These findings are different to findings of Zimmermann et al. (2004a) who found distinctive higher Pt concentrations in eel intestines than in eel liver tissues after six weeks of Pt exposure.

Highest Pt concentrations were found in acanthocephalans, which confirm the findings of previous studies with different chemical Pt species (Zimmermann et al., 2004b, 2005a; Sures et al., 2005). Nachev et al. (2010) analyzed concentrations in tissues and parasite of barbels (*Barbus barbus*) infected with *P. laevis* of the river Danube. Compared to other metals, bioconcentration factors (concentration *P. laevis*/concentration fish tissue) of Pt in *P. laevis* is lower than for Pb and Cd, but equal to Cu, Co, Mn, as well as Zn and higher than for Fe, Mo, V. Also the review of Sures (2003) confirms higher bioaccumulation factors for Pb and Cd in case of *P. laevis* living in the intestines of chub.

In the introduction it was hypothesized that an infection with acanthocephalans would reduce the Pt load in the intestinal tissues as it was already described for the accumulation of Pb by infected and uninfected chub (Sures & Siddall, 1999; Sures et al., 2003a). As a manipulation of the results through differences in abiotic or biotic conditions can be excluded (metal content of water, pH-value, temperature, conductivity as well as size and condition of fish were tested to be similar), this hypothesis holds true for fish infected with *P. terreticolis*. Significantly higher Pt concentrations were found in the intestine of infected chub at day seven after the exposure start. Also, on the other days the trend of higher Pt concentrations in uninfected fish is clearly visible. Significantly higher Pt values were not only found in the intestine tissues. Furthermore, also in the liver tissues significant lower metal concentrations can be found in infected chub on day 7 and also day 35 after the exposure start.

However, it is striking that the difference can already be seen a couple of days after the exposure start. In a study comparing the accumulation of Pb in infected and uninfected chub, Sures & Siddall (2001) considered that this effect is only visible after five weeks of exposure with Pb.

In this study, lower Pt concentrations in fish tissues were only found in fish infected with *P. tereticollis*, but not if the fish is infected with *P. laevis*. If chub are infected with *P. laevis*, concentrations in liver and intestinal tissues are comparable or in some cases slightly higher than in uninfected fish. This cannot be explained due to different abiotic conditions in which the fish were kept, as all infected fish were kept in the same treatment tank. Also the accumulation of Pt in *P. laevis* is

equally high to the accumulation by *P. tereticollis*. More over, *P. laevis* was more successful regarding the infection rate , i.e. more individuals were found in the intestine of the respective hosts. Nachev et al. (2010) did further show that the sex of the acanthocephalans and the infrapopulation within the host do not have any effect on the metal concentrations in host tissues.

Further it was unexpected that *P. laevis* does accumulate very high Pt amounts in the beginning of the experiments (13 days after exposure start) and later in the experiment reduced Pt concentrations are found in the parasites, i.e. day 28 after exposure start. One would have expected an accumulation curve with rising concentrations at the beginning followed by a steady state, as it could be seen in the accumulation kinetics of *P. tereticollis*. This does imply a different accumulation kinetic of the two *Pomphorhynchus* species in this study.

However, the accumulation kinetics of fish infected by *P. tereticollis* do indicate lower Pt concentrations compared to uninfected fish throughout the whole experiment in liver and intestinal tissues and those differences could proved to be statistically significant and simultaneous Pt concentrations are equal or higher in fish infected by *P. laevis*. Thus, it has to be concluded that the amount of metal uptake of hosts is influenced by a specific parasite infection. The results imply that different host-parasite systems do react differently according to heavy metal exposures. A difficult situation for biomonitoring studies, as it would consequently implicate an additional criterion for the choice of the biomonitor species. Consequently, if fish are used as a biomonitor not only the already well known criteria (representative, abundant, easily identified, easily sampled, long-lived and large enough for tissue analysis - see Luoma & Rainbow (2008)) have to be considered. Sures (2008) already postulated that biomonitoring programmes should take into account the influence of parasite infections on the levels of pollutants in sentinels. Based on the results of this study this statement has to be further refined. It is not only the "parasite infection" which influences the metal uptake of a host. But the species of the parasite has an influence on the uptake. These influences are already different between very similar species or even traits, as the degree of relationship for *P. laevis* and *P. tereticollis* is still under discussion (Amin et al., 2003; Perrot-Minnot, 2004). In order to avoid false negative results in biomonitoring studies, acanthocephalan species should be carefully considered if different sentinel groups are compared.

As already mentioned above, the degree of the relationship between *P. tereticollis* and *P. laevis* is still discussed (Perrot-Minnot, 2004). This is not surprising as *Pomphorhynchus* is widely spread and adult worms are very similar to each other. Amin et al. (2003) list all known species of the genus *Pomphorhynchus* and claims that due to analysis of the morphology of adult worms, no significant difference between *P. laevis* and *P. tereticollis* could be found. Consequently, they consider *P. tereticollis* as synonymous with *P. laevis*. Nowadays, the two worms can be differentiated as different species by using analysis of rDNA and COI mtDNA sequences as well as isoenzym profiles (Perrot-Minnot, 2004). Furthermore, Perrot-Minnot (2004) could show that those two genetically different groups also show morphological differences, if the larval stages are taken into account. Those larval stages did also alter the behaviour of their crustacean hosts. While *P. lae-*

vis induced a positive phototaxis, gammarids infected with *P. tereticollis* remained photophylic, like their uninfected counterfeits. Perrot-Minnot et al. (2011) could further show, that carotenoid contents in cystacanths of both groups are different. In the presented study, distinctive differences were found in the development of the cystacanths into adult worms and for the interaction of adult parasites with their final hosts (as already discussed above). The differences in the development of the cystacanths were proven by the statistically significant difference in the infection rate of both species. The hosts originated from the same offspring and fish were kept in the same tank. All fish were infected at the same day and daytime. Nevertheless, the developmental success of both species was still different. Consequently it can be assumed that this leads to a difference in success of settlement in the new habitat of *P. laevis* and *P. tereticollis* and a better adaption of *P. laevis* to *S. cephalus*. Otherwise, *S. cephalus* could also have developed a better immune reaction and therefore a better adaption to *P. tereticollis*. Those findings support the hypothesis that those two forms are different and that these are indeed two species that have to be distinguished from each other.

Genotoxicological effects In this study erythrocytes of the different treatment groups were tested for the induction of micronuclei. The hypothesis is, that Pt does induce MN production and therefore does alter the DNA structure in cell nuclei. A statistical significant effect was observed on day 21 after the exposure start for the group of fish which were solely exposed to 100 µg/L Pt and not infected with parasites. The hypothesis is therefore considered approved.

It should be pointed out, that the effect is only statistically significant when observed on day 21 after the exposure start. However, from the beginning of the exposure experiment, the frequency of micronucleated erythrocytes is rising and already at day 13 it exceeds the frequency of MN in erythrocytes of the control group. An increase of MN throughout the time of exposure is not uncommon. Nepomuceno et al. (1997) observed an increase of MN after approx. 20 days of exposure with mercury in erythrocytes of carps, Dinnen et al. (1988) found an increase of MN in erythrocytes of a rainbow trout 7-9 days after the start of a treatment with radiation and MN frequency rose to a plateau after 13-15 days after treatment start. Also Bahari et al. (1994) found an increase of MN induction 4 days after the treatment of catfish with y-radiation or mitomycin C, followed by a rapid decrease in MN frequencies a few days later. And De Flora et al. (1993) examined MN induction 7 days after the resettlement of trouts into the river Po, downstream of the confluence with the river Lambro, a small, yet, heavily polluted tributary. Dinnen et al. (1988) concluded that the time lag in between start of the treatment and observable genetic defect is due to the maturing process of the erythrocytes. In teleost fish erythrocytes are formed from hemocytoblast precursor cells in the spleen and in the kidney and then they mature in the blood stream. "Juvenile" erythrocytes were not included in the cell scoring in this study. It can therefore be concluded that on day 13 after the exposure start the first matured erythrocytes were analyzed and on day 21 the full range of MN induction became visible in matured erythrocytes. However,

Kolak et al. (1999) already found elevated MN frequencies in chub erythrocytes 6 days after the exposure with benzo(a)pyrene. This difference could be due to the faster uptake and better distribution of the organic xenobiotic compared to Pt. The decrease of the MN in the erythrocytes to values comparable to the control group on day 28 after the exposure start is striking. One would assume that the level of MN should be constant over time as the Pt concentrations in tissues stay constant, too. However, a decrease in MN kinetics was already observed in Das & Nanda (1986) and De Flora et al. (1993). De Flora et al. (1993) suggested that damaged cells are removed faster as undamaged cells. Other explanations could be that DNA repair mechanisms during the cell division are increased due to the higher frequency of MN (Bolognesi & Hayashi, 2011). For Pb it was shown that next to the increased MN induction, the synthesis of red cell precursors was inhibited, resulting in a smaller proportion of new erythrocytes (Nikinmaa, 1992). In combination with a rapid elimination of micronucleated erythrocytes the proportion of healthy cells would increase again.

If those induced MN are due to a clastogenic or a spindle poison effect can not be answered through this study. However, Migliore et al. (2002) did analyze MN induction in human lymphocytes by different species of Pt and found that MN induced by $PtCl_4$ and $PtCl_2$ did incorporate chromosomes with and without centromers, indicating that clastogenic and spindle poison mechanisms could play a significant role in MN production.

The next paragraphs will adress the third hypothesis of this chapter ("Fish infected by acanthocephalans do show a reduced genotoxic effect, compared to uninfected fish"). The comparison of the frequency of micronucleated cells in respect to the treatment groups "Pt exposed" and "Pt exposed and infected" do not show a statistically significant difference, which suggests that the hypothesis is not true. But, there is also no statistically significant difference between the treatment group "Pt exposed and infected" and the Control group. This however, does imply that there was no effect of Pt on the induction of MN. In summary, an effect of Pt on the MN induction in the treatment group which was not infected with acanthocephalans can be observed and no effect of Pt on the MN induction in the treatment group "Pt exposed and infected" could be found, which would support the hypothesis.

It needs to be pointed out, that due to the relative low number of fish sampled, the statistical test used is not sensible enough for determining if there is an actual effect on MN induction in erythrocytes of the Pt exposed and infected chub. Still, related studies have taken the approach to determine an effect based on the comparison of the spontaneous MN induction rate (i.e. control group) to the actual induction rate in the treatment group and compared those results to other genotoxic biomarker (e.g. Comet Assay) (Pavlica et al., 2010; Frenzilli et al., 2008).

In these studies, which included the use of chub as sentinels, it could be shown that the spontaneous rate of micronuclei induction in erythrocytes lie in between 0.2 to 0.9‰ (Pavlica et al., 2010; Frenzilli et al., 2008; Viganò et al., 2002; Kolak et al., 1999). In this study the basal MN

frequency is only slightly higher with 0.5 to 1.2‰ and a mean of 0.73. So the spontaneous rate in this study does fit in the overall scheme. This observation is important, as the analysis of the baseline MN frequencies in different fish species shows a large interspecies variability, ranging from 0 to 13 per 1000 cells, although the large majority of papers report data ranging from 0 to 1‰ (reviewed by Bolognesi & Hayashi, 2011).

Effects of substances on the induction of MN were often found to show a trend of an induction of MN. A clear statistical significant signal is rarely found for MN induction in chub within the literature.

Pavlica et al. (2010) and Frenzilli et al. (2008) assume that this lack of significance is due to a low mutagenic level of the observed substance or low sensitivity of the test. Frenzilli et al. (2008) suggests that the interindividual variability of MN in chub erythrocytes is too high for a statistical significant answer. Effect levels or at least visible trends were concluded at frequency levels between 0.5 and 1.9‰ (Pavlica et al., 2010; Frenzilli et al., 2008; Kolak et al., 1999; Viganò et al., 2002). However, in all cited field and laboratory studies with chub, a duplication of the mean frequency of micronucleated erythrocytes in comparison to the control values was interpreted as an effect on the MN induction, even though the difference was not proven to be statistically significant. Some of this studies supported this suggestion with results of the Comet Assay, which did find statistically significant differences between the treatment groups in question (Pavlica et al., 2010; Frenzilli et al., 2008). With this in mind it is proposed that also in the treatment group of infected and exposed chub an effect on the induction of MN was observed as the mean value of micronucleated erythrocytes is 0.7 for the control group and 1.7 in the group of infected and exposed chub.

This however, leads to the questions, if the group "infected chub" does also show the same effect? Also in this group the frequency of erythrocytes with MN is highest at day 21 and values lie in between those of the other two treatment groups. A possible conclusion is, that MN induction is not only affected by Pt but that the parasitic infection also has a genotoxic effect or an effect on the spindle formation.

A review of the literature provides evidence that several parasitic groups tend to increase DNA damage or are a principle reason for inducing cancer.

Especially trematode infections were found to cause an increase of mutation frequencies (Gentile & Gentile, 1994). Some studies reveal that also other groups, like cestodes (e.g. *Taenia solium*) do induce DNA damage in lymphocytes (Vennervald & Polman, 2009).

Several different causes are assumed to be responsible for the increasing of DNA damage by these parasitic groups. For trematodes it was observed that the infection itself induces an inflammatory response. Activated macrophages or eosinophils can form reactive oxygen species which cause damage in cells of the surrounding tissues of the parasitic infection (Gentile & Gentile, 1994;

Rosin & Anwar, 1992).

Also *Pomphorhynchus* does cause an inflammatory response, as the parasite penetrates the whole intestinal wall with its horned proboscis . Especially *P. laevis* and *P. tereticollis* then use a bulbus to anchor themself into the intestinal wall. This causes a severe damage into the cells of the intestinal wall and induces an inflammatory response (Taraschewski, 2000). Still, it seems likely that those responses could affect the DNA of nearby tissues, but it is less likely to have an effect on the DNA of erythrocytes, as observed in this study.

Nonetheless, it has also been stated that cestodes and some nematode species are thought to excrete products which increase DNA damage in different cell tissues and also in lymphocytes (Huby et al., 1995; Vennervald & Polman, 2009).

If acanthocephalans do excrete genotoxic products into the intestine or into the body cavity of their host remains subject to further investigation.

The results of this study show a clear trend that acanthocephalans do increase DNA damage in erythrocytes of their hosts, even though the effect was not statistically significant. Further research is needed to clarify the possible mechanisms behind this increase of MN as well as the impact of acanthocephalans with regard to DNA damage (or spindle poisoning). It is recommended that future biomarker studies pay attention to the infection of the sentinels, as an infection with acanthocephalans could lead to false positive responses in respect to MN induction through substances.

Chapter 6

Summary and Conclusions

Summary Since the introduction of Pt as a catalytic element in industrial processes and as an agent in automobile catalytic converters, the emission of the noble metal into the environment has been rising steadily (Rauch & Hemond, 2003; Soyol-Erdene et al., 2011). Even though Pt is not considered as a direct threat for human health, the risks for ecosystems are still not entirely estimated (SRU, 2004). Therefore, the main objective of this thesis is to extend the recent knowledge of the effects Pt has on freshwater systems. After an extensive validation of the analytical methods used, a passive monitoring study at a representative discharge location of road runoff was conducted. It provided evidence about the spatial distribution of Pt in river sediments as well as the Pt accumulation in clams. Additionally, the obtained contamination levels of Pt were compared to those of other traffic related heavy metals. A further aim of the thesis was to analyze the accumulation kinetics of Pt in the biota (e.g. bivalves, fish and fish parasites) under laboratory conditions. In exposure studies with *Corbicula* sp., the clam was tested as a sentinel for Pt. In fish exposure studies, Pt accumulation kinetics for different organs were investigated, as well as the influence of acanthocephalans on the metal metabolism of the fish. Finally, the thesis focused on genotoxic effects, which might be induced by Pt. For this purpose the DNA damage was analyzed in gill cells and hemocytes of the clam as well as in erythrocytes of fish. All results obtained in this thesis can be summarized as follows:

Chapter 2: Validation of analytical procedures The aim of the first study was to validate the analytical procedures used in the thesis (i.e. HPA/ACSV, HPA/ICP-MS, MD/AAS). Therefore, the recovery, precision and the limit of detection of the different methods were investigated. According to the results the analysis procedures were classified into three different categories, considering the analyzed metal and the respective sample matrix. Category A included the analysis of Pt in biota by HPA/ACSV and MD/AAS, of Cu and Cd in sediments by HPA/ICP-MS as well as Ag, Cd, and Cr by HPA/ICP-MS. For all these metals a recovery within the 95% confidence interval

of the respective reference material was found. Furthermore, the precision of the respective analysis procedure was equal of lower than 15%. The analysis of Zn in biota by HPA/ICP-MS was classified as class A or B, depending on the reference material used for the validation. Class B included analytical procedures, which recovery rates were between 80 and 110% and provided a precision of 15% or lower (or 20% or lower, if the concentration of the metal in the reference material was near the limit of detection). The analysis of Pt for a certified reference material containing tunnel dust by HPA/ACSV resulted in a precision of 20% and was therefore classified into the category C. Like Pt, also the analysis of Ag, Cr, Ni, Pb, and Zn in sediments by HPA/ICP-MS were classified into category C. The determined recovery rates for those heavy metals were found to be between 70 to 80%. It was suggested that those low recovery rates were the result of incomplete digestions of the silicate fraction in the sediment samples. The validation of Cu, Ni, and Pb in biota by HPA/ICP-MS gave very different results according to the two reference materials used. They were included in the data analysis within this thesis, but results of Cu and Ni could be overestimated, while the results of Pb could be underestimated, due to the results obtained in the validation. The analysis of Sb did not meet any of the quality criteria defined for the listed categories and therefore, Sb was not further considered in the thesis. In general the detection limits (LODs) of the validated analytical procedures were demonstrated to be low enough in order to detect the selected metals in field samples. LODs for sediment samples were found to be lower than the geological background concentrations defined for sediment samples by Turekian & Wedepohl (1961), while the LODs for animal samples were in the same range of LODs presented in other monitoring studies.

Chapter 3: Introduction of traffic related Platinum into river systems - Occurrence and distribution of Platinum in sediments and biota The field study at the river Alb presented in Chapter 3 provided additional information on the occurency and distribution of Pt in river systems. The sediment analysis revealed that Pt was clearly introduced via road runoff. The highest Pt concentrations in sediments were analyzed in the silt/clay fraction, while the highest Pt burden was obtained for the sand fraction, due to the dominance of the sand fraction within the sediment. Pt concentrations at the different sampling points depended on the dimensions of the drained street section as well as the distance between sampling points and discharge point. Also the stream velocity played a decisive role for the distribution of Pt in the sampling transects. Pt concentrations ranged from 1 to 30 ng/g in the sand fraction and from 7 to 30 ng/g in the silt/clay fraction. In general, the Pt concentrations in sediments at sampling sites downstream of the inlets, exceeded up to 36 times those at the reference site. Strongly increased concentrations of Pt were particularly observed near the discharge points (i.e. inlets). According to the stream velocity, increased Pt levels were found close to the discharge point or within the whole 100 m of the investigated river stretch.

Concentrations found in sediments samples corresponded also to concentrations found in soil

samples near highways. However, when Pt levels were compared at different distances from the source, it was obvious that Pt is further transported in river than in terrestrial systems.

The results also confirmed, that Pt was accumulated by *Corbicula* sp. In comparison to the Pt concentration at the reference site, Pt concentrations in clams downstream of the inlets were up to 14-times higher. In two of three transects a concentration peak in clam tissues was observed approximately 20 m downstream of the road runoff discharge. The Pt concentrations in clam tissues were found to be between 0.05 to 1.3 ng/g. Due to the fact that no correlation between sediment and clam tissue concentrations could be observed, it was assumed that Pt was accumulated in soluble form or ingested with small particles from suspended particulate matter. Due to Way et al. (1990) the particle size, which can be ingested by *Corbicula* sp., is limited to 20 µm, and therefore it might be assumed that the main load of traffic related Pt in the suspended particulate matter is not available for the clam, since particles from road runoff were proved to be of a size greater than 20 µm (Sansalone & Buchberger, 1997; Tuccillo, 2006). Similar results were observed for the other traffic related heavy metals. Pt concentrations were highly correlated to those of Cr, Cu, Ni, Pb and Zn in the sand fraction and to Cd, Cu, and Zn in the silt/clay fraction. On the other hand, no significant correlation between Pt and other traffic related heavy metals in clam tissues was found. In comparison to other traffic related heavy metals, Pt burden in sediment and clam samples were low. However, the increase compared to concentrations found at the reference site was highest for Pt, followed by Cr>Cu and Pb>Zn and Ni>Ag and Cd in sediments. Also for clams the highest relative increase was be found for Pt followed by Cr>Pb>Ag>Ni>Cd>Cu. Zn was not increased at all. Overall, with regard to the sediment classification system for the quality of aquatic systems developed by LAWA (1998) it could be concluded, that the sediments of the river Alb were between increased and highly contaminated due to the discharge of traffic related heavy metals.

Chapter 4: Accumulation of different Platinum concentrations by *Corbicula* sp. and the genotoxic effects of Platinum on gill cells and hemocytes In Chapter 4 it was clearly demonstrated, that *Corbicula* sp. accumulated Pt even if the clam is exposed to very low Pt concentrations (e.g. 10 to 100 ng/L). The accumulation of Pt by the clams was dependent on the ambient Pt concentrations. This dependency was, however, not simply linear. According to the water concentrations, *Corbicula* sp. did accumulate 1 to 53% of the overall offered Pt, whereas highest BCFs were calculated for the treatment group exposed to 10 ng/L at the very beginning of the experiment. In the later phases of the experiment approximately 10%, 7%, 5% and 2% of the offered Pt were accumulated by the treatment groups exposed to 10 ng/L, 50 ng/L, 100 ng/L and 100,000 ng/L Pt, respectively. In general, values of the calculated BCFs in respect to water were high. In comparison to other studies on *Corbicula* sp., BCFs of Pt exceed the BCFs for Zn. They were similar than BCFs found for Cd, but lower than for Cu. In comparison to other bivalves used as sentinel for Pt (e.g. *Dreissena polymorpha*), the calculated BCF for *Corbicula* sp. was higher.

The results imply that *Corbicula* sp. can be used as a bioindicator species for Pt, if there are pronounced differences in Pt concentrations between the respective monitoring sites. In the case of low differences (i.e. a few ng/L), which is actually a realistic scenario for Pt under field conditions, these differences may not be reflected in the tissue concentrations of *Corbicula* sp.

Besides the survey on accumulation kinetics, the occurrence of acute toxic effects as well as the induction of DNA damages by Pt on *Corbicula* sp. were also investigated. Pt showed no influence on the mortality of the clams. Furthermore, no Pt induced increase of the natural MN induction rate was observed for gill cells and hemocytes. However, the MN frequency of the control group has already to be rated as increased. This increase could properly be induced by an illness or a hidden substance in the water. The results are in contrast to other studies performed with mollusks. For example, Osterauer et al. (2011) found DNA damage in embryonic cells of *Marisa cornuarietis*. These differences in the findings may be attributed to different factors: (i) Gastropods react differently to Pt than bivalves; (ii) *Corbicula* sp. is not sensitive enough to detect the genotoxic potential of Pt, (iii) the induced MN frequency of the control group does hide an existing genotoxic effect of Pt, or (iv) the DNA damage detected in *Marisa cornuarietis* by the Comet Assay was in the form of a simple damage, which can be repaired by the cells repair systems. Those damages pose no threat to animals and they are not detected by the micronucleus test.

Chapter 5: The accumulation of Platinum by *Squalius cephalus* and *Pomphorhynchus* sp. and the genotoxic effect of Platinum on fish erythrocytes The accumulation kinetics of Pt was tested for fish and their intestinal parasites in the fourth study of this thesis. The results revealed that Pt was well accumulated by *Squalius cephalus*. In all tissues (i.e. muscle, liver and intestinal tissues), the steady state situations were reached on day 13 after the exposure start. The highest concentrations among fish tissues were found in the liver and intestinal samples. They were approximately ten times higher than those found in muscle tissues. Next to the fish tissues, Pt concentrations were also analyzed in parasite samples. Prior to exposure, fish were infected with two different parasite species of the genus *Pomphorhynchus* (i.e. *P. terreticollis* and *P. laevis*). *P. terreticollis* accumulated Pt in concentrations 3-, 5- and 40-fold higher than those found in liver, intestine and muscle tissues of its host, respectively. As for the fish, also for the parasite a steady state condition was found. Pt accumulation of *P. laevis*, however, was different. While Pt concentrations were found to be higher in the parasite than in the fish host at the beginning of the experiment, at the end of the exposure study, Pt concentrations in *P. laevis* were the same than in liver and intestinal tissues.

Furthermore, Pt concentrations in liver and intestine were found to be lower in fish infected with *P. tereticollis* compared to Pt concentrations in the respective organs of uninfected ones. This trend was not observed for fish infected with *P. laevis*. Those findings reveal that parasites do manipulate the Pt burden in their hosts. In the case of fish infected with *P. tereticollis*, Pt concentrations in field

conditions might be underestimated if only the Pt concentrations in fish tissues are analyzed and considered. However, such manipulation of element concentrations in the fish tissues depends on the host-parasite system. To get comparable results in fish biomonitoring studies which include different sampling sites, it is important to analyze the same fish species infected with the same parasite species at all sampling sites.

The results of this study shed light on another topic. The degree of relationship of the two acanthocephalans is still under discussion. While Amin et al. (2003) could not find any morphological differences for the adult worms, they still name both acanthocephalans *P. laevis*. Genetic analysis as well as the investigation of larval morphology and larval behavior led to the assumption that those parasites are distinctive species (Perrot-Minnot, 2004). In addition, differences for adult worms could be observed in this study. The two parasites had different infection success in the same host, they accumulated Pt to a different degree and one of the parasites did manipulate the metal metabolism of the host, while the other did not. Therefore, this study supports the hypothesis, that *P. laevis* and *P. terreticolis* are two distinct species .

Similar to the study with clams, Pt induced DNA damage was also investigated for fish. In this case, however, the analysis was focused on erythrocytes. In contrast to clams, a significant MN induction was detected on day 21 after exposure start. This MN induction was statistically significant for uninfected fish exposed to Pt. However, for two more treatment groups a trend of MN induction was observed: infected fish exposed to Pt and the non exposed, but infected treatment group. These findings reveal that Pt does induce DNA damage in fish erythrocytes and they raise the question, if an acanthocephalan infection could possibly induce the rate of MNs.

Conclusions In general it can be concluded, that traffic related Pt enters river systems. Its concentration ranges in sediment and biota samples are lower compared to other heavy metals. The relative increase of Pt concentrations, however, is higher than that of other heavy metals. The main Pt burden on sediments can be limited to river sections of approximately 40 m in length after each inlet. However, for the evaluation of the impact on an entire river ecosystem, it should be considered that there can be several point sources in form of inlets stretched along the course of the river. In addition, road runoff does not only enter the river via these inlets but there are commonly also diffuse sources such as road runoff water, that infiltrates through embankments and grass surfaces into the river. Generally, the number of discharge points and the volume of road runoff entering rivers is still unknown. Furthermore, the total discharge of Pt is still expected to increase in the future. According to Johnson Matthey (2011) the Pt demand for automobile catalyst converters is still rising, even though it is already substituted by Pd in gasoline vehicles as well as some diesel vehicles. Still, the urge to reduce CO_2 emissions forces manufacturers to search for new technologies and Johnson Matthey (2011) predicts a new demand of Pt for lean burn gasoline vehicles with lean NOx traps containing Pt. For Europe it can currently be assumed that with increasing traffic volume the pollution with Pt will increase for aquatic ecosystems.

It can be assumed that the main exposure scenarios for mussels and fish in rivers are short term exposure conditions. Soluble Pt species as well as suspended particulate matter enter the river system during precipitation events and are then directly transported downstream. Higher exposure concentrations and higher Pt uptake can therefore be assumed to be restricted to organism feeding on detritus and/or sediments (as was already shown for *Asellus aquaticus* by Rauch & Morrison (1999); Moldovan et al. (2001) and Haus et al. (2007b)).

This thesis demonstrated that, due to the relatively low exposure scenarios in the field, acute lethal or toxic effects of Pt can be ruled out for bivalves and fish. Additionally, Pt concentrations are (still) too low for many of the sublethal effects, observed in laboratory studies. However, Pt can induce DNA damage, as was shown for chub. Therefore, also low Pt concentrations in the field could possibly endanger individual organisms. It can be assumed that Pt does not pose a major threat to river ecosystems, however, it is certainly another stress factor for aquatic organism.

Research prospects Based on the findings of this thesis further research should focus on the following aspects:

- This thesis has shown that due to the mobility of Pt in river systems, mussels and fish are only subject to short term exposures. It is assumed that this will be different in lentic water bodies, like lakes or backwaters. In general, road runoff is not discharged directly into lakes, however, diffuse discharges from parking lots or nearby streets could accidentally enter the water bodies. Until now, the effect of Pt on aquatic organism in lake systems has not been investigated.
- Pt was found to induce DNA damage, but the exposure concentrations used in the experiment was higher than known Pt concentrations in the field. New experiments with lower Pt concentrations could help to better estimate the risk of possible DNA damage in the environment.
- Beside Pt, also the infection with acanthocephalans increased the number of micronuclei counted in fish erythrocytes. This should be subject to further investigation. It should be tested whether the infection with parasites does directly induce DNA damage (e.g. due to substances released by the parasite) or indirectly (e.g. through inflammatory reactions in fish which result in DNA damage).
- It was confirmed, that some acanthocephalan species reduce the metal concentrations in some host tissues. However, one aim of the thesis was to test whether toxicological effects caused by metal poisoning (i.e. the induction of MN) decrease accordingly. Due to the interference of acanthocephalans to the MN induction, this question remains unanswered. Further research is needed to distinguish between the effects of the parasite infection itself and the effects induced by metal poisoning.

Chapter 7

Zusammenfassung

Seit den 70er Jahren sind der Schutz der Fließgewässer und die Verbesserung des chemischen Zustandes Ziele der Umweltpolitik (Bartel et al., 2010). Durch das Inkrafttreten gesetzlicher Regelungen wurde die Verschmutzung der Gewässer durch Schwermetalle aus der Industrie drastisch reduziert. Insbesondere der Bau effektiver Kläranlagen und die Modernisierung industrieller Prozesse führten zu einer maßgeblichen Verbesserung der Wasserqualität europäischer Fließgewässer (Luoma & Rainbow, 2008). Die größten Reduktionen wurden für die Schwermetalle Quecksilber (99%), Blei (89%) und Nickel (47%) erzielt. Dennoch sind deutsche Flüsse heutzutage immer noch durch Schwermetalle beeinträchtigt. Regelmäßige Überwachungsprogramme zeigen, dass nur 23% der deutschen Fließgewässer Schwermetallgehalte in Sedimenten und Schwebstoffen aufweisen, die den europäischen Umweltqualitätszielen entsprechen (Arle et al., 2010). Diese Verschmutzung ist weniger auf industrielle Einleitungen zurückzuführen, sondern dem Eintrag diffuser Schwermetallquellen zuzuschreiben (Davis et al., 2001; Böhm et al., 2001; Scherer et al., 2003). Mit der Einführung neuer Technologien ändert sich regelmäßig die Komposition solcher Abflüsse. Diese Änderungen werden unter der jetzigen Umweltgesetzlage nicht berücksichtigt, da nur ausgewählte Schwermetalle einer Überwachungspflicht unterliegen. Negative Auswirkungen durch sich verändernde Schwermetalleinträge können somit erst spät entdeckt, und gesetzliche Reglementierungen noch später installiert werden. Beispiele für den in jüngerer Zeit verstärkten Eintrag von Schwermetallen, die nicht überwacht und deren Auswirkungen auf die Umwelt somit nicht eingeschätzt werden können, sind z.B. die Einträge von Platingruppenelemente und metallischer Nanopartikel.

Platin (Pt) stellt neben Iridium (Ir), Osmium (Os), Palladium (Pd), Ruthenium (Ru) und Rhodium (Rh) eines von sechs Platingruppenelementen (PGE) dar. PGE zählen zu den seltensten Schwermetallen der Erde, da ihr Anteil an der oberen Erdkruste nur 10^{-6} bis 10^{-7}% beträgt (Hoppstock & Sures, 2004). Dank ihrer sehr speziellen Eigenschaften, (z.B. ihre Korrosionsbeständigkeit und hohe Schmelzpunkte), ist die Nachfrage seitens der Industrie für PGE hoch. Der globale Bedarf,

insbesondere an Pt, Pd und Rh steigt stetig aufgrund ihres Einsatzes in der organometallischen Chemie, in der Oberflächen-, Material und Kristallherstellung, in der Foto- und Elektrochemie, in der Katalyse und organischen Synthese (Hoppstock & Sures, 2004). Der vermehrte Einsatz der PGE zog erhöhte PGE Emissionen nach sich, die bereits in mehreren Reviews dokumentiert wurde (Ek et al., 2004; Hoppstock & Sures, 2004; Ravindra et al., 2004; Zimmermann & Sures, 2004; Rauch & Morrison, 2008). Die Verbreitung von Pt in aquatischen Ökosystemen, sowie die Effekte auf Organismen wurden bisher nicht hinreichend untersucht.

Die Quellen von Pt für aquatische Ökosysteme sind vielfältig. Je 30% der jährlichen Pt Produktion werden von für industrielle Prozesse, für die Schmuckindustrie sowie für Autoabgaskatalysatoren gekauft (Johnson Matthey, 2011). In Autoabgaskatalysatoren für Dieselfahrzeuge (und zu einem kleineren Teil auch in Katalysatoren für Fahrzeuge mit Benzinmotoren), ist Pt das katalytische Element (Johnson Matthey, 2011). Pt katalysiert die Oxidation von Stickoxiden, Kohlenwasserstoffen und Kohlenmonoxid, um deren Emission zu verringern. Pt wird allerdings selber mit den Abgasen emittiert. Durch mechanische Abrasion wird es vom Katalysator gelöst und gelangt an Aluminiumoxidpartikeln gebunden in die Umwelt (Artelt et al., 1999). Die Emissionsraten eines einzelnen Fahrzeuges belaufen sich nach Studien von Artelt et al. (1999) auf einige wenige ng/km bis maximal 100 ng/km. Neben den gut kontrollier- und regelbaren Einträgen über industrielle Punktquellen, gelangt Pt also auch über Straßenabflusswässer in Fließgewässersysteme (Flemming et al., 2004). Ähnlich zu anderen verkehrsbürtigen Schwermetallen gelangt Pt über verschiedene Wege in Fließgewässer. So kann es über die Atmosphäre eingetragen werden, oder es wird mit dem Straßenabflusswasser von der Straße gespült. Insbesondere außerhalb von städtischen Gebieten wird dieses Wasser nicht der allgemeinen Kanalisation und somit den Kläranlagen zugeführt, sondern dezentral behandelt (Uhl et al., 2006). Dort kann es vor Ort straßennah versickert werden, oder wird nach einer technischen Behandlung in einen Vorfluter geleitet (Ceko & Waltz, 2011).

Die Auswirkungen von Pt auf aquatische Organismen sind bisher unzureichend untersucht. Es gibt vereinzelte Laborstudien, die allerdings mit vergleichsweise hohen Pt Konzentrationen durchgeführt wurden. Diese geben Hinweise darauf, dass die akute Toxizität von Pt weit unter der von Cd, Cr, Hg und Pb liegt (Borgmann et al., 2005). Als sublethale Effekte wurden histopathologische Veränderungen der Leber und des Darmgewebes von Fischen, sowie des Hepatopankreas, der Kiemen- und epidermalen Gewebes bei Schnecken beobachtet (Jouhaud et al., 1999a,b; Osterauer et al., 2010a). In Embryotests mit dem Zebrabärbling *Danio rerio* und der Paradiesschnecke *Marisa cornuarietis* führte die Exposition mit Pt zu verminderten Schlupfraten bei beiden Organismen und zu Missbildungen bei *Marisa cornuarietis* (Osterauer et al., 2009, 2010b). Daphnien reagierten mit verlangsamten Wachstumsraten und einer geringeren Produktion der Glutamatoxalacetat-Transaminase auf eine Pt Exposition (Biesinger & Christensen, 1972). Singer et al. (2005) konnte nachweisen, dass die Zebramuschel *Dreissena polymorpha* mit einer erhöhten Induktion der Hitzeschockproteine reagiert. Des Weiteren wird ein gentoxischer Effekt durch Pt diskutiert. In Zell-

versuchen konnten Bünger et al. (1996); Gebel et al. (1997) und Migliore et al. (2002) gentoxische Effekte für unterschiedliche chemische Pt-Speziationen nachweisen. Osterauer et al. (2011) fand Pt induzierte DNA Schäden bei Invertebraten (Schnecken), jedoch nicht bei Wirbeltieren (Fische).

Auch der Rat von Sachverständigen für Umweltfragen der Bundesrepublik Deutschland stellte in seinem vorletzten Umweltreport 2004 fest, dass der Kenntnissstand über die Schadstoffbelastung durch Pt ungenügend ist. Er empfiehlt, dass die Pt Immission zukünftig an repräsentativen Stellen in der Umwelt beobachtet werden sollte und die Pt Akkumulation in Muscheln und Fischen weiter untersucht werden sollte (SRU, 2004).

Das Ziel dieser Arbeit war es daher, den Eintrag von Pt in Fließgewässer durch den Verkehr genauer zu untersuchen. Dabei soll das Wissen über das Vorkommen, die Verteilung und die Effekte von Pt auf aquatische Ökosysteme erweitert werden, um den Einfluss von Pt als Schadstoff und sein toxikologisches Potenzial in Gewässern besser zu beurteilen.

Um diese Ziele zu erreichen, wurden vier verschiedene Studien durchgeführt. Zunächst wurden die analytischen Methoden, mit denen Pt und andere verkehrsbürtige Schwermetalle in dieser Arbeit untersucht wurden, einer ausführlichen Validierung unterzogen und die analytische Qualität der unterschiedlichen Verbundverfahren im Folgenden in drei Klassen eingeteilt (Kapitel 2). In einer zweiten Studie wurde der Frage nachgegangen, wie sich Pt an einer Einleitungsstelle im Gewässer verteilt. Es wurde untersucht in welcher Sedimentfraktion Pt akkumuliert, wie weit ist der Eintrag im Sediment nachweisbar ist und wie hoch die Pt Konzentrationen im Sediment und in aquatischen Organismen an einer Einleitungsstelle sind. Die für Pt erhobenen Daten wurden des Weiteren mit den Daten anderer verkehrsbürtiger Schwermetalle verglichen. In zwei weiteren Studien wurde die Aufnahme von Pt durch Muscheln, Fische und Fischparasiten untersucht. Dazu wurde die Körbchenmuschel *Corbicula* sp. verschiedenen umweltrelevanten Pt Konzentrationen ausgesetzt. Es wurde beobachtet, wie viel der dotierten Platinmasse im Weichgewebe wiedergefunden werden konnte und ob die Gewebekonzentrationen die Außenkonzentrationen widerspiegeln. Zusätzlich wurde ein Mikrokerntest (MN Test) durchgeführt, der Aufschluss über die Gentoxizität von Pt auf die Kiemenzellen und Hämozyten geben sollte. In der letzten Studie dieser Arbeit, wurde die Aufnahmekinetik von Pt in Fischen und Fischparasiten untersucht. Untersucht wurde die Pt-Kinetik in Leber-, Muskel-, und Darmgeweben über einen Versuchszeitraum von 35 Tagen. Zusätzlich wurde die Aufnahme von Pt durch die Parasiten (*Pomphorhynchus laevis* und *P. terreticolis*) untersucht. Der Versuchsansatz der parasitären Infektion wurde gewählt, da diese Parasitengruppe dafür bekannt ist, die Aufnahme einiger Schwermetalle durch den Wirt zu verringern. Der Versuch sollte zeigen, ob ähnliche Effekte auch für eine Pt-Aufnahme zu erwarten sind. Die Fische wurden ebenfalls einem Mikrokerntest unterzogen. Für die Fische wurde die Mikrokerninduktion in den Erythrozyten ausgewertet. Neben der generellen Frage, ob Pt Mikrokerne induziert, sollte untersucht werden, ob eine eventuell vorhandene Metallreduktion in den Geweben des Fisches durch die Parasiten, auch zu einer Verringerung des gentoxischen Effektes führt.

Die Ergebnisse der Studien, werden im Folgenden pro Kapitel zusammengefasst:

Kapitel 2: Validierung der analytischen Verbundmethoden Durch die Charakteristika der Proben aus den Studien dieser Arbeit ergab sich ein differenziertes Anforderungsprofil an die analytischen Methoden. Es lagen zum Einen unterschiedliche Probenmatrices in Form von abiotischen Sedimentproben und biotischem Gewebe vor. Hinzukam, dass diese unterschiedlichen Proben-Matrices Metallgehalte in verschiedenen Konzentrationsbereichen aufwiesen. Die Sediment- und Muschelproben aus dem Freiland wiesen die geringsten Konzentrationen auf und mussten parallel auf Pt und andere verkehrsbürtige Schwermetalle (Ag, Cd, Cr, Cu, Ni, Pb, Sb und Zn) hin analysiert werden. Aus den Expositionsstudien wurde Probenmaterial mit sehr geringen Pt-Konzentrationen (niedrig exponierte Muschelproben, die unterschiedlichen Fischgewebe, sowie die Parasitenproben aus der Fischexposition), als auch Proben mit relativ hohen Pt Konzentrationen (Muschelgewebe aus der Exposition mit 100 µg/L) gewonnen. Proben mit niedrigen Konzentrationen wurden mit einem Hochdruckaufschlussverfahren (HPA) und einer Säurekombination aus HNO_3 und HCl aufgeschlossen und im Anschluss mit der adsorptiven Voltammetrie (ACSV) auf Pt hin analysiert. Proben aus dem Freiland wurden zudem noch einer Detektion mittels ICP-MS unterzogen, um die weiteren verkehrsbürtigen Schwermetalle zu analysieren. Muschelproben mit Pt-Gehalten im µg/g Bereich, wurden einem Mikrowellenaufschluss nach Sures et al. (1995) unterzogen. Im Anschluss wurden die Pt Gehalte der Proben mittels der elektrothermalen adsorptiven atomaren Spektrometrie (ET-AAS) bestimmt. Zunächst wurden für zwei Verbundverfahren (HPA/ACSV, HPA/ICP-MS) jeweils für abiotische und biotische Proben Wiederfindungsraten, die Präzision des Verfahrens sowie die Nachweis- und Bestimmungsgrenze festgelegt. Für das Verfahren MD/AAS lagen Daten für die Wiederfindungsrate und die Präzision bereits vor (Zimmermann et al., 2003), so dass nur noch die Nachweis- und Bestimmungsgrenze bestimmt wurde. Anhand der erhobenen Daten wurde die analytische Qualität der Verbundverfahren für die einzelnen Schwermetalle in drei Klassen eingeteilt. Klasse A beinhaltete die Analyse von Pt in biologischen Proben mittels HPA/ASCV und MD/AAS. Auch die Analyse von Cu und Cd in Sedimenten und Ag, Cd und Cr in biologischen Proben mittels HPA/ICP-MS wurden mit A bewertet. Die Analyse dieser Elemente zeichnete sich dadurch aus, dass eine Analyse von Referenzmaterial in Wiederfindungsraten resultierte, die den zertifizierten Referenzbereich (Mittelwert $\pm$ 95% Konfidenzintervall) überschneidet. Des Weiteren war die Präzision dieser Verbundverfahren gleich oder kleiner 15% (im Fall von Probengehalten nahe der Nachweisgrenze, kann die Präzision auch bei 20% oder kleiner liegen). Die Analyse von Zn in biologischen Proben mittels HPA/ACSV wurde, je nach verwendetem Referenzmaterial, in die Qualitätsklasse A oder B eingeordnet. Klasse B umfasst alle Verbundverfahren, deren Wiederfindungsrate zwischen 80 und 110% liegt, bei einer Präzision von 15% oder kleiner (im Fall von Probengehalten nahe der Nachweisgrenze, kann die Präzision auch bei 20% oder kleiner liegen). Die Analyse von Pt in einem referenzierten Tunnelstaubstandard (HPA/ACSV) ergab zwar eine Wiederfindungsrate von 88%, allerdings war die Präzision mit 20% zu hoch, um das analytische Verfahren in die Klasse B einzuordnen. Auch

die Analyse von Ag, Cr, Ni, Pb und Zn in Sedimenten mittels HPA/ICP-MS konnte nur in die Kategorie C eingeordnet werden. Die Wiederfindungsraten lagen zwischen 70 und 80%. Es wird vermutet, dass diese relativ geringen Wiederfindungsraten auf einen unvollständigen Aufschluss der Silikate in den Sedimenten zurückzuführen ist. Die Ergebnisse der Validierung für Cr, Ni und Pb in biologischen Proben mittels HPA/ICP-MS waren je nach Referenzmaterial sehr unterschiedlich. Auf eine genaue Klassifizierung musste aufgrund der unterschiedlichen Ergebnisse verzichtet werden. Bei der Datenanalyse dieser Metalle sollte beachtet werden, dass die Werte für Cr und Ni eventuell überhöht, sowie die Werte von Pb unterschätzt werden könnten. Die Analyse von Sb konnte keine der Qualitätskriterien der drei Klassen erfüllen und wurde von daher aus der Datenanalyse der verschiedenen Studien ausgeschlossen. Die Nachweisgrenzen aller Verbundverfahren waren niedrig genug, um auch eine Analyse von Umweltproben sicherzustellen. Die Nachweisgrenze für Sedimentproben zeigte, dass diese unterhalb des geogenen Hintergrundwertes für die zu analysierenden Schwermetalle liegt (Turekian & Wedepohl, 1961). Für die Nachweisgrenzen der biologischen Proben konnten ähnliche Nachweisgrenzen erreicht werden, die auch für andere Monitoringstudien angegeben werden (Haus et al., 2007b; Nachev et al., 2010).

Kapitel 3 Verkehrsbürtiges Platin in Flüssen - Vorkommen und Verteilung von Platin in Sedimenten und Biota Die zweite Studie dieser Arbeit beschäftigte sich mit dem Vorkommen und der Verteilung von Platin in Fließgewässern. Als Probestelle wurde ein Flussabschnitt der Alb in der Nähe von Karlsruhe gewählt. Dieser Abschnitt wird von einer Brücke gekreuzt, die die Bundesstraße 10 trägt. Das Straßenabflusswasser der B10 wird über drei verschiedene Strecken gesammelt und über drei Einleitungsstellen dem Fluss zugeführt. Für den Flussabschnitt wurden 14 Probepunkte ausgewählt. Einer der Punkte diente als Referenzstelle und lag ca. 20 m flussaufwärts der ersten Einleitung. Nach jeder Einleitung wurde jeweils ein Transekt gelegt, welches Probepunkte bis zu 100 m flussabwärts der Einleitung beinhaltete. An jedem dieser Probepunkte wurden Sediment- und Muschelproben genommen. Dies war möglich, da die Körbchenmuschel *Corbicula* sp. gleichmäßig und in sehr hoher Dichte im Sediment aller Transekte verteilt war. Für alle Proben wurden die Konzentrationen von Ag, Cd, Cr, Cu, Ni, Pb, Pt und Zn mit den oben beschriebenen Methoden analysiert. Die Ergebnisse der Studie zeigen deutlich, dass Pt mit dem Straßenabfluss in das Gewässer gelangt. Die höchsten Pt Konzentrationen wurden in der Ton/Schluff Fraktion nachgewiesen, während die Hauptmasse des Platins, aufgrund des niedrigen Ton/Schluffgehalts im Gesamtsediment, eher in der Sandfraktion gebunden war. Die Pt-Konzentration in den Sedimentproben war abhängig von der Fläche des entwässerten Straßenabschnitts, der Distanz zwischen Einleitungs- und Probestelle und der Fließgeschwindigkeit des Wassers im Gewässerabschnitt. Die Pt-Konzentrationen lagen zwischen 1 und 30 ng/g in der Sand- und zwischen 7 und 30 ng/g in der Ton/Schluff Fraktion. Unterhalb der Einleitungsstellen erreichten sie das 36-fache der Konzentration an der Referenzstelle oberhalb der Einleitung. Die höchsten Konzentrationen wurden in direkter Nähe zu der Einleitungsstelle gemessen (0 bis 3 m Entfernung). Abhängig von der Fließgeschwindigkeit in den Transekten konnten erhöhte Pt

Konzentrationen entweder nur in direkter Nähe der Transekte oder noch in 100 m Entfernung nachgewiesen werden.

Im Vergleich zu Studien, die für terrestrische Systeme durchgeführt wurden, konnten hohe Übereinstimmungen gefunden werden. Die Pt-Konzentrationen in den Sedimenten dieser Studie glichen denen von Bodenproben, die direkt neben einer Autobahn entnommen wurden (Wichmann et al. (2007); Singer (2008)). Auch hier wurden die höchsten Konzentrationen in der Nähe der Metallquelle beobachtet und eine exponentielle Verminderung der Pt Gehalte mit der Entfernung zur Quelle festgestellt. Allerdings ist die Konzentrationsabnahme in Böden mit steigender Entfernung zur Quelle höher als in Sedimenten.

Die Ergebnisse der Studie zeigen außerdem, dass Pt durch die Körbchenmuschel im Freiland aufgenommen wird. Im Vergleich zu den Pt-Konzentrationen an der Referenzstelle, wurden an den Probestellen unterhalb der Einleitungen bis zu 14-fach höhere Konzentrationen im Muschelgewebe nachgewiesen. In zwei von drei Transekten konnte ein Konzentrationspeak 20 m unterhalb der Einleitungsstelle gefunden werden. Insgesamt lagen die Konzentrationen zwischen der Nachweisgrenze (0.05) und 1.3 ng/g. Zwischen den Pt-Konzentrationen im Sediment und den Muschelproben wurde keine Korrelation festgestellt. Von daher kann davon ausgegangen werden, dass die Muschel Pt über gelöste Spezies und/oder Schwebstoffe aufnimmt. Allerdings kann die Körbchenmuschel laut Way et al. (1990) Partikel nur bis zu einer Größe von ca. 20 µm über die Kiemen aufnehmen. Da Partikel aus Straßenabflusswässern hauptsächlich in größeren Korngrößen vorkommen (Sansalone & Buchberger, 1997; Tuccillo, 2006), kann vermutet werden, dass die Hauptlast des Pts in Schwebstaub nicht für die Muschel verfügbar sein wird.

In einem weiteren Schritt wurden die Pt Konzentrationen in Sediment- und Muschelproben zu denen anderer verkehrsbürtiger Schwermetalle in Relation gesetzt. Dabei fiel auf, dass Pt sehr stark und statistisch hochsignifikant mit Cr, Cu, Ni, Pb und Zn in der Sandfraktion und mit Cd, Cu, Ni und Zn in der Ton/Schlufffraktion korrelierte. In den Muscheln hingegen fanden sich keinerlei Korrelationen zwischen Pt und anderen verkehrsbürtigen Metallen. Im Vergleich zu den anderen verkehrsbürtigen Metallen ist die Pt-Konzentration sowohl im Sediment als auch in den Muscheln als gering einzuschätzen. Allerdings ist die relative Zunahme im Vergleich zur Referenzstelle für Pt sowohl in den Sedimenten als auch in den Muscheln am höchsten (Sediment: Pt >Cr>Cu und Pb >Zn und Ni >Ag und Cd; Muscheln: Pt >Cr >Pb >Ag >Ni >Cd >Cu). Für Zn konnte im Vergleich zur Referenzstelle keinerlei Anreicherung in den Muschelgeweben festgestellt werden, was auf die hohe Regulierung des Metalls durch die Körbchenmuschel zurückgeführt werden kann. Insgesamt kann nach dem Klassifikationssystem der LAWA (1998) die Probestelle als erhöht belastet bis hoch belastet eingestuft werden, während die Referenzstelle nur als deutlich belastet eingestuft wurde.

Kapitel 4: Die Platinaufnahme der Körbchenmuschel unter verschiedenen Konzentrationsbedingungen und der gentoxische Effekt von Platin auf Kiemenzellen und Hämozyten In einer Expositionsstudie wurde die Körbchenmuschel *Corbicula* sp. über 10 Wochen unterschiedlichen Platinkonzentrationen ausgesetzt. Neben einer Kontrollgruppe, wurden Versuchsgruppen mit einer Expositionskonzentration von 10, 50, 100 und 100,000 ng/L gehältert. Über den gesamten Versuchszeitraum wurde alle 10 Tage eine Probe genommen, um die Kinetik der Platinaufnahme beobachten zu können. An den Versuchstagen 10, 49 und 56 wurden zudem Kiemenzellen und Hämozyten aller Versuchsgruppen auf Mikrokerne untersucht. Die Ergebnisse zeigen, dass *Corbicula* sp. Pt in allen angebotenen Konzentrationen aufnimmt. Dabei war die Höhe der Aufnahme abhängig von der Höhe der Expositionskonzentration. Dieser Zusammenhang konnte allerdings nicht mit einer einfachen linearen Regression beschrieben werden. Abhängig von der Konzentration von Pt im Wasser, nahm *Corbicula* sp. zwischen 1% und 53% der im System vorliegenden Pt-Masse auf. Die höchsten Biokonzentrationsfaktoren wurden in den ersten Tagen des Versuchs für die Versuchgruppe errechnet, die der geringsten Pt-Konzentration von 10 ng/L ausgesetzt war. In der späteren Phase des Versuches reduzierte sich der Anteil des aufgenommenen Pts auf 10%, 7%, 5% und 2% jeweils für die Versuchsgruppen, die 10, 50, 100 und 100000 ng/L ausgesetzt waren. Über den gesamten Versuchzeitraum gesehen, waren die Biokonzentrationsfaktoren hoch. In anderen Akkumulationsstudien mit der Körbchenmuschel wurden geringere Biokonzentrationsfaktoren für Zn errechnet, ähnlich hohe für Cd und höhere für Cu. Im Vergleich mit anderen Bioindikatororganismen sind die Biokonzentrationen der Körbchenmuschel für Pt höher als die, die für die Zebramuschel *Dreissena polymorpha* ermittelt wurden. Es ließ sich schlussfolgern, dass *Corbicula* sp. als Bioindikatororganismus für Pt geeignet ist, wenn zwischen den untersuchten Gewässern (oder Zeitpunkten) ausgiebige Konzentrationsunterschiede vorhanden sind. Sollten sich die Unterschiede in der Pt Konzentration im Wasser oder Schwebstaub nur im Rahmen von wenigen ng/L bewegen, werden diese Unterschiede nicht in der Gewebekonzentration der Körbchenmuschel reflektiert.

Während des Versuches konnte keine Pt induzierte Mortalität festgestellt werden, auch wenn die Mortalitätsrate insgesamt (auch in der Kontrollgruppe) mit bis zu 35% sehr hoch war. Des Weiteren konnte gegenüber der Kontrollgruppe auch kein Anstieg der Mikrokernfrequenz und somit auch kein gentoxischer Effekt von Pt auf die Muschel nachgewiesen werden. Allerdings muss bereits die Mikrokernbildung in der Kontrollgruppe als erhöht angesehen werden. Dies könnte die Folge einer Krankheit sein, die die Muscheln aus dem Feld mitgebracht haben oder die Folgen einer anderen, im Hälterungswasser vorhandenen Substanz. Diese Ergebnisse stehen im Kontrast zu den Ergebnissen von Osterauer et al. (2011). In dieser Studie wurde ein gentoxischer Effekt von Pt auf Schnecken nachgewiesen. Die Gründe für die unterschiedlichen Ergebnisse können vielfältig sein: (i) Gastropoden reagieren anders auf Pt als Bivalvia; (ii) die Körbchenmuschel ist nicht sensitiv genug um einen solchen Effekt nachzuweisen; (iii) die durch andere Substanzen oder eine Krankheit hervorgerufene Mikrokerninduktion hat einen gentoxischen Effekt von Pt nicht sichtbar werden lassen, oder (iv) die von Osterauer et al. gefundenen DNA-Schäden sind reparabel. Solche

Schäden können durch den von Osterauer et al. benutzten Comet Assay entdeckt werden, während der Mikrokerntest nur irreparable DNA Schäden detektiert.

Die Akkumulation von Pt durch *Squalius cephalus* und *Pomphorhynchus* sp. und der gentoxische Effekt von Platin auf Fischerythrozyten Die Akkumulation von Pt durch den Döbel *Squalius cephalus* wurde in der vierten Studie genauer untersucht. Fünf unterschiedliche Testgruppen wurden in diesem Versuch betrachtet. Dabei wurden jeweils zwei Gruppen entweder mit dem Darmkratzer der Art *Pomphorhynchus laevis* oder *Pomphorhynchus terreticolis* vor Versuchsbeginn infiziert. Drei Gruppen (eine uninfizierte, eine mit *P. laevis* infizierte und eine mit *P. terreticolis* infizierte) wurden dann mit jeweils 100 µg/L Pt exponiert, die verbleibenden drei Gruppen (gleiche Zusammensetzung wie oben) dienten als Kontrollgruppen. Der Versuch dauerte 35 Tage, wöchentlich wurden aus jeder Gruppe 4 bis 5 Fische entnommen. Diese wurden seziert und Muskel, Darm, Leber und Parasitenproben wurden auf Pt hin untersucht. Allen Fischen wurden zudem Blutproben entnommen, die auf Mikrokerne in den Erythrozyten untersucht wurden. Dazu wurden pro Probezeitpunkt und Versuchsgruppe 10000 Erythrozyten untersucht.

In dem Versuch konnte eindeutig die Aufnahme von Pt sowohl durch den Fisch als auch dessen Parasiten gezeigt werden. In allen Fischgeweben wurde ein Fließgleichgewichtszustand (Steady-State-Situation) nach ca. 13 Tagen Exposition erreicht. Die höchsten Konzentrationen im Fisch wurden in der Leber und im Darmgewebe nachgewiesen. Diese waren ca. 10-fach höher als die Pt Konzentrationen im Muskelgewebe. Noch höhere Konzentrationen wurden allerdings in den Parasitenarten nachgewiesen. Ähnlich wie in Fischgeweben, wurde der Fließgleichgewichtszustand in *P. terreticolis* erreicht. Die Konzentrationen lagen jedoch 3-, 5-, und 40-fach höher als in den Leber, Darm und Muskelgeweben des Wirtes. Andere Ergebnisse zeigten die Versuche mit *P. laevis*. Zu Beginn des Versuches konnten auch in dieser Parasitenart höhere Konzentrationen als in den Fischparasiten gefunden werden. Nach 28 Tagen waren die Pt Konzentrationen in *P. laevis* jedoch genauso hoch wie in den Leber und Darmgeweben des Fisches.

Pt Konzentrationen in Leber und Darmgeweben von Fischen, die mit *P. terreticolis* infiziert waren, waren niedriger als die nicht infizierter Fische. Dieser Trend konnte auch schon für andere Schwermetalle in anderen Wirt-Parasit-Systemen festgestellt worden (siehe auch Sures (2008)). Für Fische, die mit *P. laevis* infiziert waren, konnte dieser Trend allerdings nicht bestätigt werden. Bei diesen Fischen wurden keine Unterschiede im Metallgehalt der Gewebe zu denen der nicht infizierten Fische gefunden. Diese Ergebnisse zeigen, dass ein Schwermetallmonitoring mit Fischen nur unter bestimmten Bedingungen durchzuführen ist. So können Schwermetallkonzentrationen im Gewässer unterschätzt werden, wenn Fische mit Darmparasiten infiziert sind. Dies gilt, wie man auch an den Ergebnissen dieser Arbeit sieht, nicht für alle Wirt-Parasit-Systeme. Um verschiedene Gewässer miteinander vergleichen zu können, sollte darauf geachtet werden an allen Probestellen diesselben Fischarten mit denselben Acanthocephalen Infektionen als Bioindikatororganismen heranzuziehen.

Auch aus einem anderen wissenschaftlichen Blickwinkel sind die Ergebnisse dieser Studie interessant. So wird immer noch der Verwandtschaftsgrad der beiden hier genutzten Acanthocephalen diskutiert. Während Amin et al. (2003) aufgrund von fehlenden morphologischen Unterschieden der adulten Kratzer *P. terreticolis* als ein Synonym für *P. laevis* ansieht, weisen genetische Untersuchungen, sowie die Larven Morphologie und von Larven herbeigeführte Verhaltensveränderungen der Zwischenwirte darauf hin, dass es sich um zwei getrennte Arten handelt (Perrot-Minnot, 2004). In dieser Studie zeigten die beiden Acanthocephalen sowohl Unterschiede in der Infektionsrate, in der Metallakkumulation und in der Manipulation des Wirtsmetallmetabolismus. Dies sind die ersten Unterschiede, die auch für adulte Kratzer gezeigt werden konnten. Damit wird die Hypothese zweier unterschiedlicher Arten weiter unterstützt.

Die Untersuchung der Erythrozyten ergab eine signifikante Erhöhung der Mikrokernfrequenz an Tag 21 in der Versuchsgruppe, die mit Pt exponiert, jedoch nicht infiziert waren. An demselben Versuchstag konnten zwar nicht signifikante dafür jedoch stark erhöhte Mikrokernfrequenzen für zwei weitere Versuchsgruppen beobachtet werden. Das war zum einen die Versuchsgruppe der infizierten, mit Pt exponierten Fische. Die Erhöhung der Mikrokernrate in beiden Pt exponierten Gruppen weist darauf hin, dass Pt gentoxische Effekte hervorruft. Allerdings wurde auch eine Erhöhung für eine nicht exponierte Gruppe gefunden. Diese war mit *P. laevis* infiziert und es stellt sich die Frage, ob auch die Infektion mit Acanthocephalen zu einem DNA Schaden in Erythrozyten führen kann.

Schlussfolgerungen Es kann festgehalten werden, dass verkehrsbürtiges Pt über das Straßenabflusswasser in Fließgewässer gelangt. Die dort vorhandenen Konzentrationen sind geringer, als die anderer Schwermetalle, der relative Anstieg zur bereits vorhandenen Schwermetallbelastung eines Gewässers ist jedoch höher. Die Hauptbelastung in den Sedimenten kann auf einen Abschnitt von ca. 40 m unterhalb der Einleitungsstelle festgelegt werden. Betrachtet man hingegen ein ganzes Flusseinzugsgebiet, sollte man bedenken, dass solche Straßenabflusseinleitungen keine singulären Ereignisse sind, sondern an mehreren Abschnitten eines Flusses vorkommen können. Hinzu kommt, dass Straßenabflusswässer nicht nur über direkte Einleitungsstellen in ein Gewässer gelangen, sondern dass sie auch diffus über die atmosphärische Deposition oder die Versickerung durch Straßenböschungen in Gewässer eingetragen werden können. Generell ist die Anzahl der Eintrittsstellen von Straßenabflusswässern und somit das Volumen, welches in die Fließgewässer gelangt, nicht bekannt. Es kann vermutet werden, dass die Menge des verkehrsbürtigen Pt in Gewässern in Zukunft weiter steigen wird. Zwar wird Pt mittlerweile in vielen Katalysatoren von Benzin und zu einem kleineren Anteil auch in Diesel betriebenen Fahrzeugen durch Pd ersetzt, jedoch sind die Prognosen für den Pt Verbrauch in europäischen Katalysatoren immer noch steigend (Johnson Matthey, 2011). Des Weiteren sieht sich Automobilbranche in Europa weiterhin unter Druck gesetzt, die Emission von CO_2 weiter zu verringern. Dies führt zur Zeit zu Experimenten mit neuartigen Verbrennungsmotoren und es zeichnet sich ein Trend bei einigen Herstellern

ab, so genannte Magermotoren einzusetzen, deren NOx Speicherkatalysatoren mit Pt benetzt sind (Johnson Matthey, 2011). Solange also die Verkehrsintensität in Europa steigt, kann auch mit einer Steigerung der Pt Emission gerechnet werden.

Auch wenn, wie gezeigt, die Akkumulationsraten von Muscheln und Fische für Pt relativ hoch sind, sind die im Freiland gefundenen Konzentrationen sehr gering. Vermutlich liegt dies an den Aufnahmewegen verkehrsbürtiger Metalle durch *Corbicula* sp. und die daraus resultierenden kurzfristigen Expositionszeiträumen in Fließgewässern. Aufgrund der Größenverteilung von Pt im Straßenstaub, kann davon ausgegangen werden, dass Pt für *Corbicula* sp. hauptsächlich in gelöster Form oder als sehr kleine suspendierte Partikel aufgenommen wird. Diese sind hauptsächlich während Niederschlagsereignissen und somit während direkter Einleitungsphasen des Straßenabflusswassers, oder während Remobilisierungsphasen aus dem Sediment in der Wassersäule verfügbar. Dies führt zu den, wie in dieser Studie sichtbar gewordenen, vergleichsweise niedrigen Pt Konzentrationen in den Freilandmuscheln. Höhere Konzentrationen können jedoch für aquatische Organismen vermutet werden, die sich von größeren Partikeln, Detritus oder auch Sedimenten ernähren. Dies konnte bereits für die Wasserassel *Asellus aquaticus* in mehreren Studien gezeigt werden (Rauch & Morrison, 1999; Moldovan et al., 2001; Haus et al., 2007b). Ein höheres Expositionrisiko für Muscheln und Fische ist vermutlich in Stillgewässern, wie Seen oder Flussaltarme, zu erwarten. In diese Gewässer eingeleitete gelöste Pt-Spezies oder kleine Schwebstaubpartikel werden nicht direkt mit einer Strömung abtransportiert und stehen den Muscheln und Fischen länger zur Aufnahme zur Verfügung.

Von daher kann die Schlussfolgerung getroffen werden, dass akut lethale oder akut toxische Effekte durch Pt für Muscheln und Fische in Flüssen zur Zeit nicht zu erwarten sind. Auch für viele der in anderen Studien im Labor beobachteten sublethalen Effekte, ist die Pt Konzentration im Freiland (noch) zu gering. Allerdings wurde in dieser Studie auch gezeigt, dass Pt gentoxische Effekte bei Fischen hervorrufen kann. Ob diese auch unter den geringen Freilandkonzentrationen vorkommen, muss noch geprüft werden. Zusammenfassend kann also festgestellt werden, dass Pt aufgrund seiner bisher niedrigen Konzentration sicherlich nicht zu den gefährlichen Schadstoffen in Fließgewässersystemen gehört. Es ist jedoch ein weiterer Stressor, der durch seine starke Nutzung in der Industrie und in Autoabgaskatalysatoren auf Organismen in Gewässern einwirkt.

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Appendices

Appendix A

A.1 Additional material for Chapter 2: Validation of analytical procedures

Table A.1: **Recovery rates determined for the analyses using HPA & ICP-MS. Values are indicated as mean values and (95% confidence interval).**

Element	Pacs-2 (µg/g)	Certified value (µg/g)	Recovery rate (%)	Dorm-2 (µg/g)	Certified value (µg/g)	Recovery rate (%)	IAEA-407 (µg/g)	Certified value (µg/g)	Recovery rate (%)
^{52}Cr	66 (7.4)	90.7 (4.6)	73 (8.2)	30 (0.9)	34.7 (5.5)	87 (3)		< NWG	
^{60}Ni	34 (6.1)	39.5 (2.3)	86 (15.3)	17 (0.5)	19.4 (3.1)	87 (2)	1.9 (0.7)	0.6 (0.05)	325 (122)
^{63}Cu	350 (38)	310 (12)	113 (12)	2.7 (0.1)	2.34 (0.08)	117 (4)	4.4 (0.08)	3.28 (0.08)	134 (4)
^{66}Zn	260 (30)	364 (24)	72 (8.1)	22 (0.9)	25.6 (2.3)	87 (4)	66 (2.0)	67.1 (0.8)	98 (3)
^{107}Ag	1.09 (0.1)	1.22 (0.14)	89 (11)	0.04 (0.01)	0.041 (0.013)	101 (24)	0.03 (0.003)	0.037 (0.004)	76 (8)
^{111}Cd	1.78 (0.2)	2.11 (0.15)	85 (9.2)	0.04 (0.01)	0.043 (0.008)	96 (15)	0.21 (0.01)	0.189 (0.04)	106 (5)
^{208}Pb	135 (9.1)	183 (8)	74 (9.1)	0.05 (0.02)	0.065 (0.007)	111 (25)	0.087 (0.01)	0.12 (0.02)	73 (15)

A.2 Additional material for Chapter 3: Introduction of traffic related Platinum into river systems - Occurrence and distribution of Platinum in sediments and biota

Table A.2: **Ratio of grain size fractions for sediment samples.**

Sampling point	Total mass	Sceletal mass < 5 mm	Ratio sceletal mass	Sand mass 2 mm to 63 µm	Ratio	Silt/clay mass	Ratio
Reference Site	1383	470	0.34	781	0.56	11	0.008
T-1/0	662	166	0.25	300	0.45	11	0.02
T-1/3	1061	435	0.41	517	0.49	18	0.02
T-1/7	750	200	0.27	438	0.58	5.5	0.007
T-1/20	1030	436	0.42	578	0.56	15	0.02
T-2/0	1685	1124	0.67	520	0.31	8.7	0.005
T-2/3	1354	951	0.70	363	0.27	1	0.0007
T-2/7	980	219	0.22	696	0.71	4.2	0.004
T-2/20	663	248	0.37	331	0.50	1.8	0.003
T-2/50	776	338	0.44	239	0.31	11	0.01
T-2/100	594	175	0.29	283	0.48	5.4	0.009
T-3/2	886	43	0.05	788	0.89	31	0.04
T-3/15	521	none		468	0.90	9.5	0.02
T-3/45	756	71	0.09	672	0.89	1.1	0.001
T-3/95	795	100	0.13	546	0.69	4.1	0.005

Table A.3: **Heavy metal concentrations in sediments (silt/clay fraction). Values are indicated as mean ± (standard deviation).**

Sampling Point	Ag µg/g	Cd µg/g	Cr µg/g	Cu µg/g	Ni µg/g	Pb µg/g	Pt ng/g	Zn µg/g
Reference Site	0.86 (0.07)	0.81 (0.05)	148 (26)	85 (4.1)	36 (3.6)	83 (4.8)	14 (2.3)	345 (15)
T-1/0	0.95 (0.3)	0.73 (0.03)	151 (26)	85 (9.2)	41 (9.2)	81 (6.5)	11 (4.6)	311 (17)
T-1/3	0.85 (0.1)	1.08 (0.2)	130 (19)	102 (19)	42 (2.0)	95 (15)	25 (3.6)	436 (147)
T-1/7	0.87 (0.1)	0.50 (0.04)	142 (14)	54 (4.0)	30 (3.5)	56 (3.4)	25 (15)	227 (24)
T-1/20	0.91 (0.2)	0.82 (0.06)	134 (7.1)	104 (7.1)	38 (3.3)	80 (10)	7.8 (1.4)	363 (34)
T-2/0	0.91 (0.09)	1.04 (0.05)	134 (23)	143 (12)	44 (2.1)	121 (7.2)	19 (6.0)	455 (15)
T-2/3	0.5 (0.2)	1.13 (0.08)	221 (39)	82 (4.3)	43 (6.3)	101 (5.0)	7.0 (5.9)	283 (14)
T-2/7	0.86 (0.04)	1.79 (0.14)	224 (55)	278 (15)	62 (5.3)	239 (25)	45 (3.1)	773 (40)
T-2/20	0.62 (0.04)	0.99 (0.09)	216 (27)	183 (11)	63 (11)	134 (9.6)	32 (11)	565 (37)
T-2/50	0.69 (0.08)	0.69 (0.07)	128 (23)	80 (4.5)	37 (5.5)	58 (8.7)	11 (3.5)	311 (11)
T-2/100	0.98 (0.2)	0.86 (0.05)	143 (12)	90 (6.7)	37 (2.1)	81 (8.7)	8.9 (3.9)	354 (9.3)
T-3/2	1.14 (0.1)	1.97 (0.09)	178 (24)	209 (5.1)	59 (2.6)	79 (3.2)	29 (3.5)	786 (60)
T-3/15	1.46 (0.3)	1.05 (0.07)	158 (4.9)	112 (2.7)	43 (3.2)	62 (30)	19 (4.6)	493 (9.6)
T-3/45	1.37 (0.8)	1.09 (0.07)	183 (25)	118 (13)	49 (5.3)	67 (28)	17 (8.5)	455 (23)
T-3/95	1.01 (0.2)	0.73 (0.04)	203 (22)	69 (8.7)	41 (5.0)	31 (3.9)	6.8 (3.7)	319 (27)

Table A.4: **Heavy metal concentrations in sediments (sand fraction). Values are indicated as mean ± (standard deviation).**

Sampling Point	Ag µg/g	Cd µg/g	Cr µg/g	Cu µg/g	Ni µg/g	Pb µg/g	Pt ng/g	Zn µg/g
Reference Site	0.19 (0.02)	< LOD	12 (9)	10 (2.0)	9 (1.6)	16 (2.7)	0.7 (0.15)	47 (3.2)
T-1/0	0.3 (0.04)	< LOD	42 (10)	21 (0.9)	13 (1.4)	24 (2.6)	6.6 (2.2)	91 (5.2)
T-1/3	0.17 (0.4)	< LOD	38 (6.4)	17 (2.1)	12 (0.2)	13 (2.2)	10 (6.4)	65 (7.7)
T-1/7	0.4 (0.004)	< LOD	29 (5.4)	19 (0.8)	11 (1.5)	21 (3.0)	1.3 (0.7)	85 (15)
T-1/20	0.22 (0.05)	< LOD	21 (2.5)	12 (2.5)	9.4 (2.0)	20 (3.2)	0.8 (0.15)	51 (4.0)
T-2/0	0.25 (0.06)	< LOD	77 (32)	178 (11)	33 (8.4)	65 (22)	3.6 (0.6)	205 (42)
T-2/3	0.23 (0.07)	< LOD	121 (61)	96 (0.7)	30 (3.7)	100 (16)	30 (12)	191 (29)
T-2/7	0.23 (0.05)	< LOD	120 (22)	55 (2.3)	28 (3.1)	81 (8.5)	32 (12)	194 (22)
T-2/20	0.16 (0.04)	< LOD	65 (12)	39 (4.5)	18 (4.3)	22 (6.3)	19 (4.0)	108 (24)
T-2/50	0.18 (0.05(	< LOD	50 (8.5)	20 (7.5)	18 (1.7)	21 (3.7)	6.5 (2.9)	111 (47)
T-2/100	0.17 (0.04)	< LOD	22 (3.6)	17 (0.9)	13 (5.5)	14 (1.8)	4.1 (1.4)	54 (8.1)
T-3/2	0.28 (0.05)	< LOD	177 (18)	51 (2.8)	21 (1.3)	91 (4.6)	24 (6.3)	176 (9.7)
T-3/15	0.22 (0.1)	< LOD	35 (5)	17 (1.7)	14 (4.7)	23 (1.0)	0.7 (0.08)	89 (10)
T-3/45	0.13 (0.007)	< LOD	18 (3.0)	9.4 (0.5)	11 (0.9)	15 (0.9)	0.7 (0.02)	56 (2.2)
T-3/95	0.17 (0.01)	< LOD	37 (3.1)	14 (0.6)	12 (1.1)	21 (3.6)	0.9 (0.3)	81 (7.3)

<LOD: Values are below the limit of detection.

Table A.5: **Heavy metal concentrations in sediments (fraction <2 mm). Values are indicated as mean ± (standard deviation).**

Sampling Point	Ag µg/g	Cd µg/g	Cr µg/g	Cu µg/g	Ni µg/g	Pb µg/g	Pt ng/g	Zn µg/g
Reference Site	0.2 (0.02)	0.15 (0.005)	14 (9.0)	11 (2.0)	9.5 (1.6)	17 (2.7)	0.88 (0.2)	51 (3.4)
T-1/0	0.3 (0.05)	0.17 (0.04)	46 (11)	23 (1.2)	14 (1.7)	26 (2.8)	6.8 (2.4)	99 (5.6)
T-1/3	0.19 (0.04)	0.16 (0.02)	41 (6.8)	19 (2.7)	13 (0.2)	17 (2.6)	11 (6.3)	78 (12)
T-1/7	0.4 (0.04)	0.15 (0.008)	30 (5.5)	20 (0.9)	11 (1.5)	21 (3.0)	1.6 (0.9)	87 (15)
T-1/20	0.25 (0.06)	0.14 (0.01)	23 (2.7)	14 (2.6)	10 (2.0)	22 (3.3)	1.0 (0.2)	59 (4.8)
T-2/0	0.26 (0.06)	0.13 (0.02)	78 (31)	178 (11)	33 (8.3)	66 (22)	3.8 (0.6)	209 (41)
T-2/3	0.23 (0.07)	0.22 (0.1)	121 (60.8)	96 (0.7)	30 (3.7)	100 (15)	30 (12)	191 (29)
T-2/7	0.24 (0.05)	0.32 (0.03)	120 (22)	56 (2.4)	28 (3.1)	82 (8.7)	32 (11)	198 (22)
T-2/20	0.16 (0.04)	0.15 (0.01)	66 (12)	40 (4.6)	18 (4.3)	23 (6.4)	19 (4.0)	110 (24)
T-2/50	0.2 (0.05)	0.22 (0.1)	53 (9.1)	23 (7.4)	19 (1.9)	23 (4.0)	6.7 (2.9)	120 (45)
T-2/100	0.19 (0.05)	0.15 (0.03)	24 (3.7)	18 (1.0)	13 (5.4)	15 (1.9)	4.2 (1.5)	60 (8.1)
T-3/2	0.31 (0.06)	0.22 (0.01)	177 (19)	57 (2.9)	23 (1.3)	90 (4.5)	24 (6.2)	199 (12)
T-3/15	0.24 (0.12)	0.15 (0.02)	38 (5.1)	19 (1.8)	15 (4.7)	23 (1.5)	1.1 (0.2)	97 (10)
T-3/45	0.14 (0.008)	0.14 (0.004)	18 (3.1)	9.6 (0.5)	11 (0.9)	15 (0.9)	0.8 (0.03)	57 (2.3)
T-3/95	0.17 (0.01)	0.14 (0.02)	38 (3.2)	14 (0.7)	12 (1.1)	21 (3.6)	0.9 (0.3)	82 (7.4)

Table A.6: **Heavy metal concentrations in *Corbicula* sp. Values are indicated as mean ± (standard deviation).**

Sampling Point	Ag µg/g	Cd µg/g	Cr µg/g	Cu µg/g	Ni µg/g	Pb µg/g	Pt ng/g	Zn µg/g
Reference Site	0.02 (0.008)	0.11 (0.02)	2.1 (0.4)	30 (2.7)	0.8 (0.03)	0.21 (0.08)	0.09 (0.06)	144 (13)
T-1/0	0.03 (0.004)	0.09 (0.01)	< LOD	30 (3.8)	0.7 (0.1)	0.26 (0.08)	0.1 (0.09)	127 (9)
T-1/3	0.03 (0.003)	0.08 (0.005)	< LOD	30 (1.9)	0.8 (0.2)	0.3 (0.17)	0.24 (0.19)	155 (14)
T-1/7	0.05 (0.003)	0.09 (0.01)	< LOD	33 (1.3)	0.7 (0.2)	0.3 (0.02)	0.19 (0.19)	137 (2.2)
T-1/20	0.04 (0.005)	0.09 (0.0005)	2.3 (0.5)	36 (2.8)	0.8 (0.1)	0.26 (0.02)	< LOD	153 (7.0)
T-2/0	0.03 (0.003)	0.09 (0.03)	2.3 (0.9)	29 (1.2)	1.4 (0.4)	0.34 (0.05)	0.14 (0.11)	148 (1.2)
T-2/3	0.03 (0.001)	0.1 (0.01)	< LOD	32 (2.3)	0.6 (0.1)	0.25 (0.1)	< LOD	144 (21)
T-2/7	0.02 (0.001)	0.11 (0.04)	< LOD	30 (1.4)	0.6 (0.2)	0.2 (0.08)	0.13 (0.06)	120 (2.5)
T-2/20	0.03 (0.006)	0.1 (0.007)	< LOD	32 (4.3)	0.7 (0.07)	0.29 (0.06)	1.3 (0.9)	131 (19)
T-2/50	0.03 (0.005)	0.1 (0.007)	< LOD	30 (2.6)	0.6 (0.1)	0.21 (0.09)	0.06 (0.03)	105 (6.4)
T-2/100	0.03 (0.003)	0.1 (0.01)	2.3 (0.08)	35 (2.1)	0.6 (0.06)	0.23 (0.05)	0.25 (0.15)	95 (2.0)
T-3/2	0.03 (0.005)	0.18 (0.02)	2.7 (0.1)	38 (1.9)	0.8 (0.04)	0.58 (0.18)	< LOD	128 (4.9)
T-3/15	0.02 (0.001)	0.2 (0.05)	2.9 (1.1)	41 (5.7)	1.4 (0.2)	0.35 (0.08)	0.9 (0.3)	126 (2.0)
T-3/45	0.02 (0.0008)	0.17 (0.009)	6.6 (1.5)	42 (1.3)	1.4 (0.2)	0.23 (0.05)	0.08 (0.05)	172 (7.7)
T-3/95	0.02 (0.003)	0.2 (0.01)	4.1 (0.8)	40 (2.7)	1.0 (0.4)	0.41 (0.2)	< LOD	121 (3.1)

<LOD: Values are below the limit of detection.

Table A.7: **Correlations of heavy metal concentrations in sediments (silt/clay fraction).**

Test	Spearman R	p-Value	Test	Spearman R	p-Value
Ag & Cd	0.12	0.3	Ni & Ag	0.04	0.8
Ag & Cr	-0.10	0.4	**Ni & Cd**	**0.71**	**5.1E-11**
Ag & Cu	0.10	0.4	**Ni & Cr**	**0.59**	**1.8E-07**
Ag & Ni	0.04	0.8	**Ni & Cu**	**0.74**	**1.7E-12**
Ag & Pb	-0.11	0.4	**Ni & Pb**	**0.40**	**0.001**
Ag & Pt	0.07	0.6	**Ni & Pt**	**0.38**	**0.006**
Ag & Zn	**0.26**	**0.04**	**Ni & Zn**	**0.75**	**1.1E-12**
Cd & Ag	0.12	0.3	Pb & Ag	-0.11	0.4
Cd & Cr	**0.44**	**0.0002**	**Pb & Cd**	**0.47**	**0.0001**
Cd & Cu	**0.80**	**4.1E-16**	Pb & Cr	0.18	0.2
Cd & Ni	**0.71**	**5.1E-11**	**Pb & Cu**	**0.59**	**8.6E-07**
Cd & Pb	**0.47**	**0.0001**	**Pb & Ni**	**0.40**	**0.001**
Cd & Pt	**0.48**	**0.0003**	**Pb & Pt**	**0.33**	**0.02**
Cd & Zn	**0.73**	**7.2E-12**	**Pb & Zn**	**0.36**	**0.005**
Cr & Ag	-0.1	0.4	Pt & Ag	0.07	0.6
Cr & Cd	**0.44**	**0.0002**	**Pt & Cd**	**0.48**	**0.0003**
Cr & Cu	**0.30**	**0.02**	Pt & Cr	0.11	0.4
Cr & Ni	**0.59**	**1.8E-07**	**Pt & Cu**	**0.57**	**0.00001**
Cr & Pb	0.18	0.2	**Pt & Ni**	**0.38**	**0.006**
Cr & Pt	0.11	0.4	**Pt & Pb**	**0.33**	**0.02**
Cr & Zn	**0.33**	**0.001**	**Pt & Zn**	**0.50**	**0.0002**
Cu & Ag	0.11	0.4	**Zn & Ag**	**0.26**	**0.04**
Cu & Cd	**0.80**	**4.1E-16**	**Zn & Cd**	**0.73**	**7.2E-12**
Cu & Cr	**0.30**	**0.02**	**Zn & Cr**	**0.33**	**0.01**
Cu & Ni	**0.74**	**1.7E-12**	**Zn & Cu**	**0.92**	**2.4E-26**
Cu & Pb	**0.58**	**8.6E-07**	**Zn & Ni**	**0.75**	**1.1E-12**
Cu & Pt	**0.57**	**0.00001**	**Zn & Pb**	**0.36**	**0.005**
Cu & Zn	**0.92**	**2.4E-26**	**Zn & Pt**	**0.50**	**0.0002**

Statistically significant correlations, with a p-level <0.05 are indicated in bold.

Table A.8: **Correlations of heavy metal concentrations in sediments (sand fraction).**

Correlation analyses in sediments for the sand fraction (63 µm to 2 mm)					
Test	**Spearman R**	**p-Value**	**Test**	**Spearman R**	**p-Value**
Ag & Cd	**0.33**	**0.004**	Ni & Ag	0.18	0.2
Ag & Cr	**0.31**	**0.01**	**Ni & Cd**	**0.31**	**0.01**
Ag & Cu	**0.38**	**0.002**	**Ni & Cr**	**0.85**	**1.4E-17**
Ag & Ni	0.18	0.2	**Ni & Cu**	**0.83**	**7.6E-16**
Ag & Pb	**0.41**	**0.001**	**Ni & Pb**	**0.57**	**9.0E-06**
Ag & Pt	0.21	0.09	**Ni & Pt**	**0.68**	**1.5E-08**
Ag & Zn	**0.34**	**0.005**	**Ni & Zn**	**0.84**	**1.7E-17**
Cd & Ag	as Cd concentrations were below the LOD, no correlation analyses were performed		**Pb & Ag**	**0.41**	**0.001**
Cd & Cr			**Pb & Cd**	**0.45**	**0.0004**
Cd & Cu			**Pb & Cr**	**0.65**	**1.6E-07**
Cd & Ni			**Pb & Cu**	**0.62**	**7.3E-07**
Cd & Pb			**Pb & Ni**	**0.57**	**9.0E-06**
Cd & Pt			**Pb & Pt**	**0.40**	**0.004**
Cd & Zn			**Pb & Zn**	**0.73**	**2.4E-10**
Cr & Ag	**0.31**	**0.01**	Pt & Ag	0.21	0.09
Cr & Cd	**0.38**	**0.002**	**Pt & Cd**	**0.32**	**0.01**
Cr & Cu	**0.86**	**2.28E-18**	**Pt & Cr**	**0.70**	**2.3E-09**
Cr & Ni	**0.85**	**1.4E-17**	**Pt & Cu**	**0.74**	**8.7E-11**
Cr & Pb	**0.65**	**1.6 E-07**	**Pt & Ni**	**0.68**	**1.5E-08**
Cr & Pt	**0.7**	**2.3E-09**	**Pt & Pb**	**0.40**	**0.004**
Cr & Zn	**0.89**	**3.0E-21**	**Pt & Zn**	**0.58**	**2.8E-06**
Cu & Ag	**0.38**	**0.002**	**Zn & Ag**	**0.34**	**0.005**
Cu & Cd	**0.36**	**0.003**	**Zn & Cd**	**0.47**	**9.5E-05**
Cu & Cr	**0.86**	**2.3E-18**	**Zn & Cr**	**0.89**	**3.0E-21**
Cu & Ni	**0.83**	**7.6E-16**	**Zn & Cu**	**0.91**	**9.6E-25**
Cu & Pb	**0.62**	**7.3E-07**	**Zn & Ni**	**0.84**	**1.7E-17**
Cu & Pt	**0.74**	**8.7E-11**	**Zn & Pb**	**0.73**	**2.4E-10**
Cu & Zn	**0.91**	**9.6E-25**	**Zn & Pt**	**0.58**	**2.8E-06**

Statistically significant correlations, with a p-level <0.05 are indicated in bold.

Table A.9: **Correlations of heavy metal concentrations in clam tissues.**

Test	Spearman	p-Value	Test	Spearman	p-Value
Ag & Cd	**-0.49**	**0.00002**	**Ni & Ag**	**-0.28**	**0.02**
Ag & Cr	**-0.34**	**0.004**	Ni & Cd	0.23	0.06
Ag & Cu	0.02	0.9	**Ni & Cr**	**0.64**	**5.2E-09**
Ag & Ni	**-0.28**	**0.02**	**Ni & Cu**	**0.32**	**0.008**
Ag & Pb	-0.06	0.6	**Ni & Pb**	**0.27**	**0.03**
Ag & Pt	0.23	0.09	Ni & Pt	-0.13	0.4
Ag & Zn	-0.06	0.6	**Ni & Zn**	**0.39**	**0.001**
Cd & Ag	**-0.49**	**0.00002**	Pb & Ag	-0.06	0.6
Cd & Cr	**0.43**	**0.0004**	Pb & Cd	0.05	0.7
Cd & Cu	**0.40**	**0.0006**	**Pb & Cr**	**0.34**	**0.005**
Cd & Ni	0.23	0.06	Pb & Cu	0.08	0.5
Cd & Pb	0.05	0.7	**Pb & Ni**	**0.28**	**0.03**
Cd & Pt	-0.11	0.4	Pb & Pt	-0.04	0.8
Cd & Zn	-0.04	0.7	Pb & Zn	0.21	0.087
Cr & Ag	**-0.35**	**0.004**	Pt & Ag	0.23	0.09
Cr & Cd	**0.43**	**0.0004**	Pt & Cd	-0.11	0.4
Cr & Cu	**0.46**	**0.00007**	Pt & Cr	-0.13	0.4
Cr & Ni	**0.64**	**5.2E-09**	Pt & Cu	-0.02	0.9
Cr & Pb	**0.34**	**0.005**	Pt & Ni	-0.13	0.4
Cr & Pt	-0.13	0.4	Pt & Pb	-0.04	0.8
Cr & Zn	0.03	0.8	Pt & Zn	-0.27	0.05
Cu & Ag	0.02	0.9	Zn & Ag	-0.06	0.6
Cu & Cd	**0.40**	**0.0006**	Zn & Cd	-0.04	0.7
Cu & Cr	**0.46**	**0.00007**	Zn & Cr	0.03	0.8
Cu & Ni	**0.32**	**0.008**	Zn & Cu	0.12	0.3
Cu & Pb	0.08	0.5	**Zn & Ni**	**0.39**	**0.001**
Cu & Pt	-0.02	0.9	Zn & Pb	0.21	0.09
Cu & Zn	0.12	0.3	Zn & Pt	-0.27	0.05

Statistically significant correlations, with a p-level <0.05 are indicated in bold.

Table A.10: **Correlations between heavy metal concentrations in different sediment fractions and clam tissues.**

Correlation analyses for clam tissue and sediments (silt/clay fraction <63 µm)		
Test	**Spearman R**	**p-Value**
Ag_sed & Ag_clam	-0.19	0.2
Cd_sed & Cd_clam	0.22	0.1
Cr_sed & Cr_clam	0.22	0.1
Cu_sed & Cu_clam	-0.09	0.5
Ni_sed & Ni_clam	0.09	0.5
Pb_sed & Pb_clam	-0.15	0.3
Pt_sed & Pt_clam	0.22	0.1
Zn_sed & Zn_clam	0.06	0.7

Correlation analyses for clam tissue and sediments (sand fraction 63 µm to 2 mm)		
Test	**Spearman R**	**p-Value**
Ag_sed & Ag_clam	**0.40**	**0.002**
Cd_sed & Cd_clam	0.21	0.1
Cr_sed & Cr_clam	-0.17	0.2
Cu_sed & Cu_clam	-0.18	0.2
Ni_sed & Ni_clam	-0.20	0.2
Pb_sed & Pb_clam	-0.15	0.3
Pt_sed & Pt_clam	-0.05	0.8
Zn_sed & Zn_clam	-0.15	0.3

Correlation analyses for clam tissue and sediments (<2 mm)		
Test	**Spearman R**	**p-Value**
Ag_sed & Ag_clam	0.08	0.6
Cd_sed & Cd_clam	**0.54**	**0.00005**
Cr_sed & Cr_clam	0.13	0.4
Cu_sed & Cu_clam	0.06	0.7
Ni_sed & Ni_clam	0.25	0.1
Pb_sed & Pb_clam	-0.05	0.7
Pt_sed & Pt_clam	0.09	0.6
Zn_sed & Zn_clam	-0.003	1.0

Statistically significant correlations, with a p-level <0.05 are indicated in bold.

Table A.11: **Length, width, height and masses determined for *Corbicula* sp. samples of the river Alb. Values are given as means ±(standard deviation).**

Sampling point	Clam length (mm)	Clam width (mm)	Clam height (mm)	Clam total mass (g)	Soft tissue mass (g)	Condition factor
Reference Site	19 (2.0)	17 (2.0)	12 (1.4)	2.6 (0.9)	0.57 (0.2)	8.5 (0.7)
T-1/0	18 (1.5)	16 (1.4)	12 (0.9)	2.5 (0.5)	0.57 (0.1)	9.0 (1.0)
T-1/3	20 (2.2)	18 (2.3)	13 (1.4)	3.0 (1.1)	0.66 (0.3)	8.2 (0.7)
T-1/7	20 (2.9)	18 (2.8)	13 (1.8)	3.2 (1.2)	0.68 (0.3)	7.6 (0.9)
T-1/20	19 (2.3)	17 (2.0)	12 (1.4)	2.7 (0.9)	0.60 (0.3)	8.2 (0.8)
T-2/0	18 (1.8)	16 (1.5)	12 (1.1)	2.4 (0.67)	0.52 (0.2)	8.6 (0.8)
T-2/3	18 (2.1)	16 (2.0)	12 (1.5)	2.5 (0.9)	0.55 (0.2)	8.8 (0.9)
T-2/7	18 (1.6)	16 (1.5)	12 (1.0)	2.4 (0.6)	0.58 (0.1)	9.7 (0.9)
T-2/20	19 (1.7)	16 (1.8)	13 (1.3)	3.0 (0.9)	0.74 (0.2)	10.7 (0.8)
T-2/50	19 (2.4)	17 (2.4)	13 (1.5)	3.1 (1.2)	0.82 (0.3)	10.8 (1.0)
T-2/100	20 (2.0)	18 (1.9)	14 (1.2)	3.6 (0.8)	0.94 (0.3)	10.7 (1.2)
T-3/2	20 (2.8)	18 (2.4)	13 (1.5)	2.9 (1.1)	0.68 (0.3)	8.6 (0.7)
T-3/15	19 (2.1)	17 (2.2)	13 (1.4)	3.2 (1.0)	0.78 (0.3)	10.3 (0.7)
T-3/45	22 (1.6)	20 (1.6)	14 (0.9)	3.8 (0.6)	0.8 (0.2)	7.2 (0.5)
T-3/95	21 (1.7)	19 (1.6)	14 (0.8)	3.8 (0.7)	0.93 (0.3)	9.7 (2.6)

Table A.12: **U-Tests for heavy metal concentrations in sediment samples (silt/clay fraction).**

Ag	Rank Sum	U	p-level
Sediment (silt/clay)			
Sampling Point			
Reference Site			
against			
T-1/0	15	5	0.9
T-1/3	20	10	1.1
T-1/7	19	9	0.6
T-1/20	18	8	1.1
T-2/0	16	6	0.7
T-2/3	**26**	**0**	**0.03**
T-2/7	18	8	0.7
T-2/20	**30**	**0**	**0.02**
T-2/50	**26**	**0**	**0.03**
T-2/100	15	5	0.3
T-3/2	**10**	**0**	**0.03**
T-3/15	**10**	**0**	**0.02**
T-3/45	21	9	0.9
T-3/95	12	2	0.1

Cd	Rank Sum	U	p-level
Sediment (silt/clay)			
Sampling Point			
Reference Site			
against			
T-1/0	22	0	0.06
T-1/3	**11**	**1**	**0.03**
T-1/7	**30**	**0**	**0.02**
T-1/20	17	7	0.9
T-2/0	**10**	**0**	**0.02**
T-2/3	**10**	**0**	**0.03**
T-2/7	**10**	**0**	**0.03**
T-2/20	**10**	**0**	**0.02**
T-2/50	24	2	0.1
T-2/100	14	4	0.2
T-3/2	**10**	**0**	**0.02**
T-3/15	**10**	**0**	**0.02**
T-3/45	**10**	**0**	**0.02**
T-3/95	**26**	**0**	**0.03**

Cr	Rank Sum	U	p-level
Sediment (silt/clay)			
Sampling Point			
Reference Site			
against			
T-1/0	18	8	1.1
T-1/3	22	4	0.3
T-1/7	23	7	0.6
T-1/20	22	4	0.3
T-2/0	21	5	0.5
T-2/3	**10**	**0**	**0.03**
T-2/7	12	2	0.06
T-2/20	**10**	**0**	**0.02**
T-2/50	24	6	0.4
T-2/100	22	8	0.7
T-3/2	15	5	0.3
T-3/15	19	9	0.9
T-3/45	14	4	0.2
T-3/95	**10**	**0**	**0.03**

Cu	Rank Sum	U	p-level
Sediment (silt/clay)			
Sampling Point			
Reference Site			
against			
T-1/0	18	4	0.6
T-1/3	13	3	0.1
T-1/7	**30**	**0**	**0.02**
T-1/20	**10**	**0**	**0.03**
T-2/0	**10**	**0**	**0.02**
T-2/3	20	6	0.7
T-2/7	**10**	**0**	**0.03**
T-2/20	**10**	**0**	**0.02**
T-2/50	22	4	0.3
T-2/100	15	5	0.5
T-3/2	**10**	**0**	**0.02**
T-3/15	**10**	**0**	**0.02**
T-3/45	**10**	**0**	**0.02**
T-3/95	**26**	**0**	**0.03**

Statistically significant correlations, with a p-level <0.05 are indicated in bold.

Table A.12: **Continued from previous page: U-Tests for heavy metal concentrations in sediment samples (silt/clay fraction).**

Ni	Rank Sum	U	p-level
Sediment (silt/clay)			
Sampling Point			
Reference Site			
against			
T-1/0	15	5	0.5
T-1/3	**11**	**1**	**0.03**
T-1/7	**29**	**1**	**0.03**
T-1/20	15	5	0.5
T-2/0	**10**	**0**	**0.03**
T-2/3	13	3	0.1
T-2/7	**10**	**0**	**0.03**
T-2/20	**10**	**0**	**0.02**
T-2/50	17	7	0.9
T-2/100	16	6	0.7
T-3/2	**10**	**0**	**0.02**
T-3/15	**11**	**1**	**0.03**
T-3/45	**10**	**0**	**0.02**
T-3/95	14	4	0.3

Pb	Rank Sum	U	p-level
Sediment (silt/clay)			
Sampling Point			
Reference Site			
against			
T-1/0	14	4	0.6
T-1/3	16	6	0.4
T-1/7	**26**	**0**	**0.03**
T-1/20	19	7	0.9
T-2/0	**10**	**0**	**0.03**
T-2/3	10	0	0.06
T-2/7	**10**	**0**	**0.03**
T-2/20	14	4	0.2
T-2/50	**30**	**0**	**0.02**
T-2/100	23	7	0.6
T-3/2	19	7	0.9
T-3/15	22	4	0.3
T-3/45	22	8	0.7
T-3/95	**26**	**0**	**0.03**

Pt	Rank Sum	U	p-level
Sediment (silt/clay)			
Sampling Point			
Reference Site			
against			
T-1/0	16	5	0.6
T-1/3	6	0	0.06
T-1/7	10	4	0.4
T-1/20	18	0	0.06
T-2/0	7	1	0.3
T-2/3	16	2	0.2
T-2/7	6	0	0.1
T-2/20	6	0	0.1
T-2/50	16	2	0.2
T-2/100	16	2	0.2
T-3/2	6	0	0.1
T-3/15	8	2	0.1
T-3/45	15	6	0.8
T-3/95	**21**	**0**	**0.04**

Zn	Rank Sum	U	p-level
Sediment (silt/clay)			
Sampling Point			
Reference Site			
against			
T-1/0	22	0	0.06
T-1/3	18	8	0.7
T-1/7	**26**	**0**	**0.03**
T-1/20	14	4	0.3
T-2/0	**10**	**0**	**0.02**
T-2/3	**26**	**0**	**0.03**
T-2/7	**10**	**0**	**0.03**
T-2/20	**10**	**0**	**0.02**
T-2/50	25	1	0.06
T-2/100	16	6	0.7
T-3/2	**10**	**0**	**0.02**
T-3/15	**10**	**0**	**0.02**
T-3/45	**10**	**0**	**0.02**
T-3/95	26	4	0.2

Statistically significant correlations, with a p-level <0.05 are indicated in bold.

Table A.13: **U-Tests for heavy metal concentrations in sediment samples (sand fraction).**

Ag Sediment (Sand) Sampling Point	Rank Sum	U	p-level
Reference Site against			
T-1/0	**15**	**0**	**0.008**
T-1/3	29	6	0.4
T-1/7	**15**	**0**	**0.008**
T-1/20	20	5	0.2
T-2/0	19	4	0.095
T-2/3	23	8	0.4
T-2/7	19	4	0.095
T-2/20	38	7	0.2
T-2/50	27	12	1.0
T-2/100	28	12	1.0
T-3/2	**15**	**0**	**0.008**
T-3/15	32	8	0.4
T-3/45	**35**	**0**	**0.016**
T-3/95	35	5	0.2

Cd Sediment (Sand) Sampling Point	Rank Sum	U	p-level
Reference Site against			
T-1/0	**30**	**10**	**0.005**
T-1/3	24	6	0.8
T-1/7	30	10	0.7
T-1/20	30	10	0.7
T-2/0	24	9	0.5
T-2/3	25	10	0.7
T-2/7	19	4	0.1
T-2/20	33	12	0.6
T-2/50	26	11	0.8
T-2/100	30	10	0.7
T-3/2	24	9	0.5
T-3/15	30	15	1.1
T-3/45	23	8	0.7
T-3/95	30	10	0.7

Cr Sediment (Sand) Sampling Point	Rank Sum	U	p-level
Reference Site against			
T-1/0	**10**	**0**	**0.03**
T-1/3	10	0	0.06
T-1/7	**10**	**0**	**0.02**
T-1/20	13	3	0.2
T-2/0	**10**	**0**	**0.03**
T-2/3	**10**	**0**	**0.02**
T-2/7	**10**	**0**	**0.02**
T-2/20	**10**	**0**	**0.02**
T-2/50	**10**	**0**	**0.03**
T-2/100	12	2	0.1
T-3/2	**10**	**0**	**0.02**
T-3/15	**10**	**0**	**0.02**
T-3/45	16	6	0.7
T-3/95	**10**	**0**	**0.03**

Cu Sediment (Sand) Sampling Point	Rank Sum	U	p-level
Reference Site against			
T-1/0	**10**	**0**	**0.03**
T-1/3	**10**	**0**	**0.03**
T-1/7	**10**	**0**	**0.02**
T-1/20	15	5	0.3
T-2/0	10	0	0.06
T-2/3	10	0	0.06
T-2/7	**10**	**0**	**0.02**
T-2/20	**10**	**0**	**0.02**
T-2/50	12	2	0.06
T-2/100	**10**	**0**	**0.03**
T-3/2	**10**	**0**	**0.03**
T-3/15	**10**	**0**	**0.02**
T-3/45	19	7	0.9
T-3/95	**10**	**0**	**0.03**

Statistically significant correlations, with a p-level <0.05 are indicated in bold.

Table A.13: **Continued from previous page: U-Tests for heavy metal concentrations in sediment samples (sand fraction).**

Ni	Rank Sum	U	p-level
Sediment (Sand)			
Sampling Point			
Reference Site			
against			
T-1/0	11	1	0.06
T-1/3	10	0	0.1
T-1/7	13	3	0.1
T-1/20	18	8	0.7
T-2/0	**10**	**0**	**0.03**
T-2/3	**10**	**0**	**0.03**
T-2/7	**10**	**0**	**0.02**
T-2/20	**10**	**0**	**0.02**
T-2/50	**10**	**0**	**0.03**
T-2/100	15	5	0.3
T-3/2	**10**	**0**	**0.02**
T-3/15	**10**	**0**	**0.02**
T-3/45	13	3	0.2
T-3/95	11	1	0.06

Pb	Rank Sum	U	p-level
Sediment (Sand)			
Sampling Point			
Reference Site			
against			
T-1/0	10	0	0.06
T-1/3	22	0	0.06
T-1/7	12	2	0.06
T-1/20	13	3	0.1
T-2/0	14	4	0.2
T-2/3	10	0	0.06
T-2/7	**10**	**0**	**0.02**
T-2/20	18	8	0.5
T-2/50	10	0	0.06
T-2/100	21	1	0.1
T-3/2	**10**	**0**	**0.03**
T-3/15	**10**	**0**	**0.03**
T-3/45	14	4	0.6
T-3/95	10	0	0.06

Pt	Rank Sum	U	p-level
Sediment (Sand)			
Sampling Point			
Reference Site			
against			
T-1/0	10	0	0.06
T-1/3	10	0	0.06
T-1/7	16	6	0.7
T-1/20	17	7	0.9
T-2/0	**10**	**0**	**0.03**
T-2/3	10	0	0.06
T-2/7	**10**	**0**	**0.03**
T-2/20	**10**	**0**	**0.02**
T-2/50	**10**	**0**	**0.02**
T-2/100	15	5	0.3
T-3/2	**10**	**0**	**0.03**
T-3/15	21	9	0.9
T-3/45	16	6	0.7
T-3/95	19	9	0.9

Zn	Rank Sum	U	p-level
Sediment (Sand)			
Sampling Point			
Reference Site			
against			
T-1/0	**10**	**0**	**0.03**
T-1/3	**10**	**0**	**0.03**
T-1/7	**10**	**0**	**0.02**
T-1/20	13	3	0.1
T-2/0	**10**	**0**	**0.03**
T-2/3	**10**	**0**	**0.03**
T-2/7	**10**	**0**	**0.02**
T-2/20	**10**	**0**	**0.02**
T-2/50	13	3	0.1
T-2/100	13	3	0.2
T-3/2	**10**	**0**	**0.03**
T-3/15	**10**	**0**	**0.02**
T-3/45	**10**	**0**	**0.03**
T-3/95	**10**	**0**	**0.03**

Statistically significant correlations, with a p-level <0.05 are indicated in bold.

Table A.14: **U-Tests for heavy metal concentrations in clam tissues.**

Ag	Rank Sum	U	p-level
Clam tissue			
Sampling Point			
Reference Site			
against			
T-1/0	26	5	0.08
T-1/3	26	5	0.08
T-1/7	**21**	**0**	**0.004**
T-1/20	**22**	**1**	**0.02**
T-2/0	27	6	0.1
T-2/3	26	5	0.08
T-2/7	27	6	0.1
T-2/20	27	6	0.1
T-2/50	28	7	0.09
T-2/100	26	5	0.08
T-3/2	27	6	0.1
T-3/15	25	4	0.3
T-3/45	29	8	0.5
T-3/95	32	11	0.9

Cd	Rank Sum	U	p-level
Clam tissue			
Sampling Point			
Reference Site			
against			
T-1/0	37	3	0.06
T-1/3	**40**	**0**	**0.008**
T-1/7	35	5	0.2
T-1/20	29	6	0.4
T-2/0	30	5	0.3
T-2/3	32	8	0.4
T-2/7	29	11	0.8
T-2/20	32	8	0.4
T-2/50	37	8	0.2
T-2/100	30	10	0.7
T-3/2	**15**	**0**	**0.008**
T-3/15	**15**	**0**	**0.04**
T-3/45	**15**	**0**	**0.02**
T-3/95	**15**	**0**	**0.02**

Cr	Rank Sum	U	p-level
Clam tissue			
Sampling Point			
Reference Site			
against			
T-1/0	25	5	0.3
T-1/3	28	2	0.06
T-1/7	22	4	0.3
T-1/20	16	6	0.7
T-2/0	19	9	0.9
T-2/3	**29**	**1**	**0.03**
T-2/7	23	7	0.6
T-2/20	22	8	0.7
T-2/50	29	5	0.2
T-2/100	14	4	0.3
T-3/2	12	2	0.06
T-3/15	12	2	0.2
T-3/45	**10**	**0**	**0.03**
T-3/95	**10**	**0**	**0.03**

Cu	Rank Sum	U	p-level
Clam tissue			
Sampling Point			
Reference Site			
against			
T-1/0	38	13	0.8
T-1/3	40	11	0.5
T-1/7	26	5	0.08
T-1/20	**22**	**1**	**0.02**
T-2/0	44	7	0.2
T-2/3	32	11	0.5
T-2/7	40	11	0.5
T-2/20	35	14	0.9
T-2/50	36	15	0.7
T-2/100	**22**	**1**	**0.009**
T-3/2	**21**	**0**	**0.004**
T-3/15	**21**	**0**	**0.03**
T-3/45	**21**	**0**	**0.004**
T-3/95	**21**	**0**	**0.01**

Statistically significant correlations, with a p-level <0.05 are indicated in bold.

Table A.14: **Continued from previous page: U-Tests for heavy metal concentrations in clam tissues.**

Ni	Rank Sum	U	p-level
Clam tissue			
Sampling Point			
Reference Site			
against			
T-1/0	30	5	0.3
T-1/3	25	10	0.7
T-1/7	30	10	0.7
T-1/20	22	7	0.6
T-2/0	**15**	**0**	**0.008**
T-2/3	37	3	0.06
T-2/7	30	5	0.3
T-2/20	32	8	0.4
T-2/50	40	5	0.08
T-2/100	35	5	0.2
T-3/2	**17**	**2**	**0.03**
T-3/15	**15**	**0**	**0.04**
T-3/45	**15**	**0**	**0.008**
T-3/95	21	6	0.4

Pb	Rank Sum	U	p-level
Clam tissue			
Sampling Point			
Reference Site			
against			
T-1/0	31	10	0.4
T-1/3	29	8	0.3
T-1/7	31	10	0.4
T-1/20	28	7	0.4
T-2/0	28	7	0.4
T-2/3	39	12	0.7
T-2/7	33	12	1.0
T-2/20	26	5	0.08
T-2/50	40	17	0.9
T-2/100	35	10	0.8
T-3/2	**23**	**2**	**0.02**
T-3/15	23	2	0.1
T-3/45	28	7	0.2
T-3/95	33	12	1.0

Pt	Rank Sum	U	p-level
Clam tissue			
Sampling Point			
Reference Site			
against			
T-1/0	28	13	0.8
T-1/3	19	4	0.2
T-1/7	24	9	0.4
T-1/20	25	10	0.7
T-2/0	27	12	0.8
T-2/3	34	11	0.5
T-2/7	22	7	0.3
T-2/20	22	7	0.2
T-2/50	29	11	0.8
T-2/100	22	7	0.1
T-3/2	27	9	0.7
T-3/15	**15**	**0**	**0.02**
T-3/45	33	7	0.3
T-3/95	32	9	0.4

Zn	Rank Sum	U	p-level
Clam tissue			
Sampling Point			
Reference Site			
against			
T-1/0	47	4	0.05
T-1/3	33	12	0.6
T-1/7	45	6	0.1
T-1/20	27	6	0.3
T-2/0	33	6	0.5
T-2/3	39	12	0.6
T-2/7	**48**	**3**	**0.03**
T-2/20	43	8	0.2
T-2/50	**69**	**0**	**0.0007**
T-2/100	**51**	**0**	**0.004**
T-3/2	43	8	0.2
T-3/15	36	3	0.2
T-3/45	**21**	**0**	**0.01**
T-3/95	42	3	0.07

Statistically significant correlations, with a p-level <0.05 are indicated in bold.

Table A.15: **Bioconcentration factors ($BCF_{sediment}$) for heavy metals in clam tissue and sediment <2 mm.**

	Ag	Cd	Cr	Cu	Ni	Pb	Pt	Zn
Reference Site	0.11	0.8	0.17	**3.5**	0.08	0.01	0.13	0.1
T-1/0	0.11	0.6	0.03	**1.5**	0.05	0.01	0.02	**1.4**
T-1/3	0.2	0.6	0.04	**2.1**	0.06	0.02	0.02	**2.4**
T-1/7	0.12	0.6	0.05	**2.0**	0.06	0.01	0.14	**1.6**
T-1/20	0.17	0.7	0.1	**3.2**	0.08	0.01	0.06	**3.0**
T-2/0	0.1	0.7	0.03	0.18	0.04	0.005	0.04	0.72
T-2/3	0.12	0.5	0.01	0.38	0.02	0.002	0.002	0.76
T-2/7	0.1	0.4	0.01	0.64	0.02	0.002	0.004	0.62
T-2/20	0.18	0.7	0.02	0.96	0.04	0.01	0.07	**1.2**
T-2/50	0.17	0.5	0.03	**1.6**	0.03	0.01	0.009	0.94
T-2/100	0.17	0.7	0.1	**2.2**	0.05	0.02	0.06	**1.8**
T-3/2	0.1	**1.2**	0.02	0.8	0.04	0.006	0.002	0.72
T-3/15	0.11	**1.5**	0.08	**2.8**	0.1	0.02	**1.3**	**1.4**
T-3/45	0.16	**1.2**	0.04	**4.4**	0.1	0.01	0.33	**1.7**
T-3/95	0.12	**1.4**	0.1	**3.0**	0.09	0.02	0.06	**1.5**

A.3 Additional material for Chapter 4: Accumulation of different Platinum concentrations by *Corbicula* sp. and the genotoxic effects of Platinum on gill cells and hemocytes

Table A.16: **Length, width, height, masses and condition factor determined for *Corbicula* sp. samples of the exposure study determined for *Corbicula* sp. samples of the exposure study. Values are given as means ±(standard deviation).**

Treatment group	Days after exposure start	Clam length (mm)	Clam width (mm)	Clam height (mm)	Clam total mass (g)	Soft tissue mass (g)	Condition factor
All	0	19.8 (2.9)	17.9 (2.6)	13 (1.7)	3.5 (1.5)	0.56 (0.3)	6.9 (0.7)
Control	12	19.0 (2.2)	16.5 (1.9)	12.4 (1.3)	3.1 (0.9)	0.36 (0.12)	5.1 (0.7)
Control	20	18.9 (2.2)	16.8 (2.0)	12.2 (1.3)	2.7 (0.8)	0.33 (0.1)	4.7 (0.5)
Control	30	19.8 (2.4)	17.5 (2.3)	12.8 (1.4)	3.0 (1.0)	0.38 (0.2)	4.7 (0.7)
Control	40	19.6 (2.8)	17.4 (2.4)	12.4 (1.5)	2.8 (1.0)	0.37 (0.1)	4.7 (0.7)
Control	60	18.5 (2.0)	16.4 (2.0)	12.0 (1.2)	2.5 (0.7)	0.29 (0.1)	4.4 (0.6)
Control	70	18.6 (1.7)	16.1 (1.4)	11.9 (0.9)	2.4 (0.5)	0.26 (0.1)	4.0 (0.8)
10 ng/L	12	18.5 (2.1)	16.3 (1.7)	11.6 (1.2)	2.5 (0.6)	0.33 (0.1)	5.0 (0.6)
10 ng/L	20	18.4 (1.8)	16.0 (1.8)	11.5 (1.3)	2.4 (0.6)	0.30 (0.1)	4.7 (0.6)
10 ng/L	30	20.2 (3.2)	17.3 (1.9)	12.3 (1.2)	2.9 (0.8)	0.39 (0.1)	4.6 (0.7)
10 ng/L	40	19.6 (2.6)	17.2 (2.4)	12.3 (1.9)	3.3 (2.9)	0.39 (0.2)	4.9 (0.7)
10 ng/L	50	19.2 (2.0)	16.7 (1.7)	11.9 (1.1)	2.7 (0.7)	0.33 (0.1)	4.5 (0.5)
10 ng/L	60	18.8 (1.8)	16.3 (1.4)	11.9 (1.1)	2.5 (0.6)	0.3 (0.1)	4.5 (0.77)
10 ng/L	70	19.0 (1.9)	17.1 (1.7)	12.2 (1.1)	2.4 (0.7)	0.24 (0.1)	3.4 (0.57)
50 ng/L	12	19.6 (1.9)	17.3 (1.5)	12.8 (1.2)	2.8 (0.6)	0.35 (0.1)	4.5 (0.5)
50 ng/L	20	19.2 (2.2)	16.5 (1.6)	11.8 (1.3)	2.7 (0.8)	0.35 (0.1)	4.8 (0.6)
50 ng/L	30	19.7 (2.2)	17.3 (2.0)	13.0 (1.49	2.9 (0.8)	0.35 (0.1)	4.4 (0.7)
50 ng/L	40	19.2 (1.9)	16.8 (1.7)	11.8 (1.2)	2.7 (0.6)	0.35 (0.1)	4.8 (0.6)
50 ng/L	50	19.2 (2.4)	16.9 (2.1)	12.1 (1.4)	2.7 (0.7)	0.33 (0.1)	4.6 (0.7)
50 ng/L	60	18.7 (2.0)	16.1 (2.2)	11.8 (1.1)	2.5 (0.6)	0.27 (0.1)	4.0 (0.5)
50 ng/L	70	17.9 (1.9)	15.7 (1.6)	11.1 (1.1)	2.3 (0.5)	0.25 (0.1)	4.2 (0.7)
100 ng/L	12	19.3 (1.9)	17.0 (1.7)	12.2 (1.3)	2.9 (0.8)	0.36 (0.1)	4.9 (0.7)
100 ng/L	20	19.9 (2.2)	17.4 (2.0)	12.2 (1.3)	3.0 (0.9)	0.38 (0.1)	4.7 (0.6)
100 ng/L	30	19.6 (1.9)	17.1 (1.6)	11.9 (1.1)	2.7 (0.6)	0.36 (0.1)	4.7 (0.5)
100 ng/L	40	19.3 (1.9)	16.7 (1.7)	12.2 (1.2)	2.7 (0.6)	0.34)0.1)	4.7 (0.6)
100 ng/L	50	19.5 (2.4)	17.1 (2.2)	11.9 (1.5)	2.8 (0.8)	0.35 (0.1)	4.5 (0.6)
100 ng/L	60	18.6 (0.8)	16.2 (0.8)	11.7 (0.8)	2.5 (0.8)	0.28 (0.8)	4.3 (0.8)
100 ng/L	70	18.2 (2.2)	15.9 (1.8)	11.4 (1.3)	2.2 (0.6)	0.24 (0.1)	4.0 (0.6)
100 µg/l	12	19.1 (2.3)	16.7 (2.0)	12.5 (1.3)	2.7 (0.8)	0.34 (0.1)	4.8 (0.7)
100 µg/l	20	19.5 (2.5)	17.2 (2.3)	12.2 (1.6)	2.9 (1.0)	0.37 (0.2)	4.8 (0.6)
100 µg/l	30	19.4 (2.3)	17.1 (2.1)	12.4 (1.3)	2.8 (0.8)	0.37 (0.1)	4.9 (0.6)
100 µg/l	40	19.3 (1.8)	16.9 (1.4)	12.0 (1.1)	2.6 (0.6)	0.33 (0.1)	4.6 (0.5)
100 µg/l	50	18.8 (2.7)	16.2 (1.9)	11.6 (1.3)	2.5 (0.7)	0.30 (0.1)	4.7 (0.9)
100 µg/l	60	19.0 (1.9)	16.6 (1.5)	11.7 (1.1)	2.5 (0.6)	0.31 (0.1)	4.4 (0.5)
100 µg/l	70	21.0 (1.9)	18.9 (1.6)	13.1 (1.0)	2.8 (0.6)	0.33 (0.1)	3.5 (1.0)

A.4 Additional material for Chapter 5: The accumulation of Platinum by *Squalius cephalus* and *Pomphorhynchus* sp. and the genotoxic effect of Platinum on fish erythrocytes

Table A.17: **Size, sex and infection rates for individual fish.**

Treatment group	Days after exposure start	Fish number	Infected with	Number of parasites in the intestine	Sex	Total mass (g)	Length (cm)	Mass liver (g)
Control uninfected	0	1	none	0	male	9	11.1	0.057
Control uninfected	0	2	none	0	male	9	11.7	0.054
Control uninfected	0	3	none	0	male	5	9.9	0.051
Control uninfected	0	4	none	0	female	8	10.3	0.047
Control uninfected	0	5	none	0	female	8	10.3	0.056
Control uninfected	0	6	none	0	female	6	9.6	0.0445
Control uninfected	7	7	none	0	male	10	11.6	0.064
Control uninfected	7	8	none	0	female	8	10.3	0.035
Control uninfected	7	9	none	0	male	7	9.9	0.031
Control uninfected	7	10	none	0	female	6	10.1	0.047
Control uninfected	7	11	none	0	female	10	10.9	0.042
Control uninfected	7	12	none	0	female	7	9.4	0.034
Control uninfected	13	31	none	0	female	11	11.1	0.046
Control uninfected	13	32	none	0	female	9	11.2	0.055
Control uninfected	13	33	none	0	female	7	9.6	0.052
Control uninfected	13	34	none	0	female	7	9.7	0.063
Control uninfected	13	35	none	0	female	6	9.5	0,.023
Control uninfected	21	51	none	0	female	8	10.7	0.060
Control uninfected	21	52	none	0	female	8	10.3	0.085
Control uninfected	21	53	none	0	female	8	10.2	0.039
Control uninfected	21	54	none	0	female	8	10	0.081
Control uninfected	21	55	none	0	female	7	10	0.067
Control uninfected	21	56	none	0	female	8.5	10.8	0.086
Control uninfected	28	75	none	0	female	5	9.7	0.068
Control uninfected	28	76	none	0	female	7.5	10.6	0.068
Control uninfected	28	77	none	0	female	8	10.6	0.072
Control uninfected	28	78	none	0	female	10.5	11	0.088
Control uninfected	28	79	none	0	female	7.5	10.1	0.086
Control uninfected	35	107	none	0	female	8.5	10.1	0.103
Control uninfected	35	108	none	0	male	8.5	10.4	0.121
Control uninfected	35	109	none	0	male	11	11.4	0.155
Control uninfected	35	110	none	0	female	10.5	10.9	0.099
Control uninfected	35	111	none	0	female	8	10.7	0.093
Control uninfected	35	112	none	0	female	7.5	10.3	0.105

Table A.17: **Continued from previous page: Size, sex and infection rates for individual fish.**

Treatment group	Days after exposure start	Fish number	Infected with	Number of parasites in the intestine	Sex	Total mass (g)	Length (cm)	Mass liver (g)
Pt uninfected	7	13	none	0	female	10	11.3	0.070
Pt uninfected	7	14	none	0	male	5	9.6	0.042
Pt uninfected	7	15	none	0	male	9.5	11	0.055
Pt uninfected	7	16	none	0	male	7	10.2	0.031
Pt uninfected	7	17	none	0	male	12	11.9	0.041
Pt uninfected	7	18	none	0	female	12	12.2	0.087
Pt uninfected	13	36	none	0	female	8	10.2	0.042
Pt uninfected	13	37	none	0	female	6.5	10.2	0.033
Pt uninfected	13	38	none	0	female	10	11.6	0.065
Pt uninfected	13	39	none	0	female	7	9.6	0.036
Pt uninfected	13	40	none	0	female	9	10.7	0.068
Pt uninfected	21	57	none	0	female	8.5	10.3	0.048
Pt uninfected	21	58	none	0	female	7.5	10.2	0.059
Pt uninfected	21	59	none	0	female	9	10.6	0.056
Pt uninfected	21	60	none	0	female	9	11.1	0.048
Pt uninfected	21	61	none	0	female	6	9.2	0.04
Pt uninfected	21	62	none	0	female	8	10.1	0.075
Pt uninfected	28	80	none	0	female	10	11.2	0.1
Pt uninfected	28	81	none	0	female	9.5	10.5	0.121
Pt uninfected	28	82	none	0	female	7	9.2	0.089
Pt uninfected	28	83	none	0	female	5	8.9	0.057
Pt uninfected	28	84	none	0	female	11	11.4	0.096
Pt uninfected	35	113	none	0	female	11.5	11.6	0.129
Pt uninfected	35	114	none	0	female	8	10.4	0.128
Pt uninfected	35	115	none	0	female	8	10.6	0.101
Pt uninfected	35	116	none	0	female	10.5	11.4	0.142
Pt uninfected	35	117	none	0	female	8.5	10.2	0.067
Control infected	7	19	*P. tereticollis*	2	female	10	10.9	0.047
Control infected	7	20	*P. tereticollis*	1	female	10	11.5	0.079
Control infected	7	21	*P. tereticollis*	3	female	9	10.6	0.042
Control infected	7	22	*P. tereticollis*	4	female	9	10.5	0.034
Control infected	7	23	*P. tereticollis*	1	female	12	12	0.054
Control infected	7	24	*P. tereticollis*	2	male	10	10.9	0.042
Control infected	13	41	*P. laevis*	3	female	15	13.3	0.089
Control infected	13	42	*P. laevis*	1	female	10	11.1	0.06
Control infected	13	43	*P. laevis*	3	female	8	10.1	0.03
Control infected	13	44	*P. laevis*	4	female	7.5	10.6	0.041
Control infected	13	45	*P. laevis*	4	female	7	9.7	0.029
Control infected	21	63	*P. tereticollis*	1	female	7	9.8	0.016
Control infected	21	64	*P. tereticollis*	4	male	7.5	10.7	0.046
Control infected	21	65	*P. tereticollis*	4	female	7	10.3	0.046
Control infected	21	66	*P. tereticollis*	1	female	8	10.7	0.033
Control infected	21	67	*P. tereticollis*	1	female	9	10.8	0.070
Control infected	21	68	*P. tereticollis*	4	female	7	9.9	0.027
Control infected	28	85	*P. laevis*	5	female	11.5	11.8	0.069
Control infected	28	86	*P. laevis*	4	female	8.5	10.7	0.086
Control infected	28	87	*P. laevis*	1	female	11	11.9	0.088
Control infected	28	88	*P. laevis*	4	female	8	10.1	0.074
Control infected	28	89	*P. laevis*	5	female	9	10.8	0.077
Control infected	35	119	*P. tereticollis*	2	female	11	11.2	0.07
Control infected	35	120	*P. tereticollis*	0	male	13.5	13	0.118
Control infected	35	121	*P. tereticollis*	2	female	9.5	11.2	0.123
Control infected	35	122	*P. tereticollis*	0	female	10	11.4	0.115
Control infected	35	123	*P. tereticollis*	2	female	10.5	11.3	0.114
Control infected	35	124	*P. tereticollis*	5	female	8	10.5	0.112

Table A.17: **Continued from previous page: Size, sex and infection rates for individual fish.**

Treatment group	Days after exposure start	Fish number	Infected with	Number of parasites in the intestine	Sex	Total mass (g)	Length (cm)	Mass liver (g)
Pt infected	7	25	*P. tereticollis*	1	female	8	10.7	0.045
Pt infected	7	26	*P. tereticollis*	3	female	7	10.6	0.051
Pt infected	7	27	*P. tereticollis*	1	female	10	11	0.051
Pt infected	7	28	*P. tereticollis*	2	female	12	11.4	0.068
Pt infected	7	29	*P. tereticollis*	2	male	9	11.9	0.029
Pt infected	7	30	*P. tereticollis*	2	female	8	10.5	0.029
Pt infected	13	46	*P. laevis*	3	female	8	10.3	0.03
Pt infected	13	47	*P. laevis*	5	female	7	10.3	0.038
Pt infected	13	48	*P. laevis*	4	female	12.5	12.2	0.033
Pt infected	13	49	*P. laevis*	3	female	11.5	12	0.056
Pt infected	13	50	*P. laevis*	3	male	8	10.2	0.054
Pt infected	21	69	*P. tereticollis*	3	female	8.5	10.8	0.061
Pt infected	21	70	*P. tereticollis*	2	female	9	10.9	0.074
Pt infected	21	71	*P. tereticollis*	4	female	7	10	0.038
Pt infected	21	72	*P. tereticollis*	4	female	7.5	10.3	0.056
Pt infected	21	73	*P. tereticollis*	3	female	6	10.1	0.052
Pt infected	21	74	*P. tereticollis*	4	female	6	9.9	0.046
Pt infected	28	90	*P. laevis*	5	female	11	11.5	0.087
Pt infected	28	91	*P. laevis*	4	female	9.5	10.8	0.059
Pt infected	28	92	*P. laevis*	4	female	7.5	10.1	0.037
Pt infected	28	93	*P. laevis*	4	female	10.5	11.1	0.082
Pt infected	28	94	*P. laevis*	4	female	7	10.1	0.091
Pt infected	35	125	*P. tereticollis*	4	female	10	11.3	0.148
Pt infected	35	126	*P. tereticollis*	4	female	7.5	9.6	0.067
Pt infected	35	127	*P. tereticollis*	1	female	8.5	10.1	0.078
Pt infected	35	128	*P. tereticollis*	2	male	11.5	11.8	0.17
Pt infected	35	129	*P. tereticollis*	0	female	11.5	11.3	0.153
Pt infected	35	130	*P. tereticollis*	2	female	15	12.9	0.134