Bioavailability of Pb, Sb, Cu and Zn in a rifle-range runoff: Accumulation and biomarker response in Brown trout *(Salmo trutta L)*

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8) ABSTRACT

The present knowledge on environmental effects on aquatic biota caused by heavy metal efflux from rifle ranges is insufficient in order to predict the effects on the ecosystem. The goal of this work has been to provide data on speciation of lead (Pb), antimony (Sb), copper (Cu) and zinc (Zn) in a rifle range runoff, and to document accumulation and toxic interactions of theses metals in fish. We found that the efflux of Pb and Cu differed significantly during a snow melting event, while Sb and Zn did not. Dissolved heavy metals caused acute and evident osmoregulatoric dysfunction in juvenile trout exposed to the shooting range effluent. Contrary to our hypotheses, correlations between cationic heavy metals in the low molecular mass fraction (<10 kDa) and accumulation of heavy metals in fish liver and gills were weak or absent, even when Ca and pH were taken into consideration. Accumulation of Sb in fish gills and livers was detected, however only temporary. Pb was proved to inhibit the heme-synthesis in red blood cells, but it was not possible to connect (or exclude) accumulation of Cu, Zn and Sb to any specific toxic effect. We found indications that flushing events in shooting ranges potentially may cause chronic accumulation of Pb rather than Cu, Zn and Sb. Other findings of interest were lethal respiratory dysfunction caused by precipitation of Al on gills in a uncontaminated control stream, and acute mortality caused by ferrous iron detected in a pre-survey of the shooting range. In aquatic risk assessment of pollution from rifle ranges established on peat, it is necessary to incorporate focus on these metals as well.

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1 INTRODUCTION

Deposition of heavy metals in rifle ranges represents a potential environmental risk, both terrestrial and aquatic. Contamination of the aquatic environment has traditionally been of most public interest, because runoff may pollute areas outside the shooting range itself. The heavy metal pollution is possible to monitor, but the ecological risk posed by metals is however difficult to document. The toxicity of various metal species differs significantly and the responses among organisms and life stages are not equal. Furthermore, the environmental influences and effects are complex, and it is therefore difficult to reveal significant toxic effects of pollutants in natural water. By means of a broad investigation of aquatic and biological parameters, we try to provide data on distribution of heavy metals in a rifle range runoff, and to document accumulation and toxic interactions of these metals in fish.

1.1 Heavy metals in Norwegian military areas for small arms training

The use of small arms ammunition causes significant deposits of the heavy metals lead (Pb), antimony (Sb), copper (Cu) and zinc (Zn) in firing ranges. In the Norwegian military areas for small arms training, the deposition of heavy metals in 2006 was approximately 126 tons of Pb, 14 tons of Sb, 55 tons of Cu and 6 tons of Zn (Christiansen et.al 2007). Depending on the physical and chemical properties of the soil, heavy metals deposited in the top soil (*e.g.* backstop berms) may be mobilized from solid phases to dissolved phases or associate to mobile structures. Deposited heavy metals may then contaminate receiving water recipients.

During a 15-year surveillance program of aquatic metal pollution in military training areas, levels of Pb, Zn, Cd, and Sb are registered above background levels. In small streams near the deposits, the concentration of the metals exceeds water quality guidelines established by The Norwegian Pollution Control Authority (Rognerud 2006). High concentrations in effluents are mainly found in shooting ranges established on marshy ground (peat) where the corrosion rate is high due to acidic conditions, and the mobility of heavy metals is high due to chelating humic ligands.

1.2 Runoff events

In most parts of Norway, rainfall- and snow melting events cause alternating wet and dry conditions, followed by a variation in discharge and changes in surface water chemistry (e.g. pH, conductivity and content of organic matter). Runoff events are often reported to be essential for the efflux of heavy metals from a wide range of contaminated areas, *e.g*. mines, waste disposal sites, roadways, urban areas and shooting ranges (i.a. Sansalone & Buchberger 1997, Westerlund & Viklander 2006, Kim *et al*. 2005, Gundersen *et al*. 2001, Olsvik et. al 2000, Johnson *et al*. 1999). The scale and duration of peaks in flow rate depend on the size of the drainage basin; the greater variation in flow rate, the more significant is the runoff dips and peaks (Allan 1995). The effects of runoff events are however complex and will depend on climatic factors, physical and chemical properties of the soils, and the catchment configuration. Hence, it is not possible to establish an overall model of how flushing events influence the discharge of different metal species.

Heier *et al.* (2004) reported that the concentrations of heavy metals in a Norwegian shooting range effluent were doubled during rainfall runoff, compared with the base flow concentrations. In this case, a great part of the increase could be explained by flushing of organic matter and particular material. However, it is not clarified how such runoff regimes affect the bioavailability of metals in shooting range effluents.

1.3 Speciation and bioavailability of heavy metals

1.3.1 General

In the aquatic environment, trace elements exist in different physico-chemical forms, called chemical species (different isotopic composition, electronic or oxidation state, and/or complex or molecular structure). Depending on the water composition, the elements of interest can be found in different forms with various size and charge properties (i.e. chelating ligands with low molecular mass, colloids, particles, ions etc).

Metals are shown to affect different biological receptors. The availability of a metal to a biological receptor depends on its chemical species (bioavailable). The bioavailability of a metal is generally reported to correlate with its free ion concentration (Klaassen 2001, Walker 2003). Water acidity is a major factor influencing the speciation of metals, and consequently the occurrence of cationic metal species. In natural freshwater, cationic species of Pb, Zn and Cu are generally predominant at pH<6.0, 5.5 and 6.5, respectively. If pH is higher than 8.0, anionic species (e.g. metal hydroxides) dominate (Stumm and Morgan 1996). Thus, the acidity is important for the bioavailability of metals.

Furthermore, different biological receptors, affected by metals, are not necessarily element-specific. The bioavailability of heavy metals (both essential and nonessential) is reported to correlate negatively with the content of other cations, primarily Calcium (Walker 2003, Lydersen *et al*. 2002, Galvez *et al*. 1998). In addition to competitive inhibition of uptake, presence of a metal may also inhibit uptake of another through uncompetitive interactions (Rogers 2006).

1.3.2 Bioavailability and uptake in fish

There are three possible routes for uptake of metal in fish:

- branchial uptake (water-to-gill by ion exchange/respiration)
- gastro-intestinal uptake (in stomach/gut)
- dermal uptake

In any case, the crucial qualifications for biological uptake of heavy metals are contact between the metal species and epidermal cells, and the species' ability to cross or interact with the cellular membrane. Dermal uptake of metals (and other non-fatty substances) is not well characterized compared with the two other routes. In fish, dietary uptake of Pb, Cu and Zn is commonly considered less important than branchial uptake, because heavy metals generally do not biomagnify (except from alkylated mercury). In this study we have focused on the branchial uptake of the studied metals.

The epithelial cells in fish gills are adapted to provide the appropriate transport mechanisms, depending upon whether the fish is in freshwater or seawater. In freshwater, the concentration of ions in the fish is higher than in the surroundings (hyperosmotic). To maintain body fluid and mineral homeostasis freshwater fish compensate for diffusive ion loss and osmotic gain of water by active absorption of Ca^{2+}, Na^{+} and Cl^{-}, and by producing large volumes of dilute urine. Fish is poikilothermic and temperature may therefore affect the active uptake of solutes.

The most important epithelial structure for osmoregulation in fish is large mitochondria-rich cells called chloride cells. In fact, Foskett *et al*. (1983) found that the area-specific surface current and conductance of chloride cells ranked them as one of the most active transporting and conductive cells known. In post-embryonic stages of fish, these cells are primarily evident in gills (Rombough 1999). Chloride cells have an apical pit, which in freshwater species have tubular structures (microvilli) with large absorptive surface area. In short, fish gills have a negative surface charge, and hence affinity to electrophilic structures.

1.3.3 Bioavailability of Pb, Cu, Zn and Sb

The bioavailable species of Pb are within the dissolved fraction. Hence, the availability of lead to organisms is limited by its strong adsorption to environmental components, such as soil, sediment, organic matter and biota. It is accepted that biomagnification of Pb does not take place, since no increase in concentration of the metal is observed in food chains. However, environmental contamination with Pb is widespread, and organisms do accumulate high body burdens.

Previous studies on the speciation of Cu in a shooting field runoff demonstrated that Cu was mainly found in the colloidal fraction (on average 60%). Approximately 25% was found in the low molecular fraction (<10KDa) and the remaining 15% as particulate material (Heier *et al*. 2004). In general the most bioavailable forms of metals are the positively charged low molecular forms, but the uptake in organism was not studied in this study. The toxic forms of Cu are mainly coupled to the Cu(II) ion, free or as hydroxide complexes. Although Cu speciation is important for the bioavailability in fish, Erickson *et al*. (1996) observed that Cu speciation alone could not explain observed variation in Cu toxicity in fathead minnow. The toxicity decreases with water hardness, alkalinity and pH due to the decreased Cu permeability across gill membranes and the formation of less toxic hydroxides and carbonate complexes in water. In general, the bioavailability and thus the toxicity decreases with increased ionic strength and complexation to organic or inorganic ligands (*e.g.* humic compounds) (Lydersen 2002).

Zn is primarily found as the positively charged Zn^{2+} ion, and generally less associated to colloids and particles than Pb and Cu. When examined, this pattern has been found in all previous studies of Norwegian shooting ranges. As for other metals, water quality parameters will affect the speciation of Zn and thus its bioavailability.

Studies of Sb in shooting ranges has shown that in spite of a high variability in geology, soil pH (3.1-7.5), Sb concentrations and shooting range history, only two Sb species were identified; metallic Sb, and Sb(V) absorbed to Fe- or Mn-oxides (Scheinost et al., 2004). Modelling of antimony speciation in aquatic environment shows that antimony is exclusively present as Sb(OH)6- in oxic conditions, and as Sb(OH)3 in anoxic conditions, at all pH values

of environmental relevance for aquatic systems (Filella and May, 2003). This is consistent with observations (Filella et al., 2002a; Filella et al., 2002b). In natural waters Sb is found mostly in the dissolved phase, which can be explained by the hydroxide species (Filella and May, 2003). It is not known whether dissolved hydroxide species are available for uptake in aquatic organisms. There is little evidence of biomagnification of antimony in food chains represented by soil–vegetation–invertebrate–insectivore pathway of grasslands (Ainsworth et al., 1990; Gál et al., 2007), and little indication of significant accumulation by herbivorous mammals despite marked contamination of their diet (Ainsworth et al., 1990). No information is available regarding accumulation of Sb in fish.

1.4 Toxicity and effects of metals to fish

1.4.1 General

In brief, the toxicity of metal species, depends on the dose (concentration and exposure time). Rates of uptake and excretion are therefore important host factors, together with host sensibility (life stage, preliminary exposure etc.). The most important external factors affecting metal toxicity are chemical speciation, concentration, time of exposure and route of entrance (Klaassen 2001, Clearwater *et al*. 2002). In addition, toxicity of metals correlates with several physical and chemical properties of ions, mostly cations (Cationic Activity Relationships) (Walker 2003).

Differences in toxicity result from differences in cationic metal binding to biological molecules (ligand-binding). Generally, high concentrations may give acute respiratory, osmoregulatory and immunologic dysfunctions. Acute respiratory effects are caused by increased mucus secretion on epithelum and thickening of blood-water barrier. Coagulated mucus on gills may block the gas exchange and cause death by hypoxia. Such effects may be manifested as low pO_2 and high pCO_2 in arterial blood leading to blood acidosis, accompanied with an increase in blood glucose and hematocrit depletion. Precipitation of cationic heavy metals on gills may give acute osmoregulatory effects, such as increased permeability of water, decreased uptake of ions or renal dysfunction (reduced urine dilution and blood buffering). These effects are manifested as a decrease in blood concentration of Na^+, Cl^- and HCO_3^- (and hence decline in blood pH).

1.4.2 Pb

Fish exposed to Pb may develop acute dysfunctions in respiration and osmoregulation, as mentioned above. The major factor affecting Pb-toxicity to fish is alkalinity due to an antagonistic uptake of Pb and Ca. Rogers *et al*. (2006) concluded that Pb exposure may inhibit influx of Na^+ and Cl^-. Pb-induced disruption of Na^+ and Cl^- homeostasis is in part a result of rapid inhibition of carbonic anhydrase activity and binding of Pb to Na^+-K^+-ATPase, causing noncompetitive inhibition of Na^+ and Cl^- influx. Na^+-K^+-ATPase is one of seven channels and transporters that are active in ion uptake by the chloride cells and pavement cells (Hirose *et al*. 2003). Both inorganic and organic species are toxic, and the latter is the most toxic (i.e. alkylated Pb, previous used as additive in gasoline) (Klaassen 2001). The toxicity of Pb complexed to natural organic acids has not been examined (Lydersen *et al*. 2002). Concentrations above 10 μg Pb/L are expected to provide severe long-term effects on fish. The acute toxicity (4 day, LC_{50}) has been reported between 700 and 4100 μg Pb/L for different

salmonid species (Atlantic salmon, rainbow trout and brook trout) and different water hardness (Lydersen *et al*. 2002).

1.4.3 Sb

Sb is considered as a non-essential metal to fish. In mammals, Sb may be absorbed in the gastrointestinal tract and lung (Klaassen 2001). Toxic effects of Sb are not well described, but in mammals Sb seems to follow the same metabolic pathways as arsenic (As). There are few published data on the uptake and effect of Sb in fish. Trivalent forms are reported to be more toxic than pentavalent forms (Filella *et al*. 2002). However, it has not been possible to find any data on uptake in freshwater fish and critical concentrations of antimony species in the literature.

1.4.4 Zn

Zinc and copper are essential trace metals, as they are key components in enzymes needed to sustain all life. For example, zinc is a functional component of carbonic anhydrase (maintains acid-base balance in blood and other tissues by interconversion of CO_2 and HCO_3^-) and the functional site in many proteins playing a role in intracellular signaling (many transcription factors and regulatory proteins contain functional structures called zinc-fingers that interact with DNA). In mammalian, neural cells, cells in the immune system and intestine cells all secrete zinc. The primary acute effect of Zn in fish is (as mentioned above) increased mucus production in gill epithelium, and blockage of gas exchange. Chronic exposure may affect the reproduction, due to reduced egg production and larvae with cellular disruption (Somasundaram 1985, In: Lydersen *et al*. 2002). Freshwater fish are generally more sensitive to Zn than marine species. For freshwater fish, behavioral modifications have been reported at 5-6 µg Zn/L, chronic effects at 10-25 µg Zn/L, and acute lethal/sublethal effects at 50-340 µg Zn/L (Lydersen *et al*. 2002).

1.4.5 Cu

Copper is found in a variety of enzymes, including the copper centers of cytochrome c oxidase (electron transfer chain) and the enzyme superoxide dismutase, containing copper and zinc (catalyzes the reduction of free radicals such as superoxide anions to hydrogen peroxide). In addition to its enzymatic roles, copper is used for biological electron transport. Despite the need for Cu to maintain cellular functions, fish are relatively sensitive to high Cu-concentrations. At the cellular level Cu-excess may affect the enzymatic activity, causing reduced growth. The acute effects are similar as for zinc (hypoxia), but Cu has effect at lower concentrations. Grande (1991) found a critical level for negative effects of Cu on the fish communities in 27 Norwegian rivers at 20 µg/L (In: Lydersen 2002). Freshwater fish are generally more tolerant to Cu than marine species. For freshwater fish, behavioral modifications have been reported at 4 µg Cu/L. As for Zn, the toxicity of Cu decreases with increasing ionic strength (Erickson *et al*. 1997). In soft waters, lethal effects in juvenile salmonids (96h LC_{50}) have been reported in the range of 18-25 µg Cu/L (Lydersen *et al*. 2002).

1.5 The ALA-D biomarker

Pb is known to inhibit the synthesis of heme, due to interaction with δ-aminolevulininc acid (ALA-D), an enzyme found in erythrocytes. In mammals this may cause anemia, but this has

not been reported to arise in fish. In fish, ALA-D is nevertheless considered as a selective and fast responding biomarker of Pb toxicity. Significant decreases in the activity of ALA-D in rainbow trout were observed after a 29-day exposure to 121 and 201 μg Pb/L, but not after exposure to 29 or 48 μg Pb/L (Burden *et al*. 1998).

1.6 Objectives and hypotheses in the present work

Norwegian Defence Research Establishment (FFI) and University of Life Science (UMB) established a cooperation project with the aim to investigate if heavy metals in shooting range effluents represent an environmental risk. The link between the speciation of metals in water (Pb, Sb, Cu, and Zn) and the uptake/effects in aquatic organisms has been particularily scarce documented, and it has also been of interest to provide data on how changes in water quality during runoff events affect the speciation and the uptake of the studied elements. Brown trout was selected as a model organism because it is a well-studied and abundant species, easy to obtain and handle, and of public interest. Based on previous studies and literature data, the following hypotheses were made:

- Pb, Cu and Zn accumulate on fish gills and accumulate in the liver.
- Bioaccumulation factors (BAF) of Pb, Cu and Zn in fish gills correlate negatively with pH and [Ca]. Regression models predicting BAF of cationic and low-molecular-mass species have stronger correlation coefficients than models predicting BAF from other fractions or the total metal concentration.
- Sb will not accumulate in fish.
- Accumulated Pb inhibits ALA-D activity in red blood cells.
- Exposed fish will develop respiratory and osmoregulatory dysfunctions, measured as abnormal levels of glucose, Hct, Na and Cl in whole blood.

2 METHODS

2.1 Field sites

A field study was conducted at Avgrunnsdalen military training area, 5-28 December 2005 (Fig 2.1). Brown trout (*Salmo trutta L.*), age 1+, weight 15-70 g, were exposed to a shooting range effluent (Fig 2.1 A) and water from a clean brook nearby (Fig 2.1 B). The drainage basins are approximately 0.5 km^2 each, and the annual mean discharges are 8 L/s in both streams (Beldring *et al*. 2002). The geology is dominated by coarse granite (higher areas), marshy soils (lower areas) and dispersed deposits of loam and glacial drift.

2.2 Experimental design

Water was pumped from the small streams into fish tubs, in a flow-through system as illustrated in Fig 2.2. A pre-survey of the study area in September 2005 showed that the control site was more acidic and had higher Al content than the shooting range effluent. Therefore, water from the control stream was limed (~2 pH units) with coquina (with low Cd-content) (Fig. 2.2 A). To avoid toxic polymerization of Al in the fish tubs (Fig 2.2 D), a reservoir tank (2.2 C) was

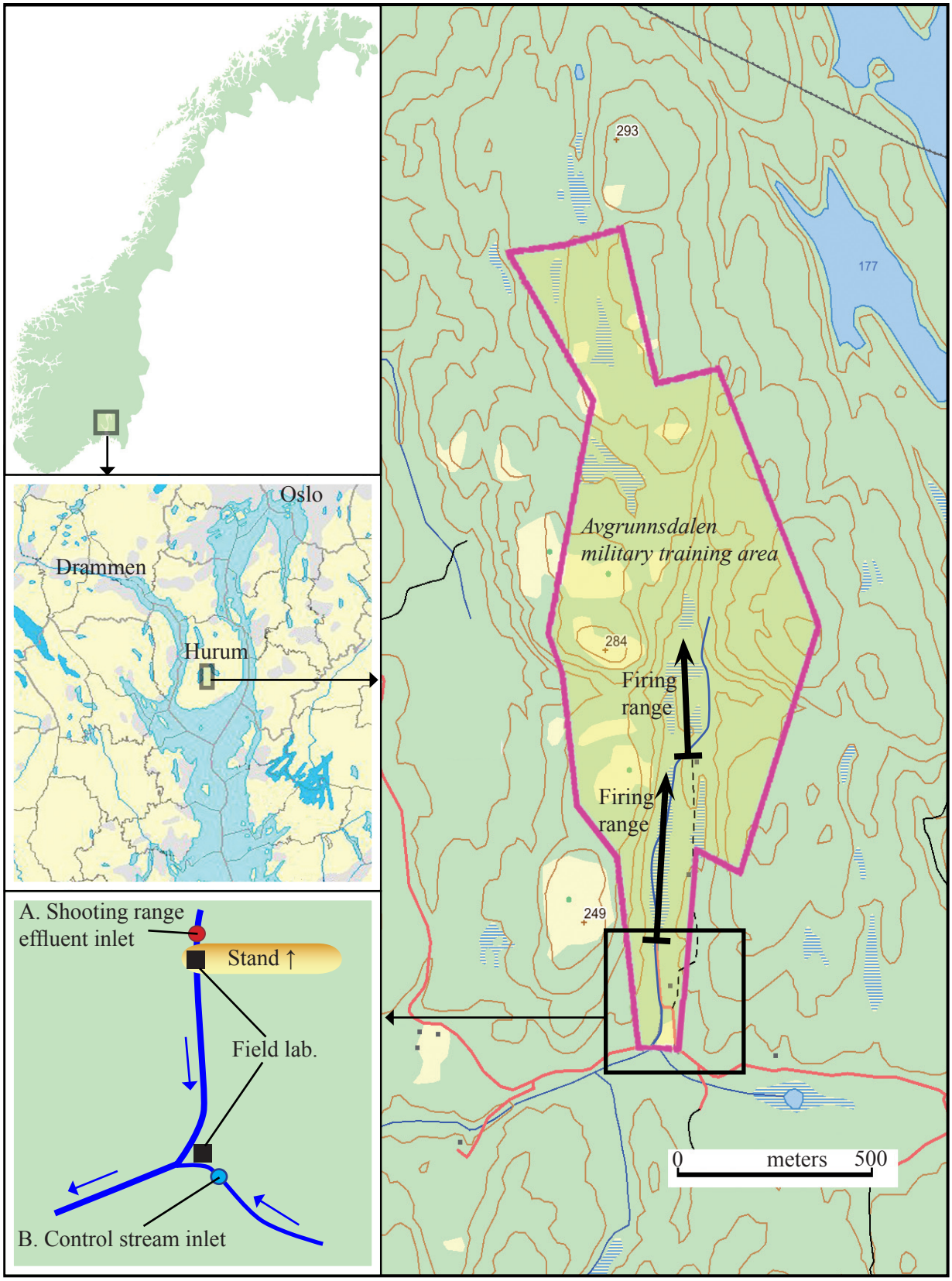


Figure 2.1. *Location of Avgrunnsdalen military training area, Hurum, Southern Norway. The facility (91 ha) was established in 1917 for small arms training. Sites for water intake are marked A (shooting range effluent) and B (control site).*

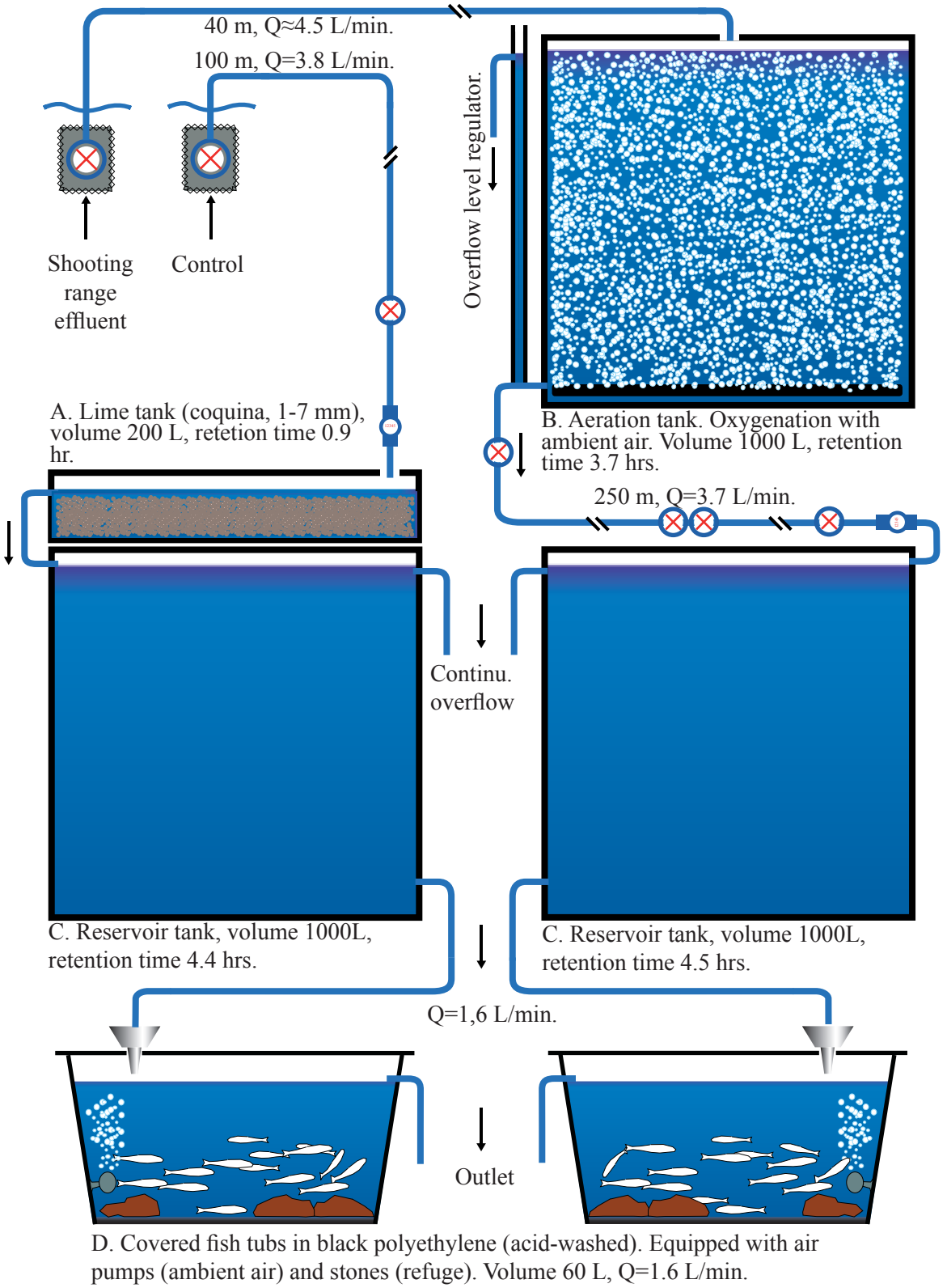


Figure 2.2. Experimental flow-through system. The input water was aerated with ambient air (B) and limed (C) pumped into reservoir tanks with continuous overflow to keep the flow rate constant.

Water hoses, Ø=1/2″ — *Impeller flow meter.*

Supply pumps with inlet filter (~1mm). — *Support pumps.*

placed subsequent to the lime tank. Total retention time after liming was 5.3 hrs. The pre-investigation also showed that the shooting range effluent was rich in Fe(II), which may lead to toxic Fe-precipitation on gills (causing hypoxia). In order to oxidize Fe(II) to Fe(III), water from the effluent was oxygenated with ambient air in an aeration tank nearby the intake (fig. 2.2 C). Due to ambient temperatures below 0°C, all water containers and pipelines above water surface were insulated and heated with self-adjusting heater cables. The fish tubs were placed in a heated container, designed for in-door sampling (Fig 2.3).
To minimize confounding stress factors, feeding of fish was stopped 3 days prior to transport from hatchery. Fish were carefully transported (1 hr) in a 200 L oxygenation tank aerated with 100 % O_2, and transferred into fish tubs keeping the same water temperature. Fish tubs were designed to minimize stress due to light (tight covers), social interactions (stone bedding) and insufficient saturations of O_2/CO_2 (air pump with submerged nozzles). All equipment in contact with water were checked for liberation of heavy metals.

Figure 2.3. Custom-built transport container (A), containing fish tubs, power supply, automatic water sampler/logger (B: ISCO Model 6700 Portable sampler/Ysi multiprobe 600R) and facilities for in situ sampling (water fractionation, blood testing, organ sampling) and storage facilities. Photo: A E Strømseng.

2.3 Sampling procedures

Samples of water and fish were collected 7 times during the experiment (at start of experiment, and after 2, 4, 7, 9, 11 and 23 days of exposure). Water samples were collected directly from the fish tubs. Samples requiring storage at -80°C were temporarily put on liquid nitrogen, others were brought to a storage facility within 4 hrs.

2.3.1 Sampling of water

At each sampling date, total and fractionated water samples were collected for determination of the parameters listed in Tab. 2.1. In addition, pH, temperature and ionic strength were measured manually (in addition to the automatic logging). Water samples were fractionated *in situ* according to size and charge properties. Size-fractionation was performed using filtration (membrane filter, 0.45µm) and ultrafiltration (hollow fibre, nominal cut of 10 KDa) (principle in fig 2.4). Charge-fractionation was performed using ionic exchange chromatography. To quantify positively charged trace element species, the cation-exchange resin Chelex-100 was used. For quantification of negatively charged trace element species, the anion-exchange resin AG1-X8 was used.

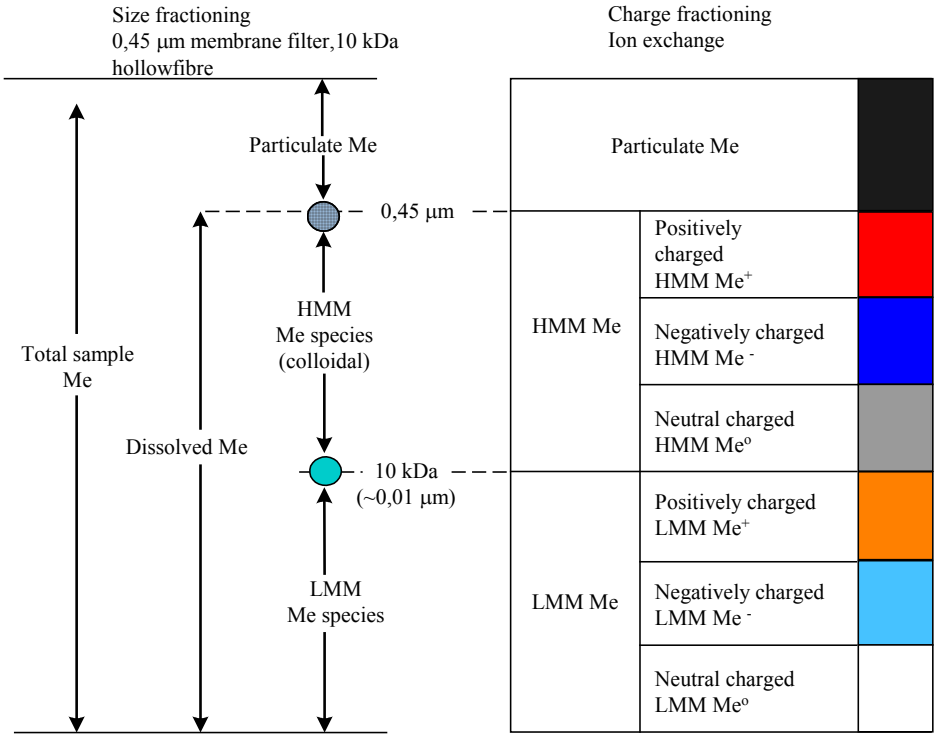


Figure 2.4. Metal fractions obtained using 0,45µm membrane filteration and hollow fibre ultrafiltration (10 kDa) interfaced with ion chromatography and liquids extraction.

To obtain an efficient size- and charge-fractionation, a combined fractionation system was used. The water was size- and charge-fractionated simultaneously using a combined interphased fractionation system. When interphased, the continuously size-fractionated sample is transferred directly to the column for charge-fractionation without any storage. The method is also described in Heier *et al*. (2004).

Water samples were collected in 100 mL plastic bottles. Water samples for trace element analysis were acidified with suprapure nitric acid (0.5%). For determination of Fe^{2+} in the water, 20 mL of water were added to a ferrous iron reagent (1 g of 1,10-phenanthroline monohydrate 1-10%). All samples were stored cold and dark.

Figure 2.5. *A syringe (1 mL) with luer needle (0.5x16mm) was used to draw blood from the caudal vein (inserted at the gut fin towards the ventral side of the spine). Photo: A E Strømseng.*

Table 2.1 Fractions of water sampled for determination of different analytes.

Fraction	**To be analysed for**
Total	Metals, major ions
Total	Organic carbon
Particular (0.45 µm membrane filter)	Particle characterization
Dissolved (0.45 µm filtrate)	Metals
Dissolved (0.45 µm filtrate)	Organic carbon
Dissolved cationic (0.45 µm filtrate, chelex-100 eluate)	Metals (eluent ÷ eluate)
Dissolved anionic (0.45 µm filtrate, AG1-X8 eluate)	Metals (eluent ÷ eluate)
Low molecular mass (10 KDa hollowfibre filtrate)	Metals
Low mol. mass (10 KDa hollowfibre filtrate)	Organic carbon
Low mol. mass cationic (10 KDa hollowfibre filtrate, chelex-100 eluate)	Metals (eluent ÷ eluate)
Low mol. mass anionic (10 KDa hollowfibre filtrate, AG1-X8 eluate)	Metals (eluent ÷ eluate)
Total (complexed with 1,10-phenanthroline-1-hydrate 1-10%)	Fe^{2+}

2.3.2 Sampling of fish

At each sampling date, 5 fishes were collected (one by one) for determination of metal accumulation (gill and liver) and biomarker response (blood and liver) (Tab. 2.2) by the following procedure: Fish were killed by a hit to the head, weighted and measured. Blood was sampled immediately from the caudal vein (fig. 2.5). Whole blood was directly analyzed for blood gases, plasma ions and acid/base parameters using an I-STAT portable Clinical analyzer with EC8+ cassettes from Abbot Inc. The remaining blood (ca 0.2 mL) was filled on Eppendorf microtubes and centrifuged for 5 minutes. After removal of blood plasma, the microtubes were perforated and put on liquid nitrogen for ALA-D quantification. The 2nd gill arch (on the right side) was then excised and placed in a vial for determination of gill reactive metals. The abdomen was opened, and the liver was carefully separated from the bile bladder. The distal part (approximately 0.25 g) was put on a pre-weighted evaporation-safe plastic vial for metal burden anaylsis.

Table. 2.2. Storage of fish samples.

Tissue	Sample containers	Storage temp. (field/lab)
Whole blood	Analyzed *in situ*	-
Erythrocytes	Eppendorf microtubes 1.5 mL	Liquid N/-80°C
Gill (2nd right arch)	Plastic vials acid-washed, 25 mL	+4°C/-20°C
Liver (distal)	Vials as for gills, pre-weighted	+4°C/-20°C
Liver (proximal)	cryo-tubes, 1 mL	Liquid N/-80°C

2.4 Data logging

Each sampling, pH, temperature, conductivity, gas saturation (O_2/CO_2) and flow rates were measured in the fish tubs. Water levels were measured in the two streams in order to calculate the flow. In addition, the shooting field effluent was continuously logged for pH, temperature and water level, using a multi-probe/bobble flowmeter connected to a logger unit (Tab 2.3).

2.5 Analyses

Water samples, fish gills and liver were analyzed at University of Life Sciences. ALA-D was analyzed at Norwegian Institute for Water Research (NIVA). Analyses preformed on the different samples are listed in Tab. 2.4.

Table 2.3 Manual and automatic data registration.

Data logged	Instruments
pH	Manually: Hanna Instruments HI 9224 Automatically: YSI multi-probe 600R*
Conductivity	Manually: WTW Multi 340i
O_2-saturation	Manually: WTW Multi 340i
CO_2-concentration	Manually: Oxyguard
Water level	Manually: Millimeter rule Automatically: ISCO bubble-flowmeter*

* Connected to ISCO Model 6700 Portable Sampler logger unit.

Table 2.4. Parameters determinated in water and tissue samples

Matrix	Parameters
Water	Trace elements (Pb, Cu, Zn, Sb, Al, Fe, Mn) Major ions (Ca, Mg, K, F, Cl, SO_4^{2-} , NO_3^-)
Fish blood	Whole blood: Na, K, Cl, glucose, Hct, pH, pCO_2 Erythrocytes: ALA-D
Fish gills	Pb, Cu, Zn, Sb, Fe, Mn, Al
Fish liver	Pb, Cu, Zn, Sb, Fe, Mn, Al

2.5.1 Water chemistry

Organic carbon was measured in total and fractionated water samples using a Shimadzu TOC-V cpn Total organic carbon analyzer. Anions (Cl^-, SO_4^{2-}, F^- and NO_3^-) were measured in total water samples using an Iachat IC5000 Ion chromatograph. Pb, Cu, Zn, Sb, Al, Fe and Mn in fractionated water samples and digested liver and gill samples were measured using a Perkin Elmer Sciex ELAN 6000 (ICP-MS). Al, Fe, Mn, Zn, Ca, Na, Mg, K and Si were also measured using Perkin Elmer Optima 5300DV (ICP-OES). Blanks and quality control standards were used in all analyses. Fe^{2+} samples were measured using a Hach DR2000 spectrophotometer (λ=510nm), within a few hours after sampling.

2.5.2 Metal accumulation in gills and liver

Gills were freeze dried, weighed and digested in HNO_3 and H_2O_2, then diluted with MQ water processed by MilliQ water system (by Millipore) to a 2% HNO_3 solution. Internal standards for ICP-MS measurements were added before the gills were digested. Liver samples were treated in the simular way, except from being freeze dried. The metal concentration in the gill and liver digested samples were determined using ICP-MS. The results are reported as μg/g dry weight for gills and μg/g wet weight for livers.

2.5.3 Biomarker quantification

ALA-D were measured at NIVA according to their procedures. The ALA-D activity was determined in red blood cells, basically as described by Hodson *et al.*, (1984) modified as in Hylland (2004). Samples were coded internally and analyses performed blind. The results are reported as ng PBG/min/mg protein.

2.5.4 Statistical analysis

Statistical significance of time course changes in metal accumulation and biomarker response were assessed by one-way ANOVA (post hoc analysis verified by LSD test). BAF-correlations were tested by linear regression (Ca and pH as independent variables). Preliminary to statistical analysis, data were checked for normality (qq-plots) and tested for variance equality (Levene's test). Analyses were preformed using the data statistical computing program SPSS 13.0.

3 RESULTS

3.1 General water data

Estimated water flow varied from 1-5 L/s (water level 22-40 cm, Fig 3.1) in both streams. A peak in discharge was registered at Dec. 12th, due to increased melting water runoff. At the same time a drop in conductivity (-55%) was registered in both streams (control ≈ 40-90 µS/cm, shooting range effluent ≈ 20-45 µS/cm). The conductivity stayed at this level the next 4 days. During the following 2 weeks, the conductivity increased at a constant rate back to the base flow level (Fig 3.1). In the shooting range effluent pH varied inversely to water flow in the range from 5,4-5,9.

The limed control water was alkaline, with a pH range from 7.2-7.7 (Fig 3.1). Water temperatures (in fish tubs) were stable (no day/night variation), ranging from 1.5-4°C (Fig 3.1).

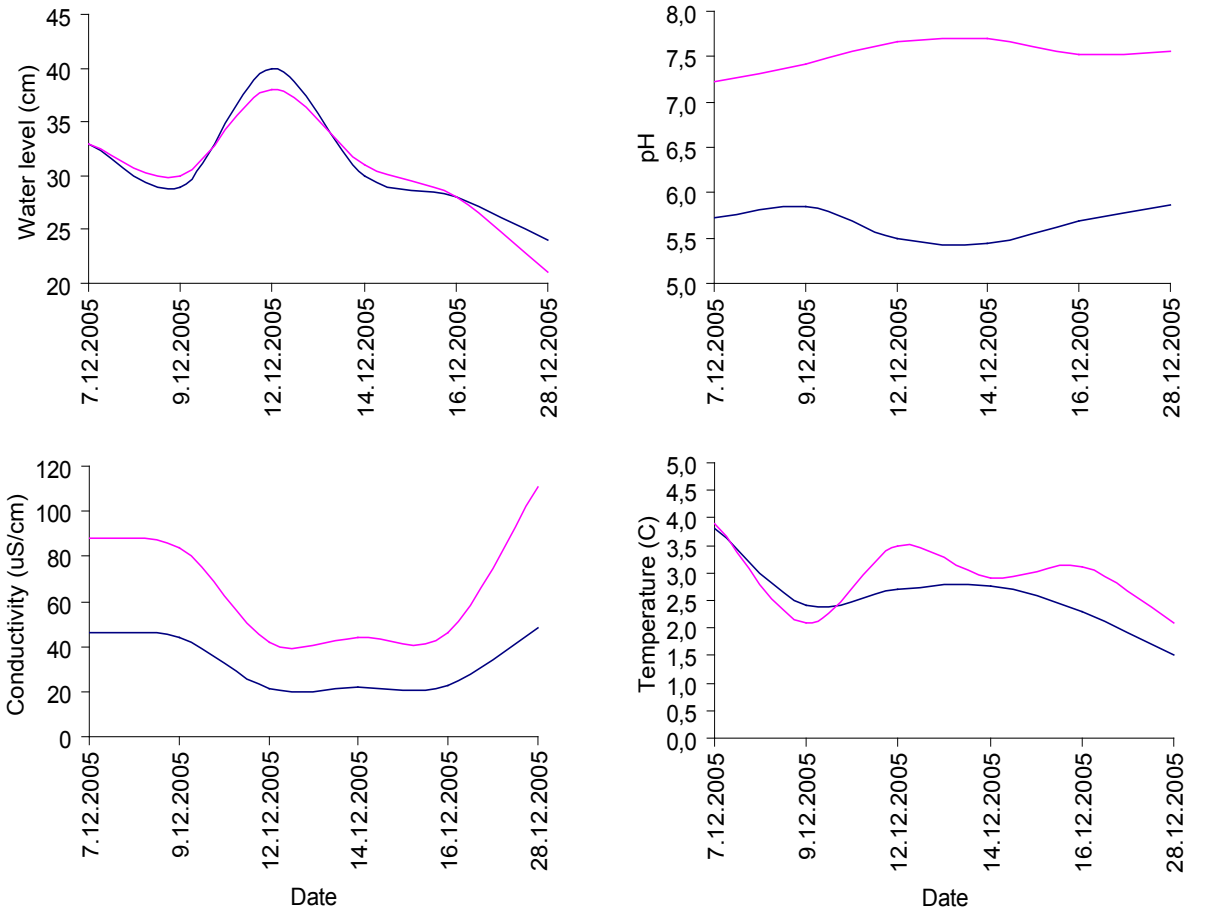


Figure 3.1. Water data from the shooting range effluent and the control stream. Water level was registered in the two streams, while pH, conductivity and water temperature were measured in the fish tubs.

3.2 Discharge and speciation of heavy metals

Total concentration of Pb in the shooting range effluent was correlated to flow rate, ranging from 15-45 μg/L. Pb was almost exclusively found in the dissolved fraction, contrary to earlier findings (where Pb mainly has been found associated to particles >0.45μm). Generally, ~50% of the total Pb was found as cationic species, mainly associated to the high molecular mass (HMM) fraction (10 KDa<HMM<0.45 μm). This fraction (HMM^+) was the most responsive during the snow melt event (fig. 3.2a). Sb was exclusively detected in the dissolved fraction, ranging from 2-3 μg/L (Fig 3.2b). Sb was mainly found as anionic ions associated to molecules of low molecular mass (LMM<10 KDa). The concentration of Sb did not respond to changes in runoff. Cu was detected in the range from 11-18 μg/L, and the discharge of Cu-species followed the same pattern as Pb (mainly HMM^+). However, a greater part of Cu was non-reactive (strictly combined or electro-neutral species) compared to Pb (Fig 3.2c). Zn was exclusively found as dissolved cationic ions (Fig. 3.2d) in concentrations from 26-35 μg/L, mainly in the LMM^+ fraction. The Zn-concentration showed a minor response to changes in water flow fraction, as the HMM^+ and non-labile LMM^0 fraction increased during snow melting. Fe was found in concentrations from 318-417 μg/L, mainly as non-labile species associated to molecules with high molecular mass (HMM). Nearly all of the detected Mn (totally 56-83 μg/L) was found as cationic species in the dissolved fraction (18-55% HMM^+, 51-67% LMM^+). As with Pb and Cu, the concentration of Al increased during snow melting, ranging from 360-550 μg/L. Al in all fractions ($HMM^{+,-,0}$ and $LMM^{+,-,0}$) responded positively to water flow, except the particular fraction which was absent during snow melting.
The concentrations of heavy metals in the control stream were 0.2-0.3 μg Pb/L, 66-86 ng Sb/L, 0.4-1.4 μg Cu/L, 8-10 μg Zn/L, 162-208 μg Fe/L and 19-130 μg Mn/L (fig. 3.2a-d). Al was found in concentrations from 295-487 μg/L. The result from the speciation shows that Zn in the control was similar to the shooting range effluent. However, Zn did not dominate in the LMM^+-fraction in the control. As for Zn, there were minor differences in speciation of Fe, Mn and Al between the two streams. In the control, Fe was not found in the LMM fraction, Mn was more prominent in the LMM^+- and LMM^0-fractions and a greater part of Al was found as anionic species.

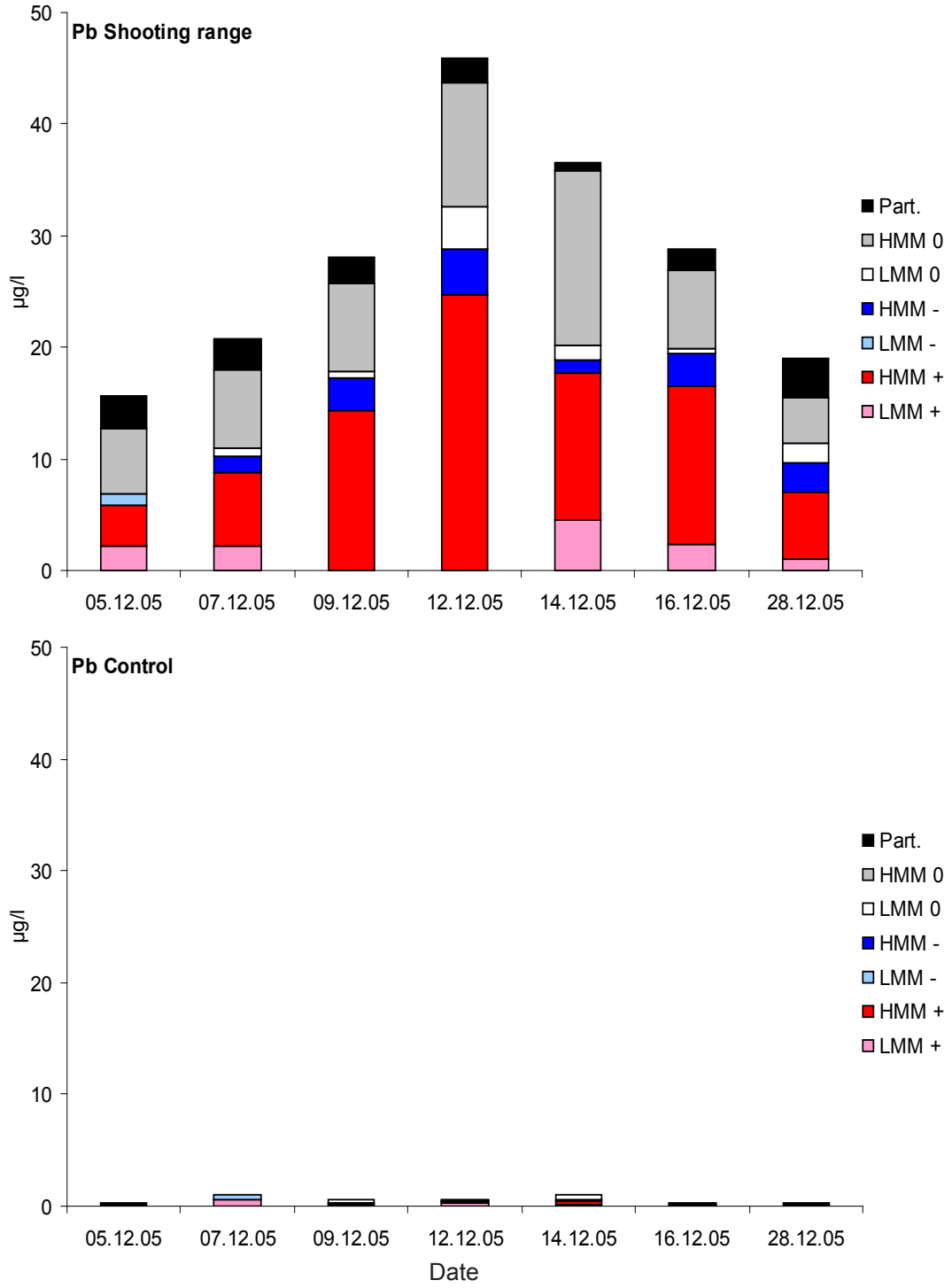


Figure 3.2a. *Concentration of Pb species found in the shooting range effluent and the uncontaminated stream (control) at each sampling. Pb speciation is presented as cationic (+), anionic (-) and non-labile (0) ions found in the low molecular mass fraction (LMM<10 KDa), in the high molecular mass fraction (10 KDa<HMM<0,45µm), or assoicated to particles (Part.>0,45µm).*

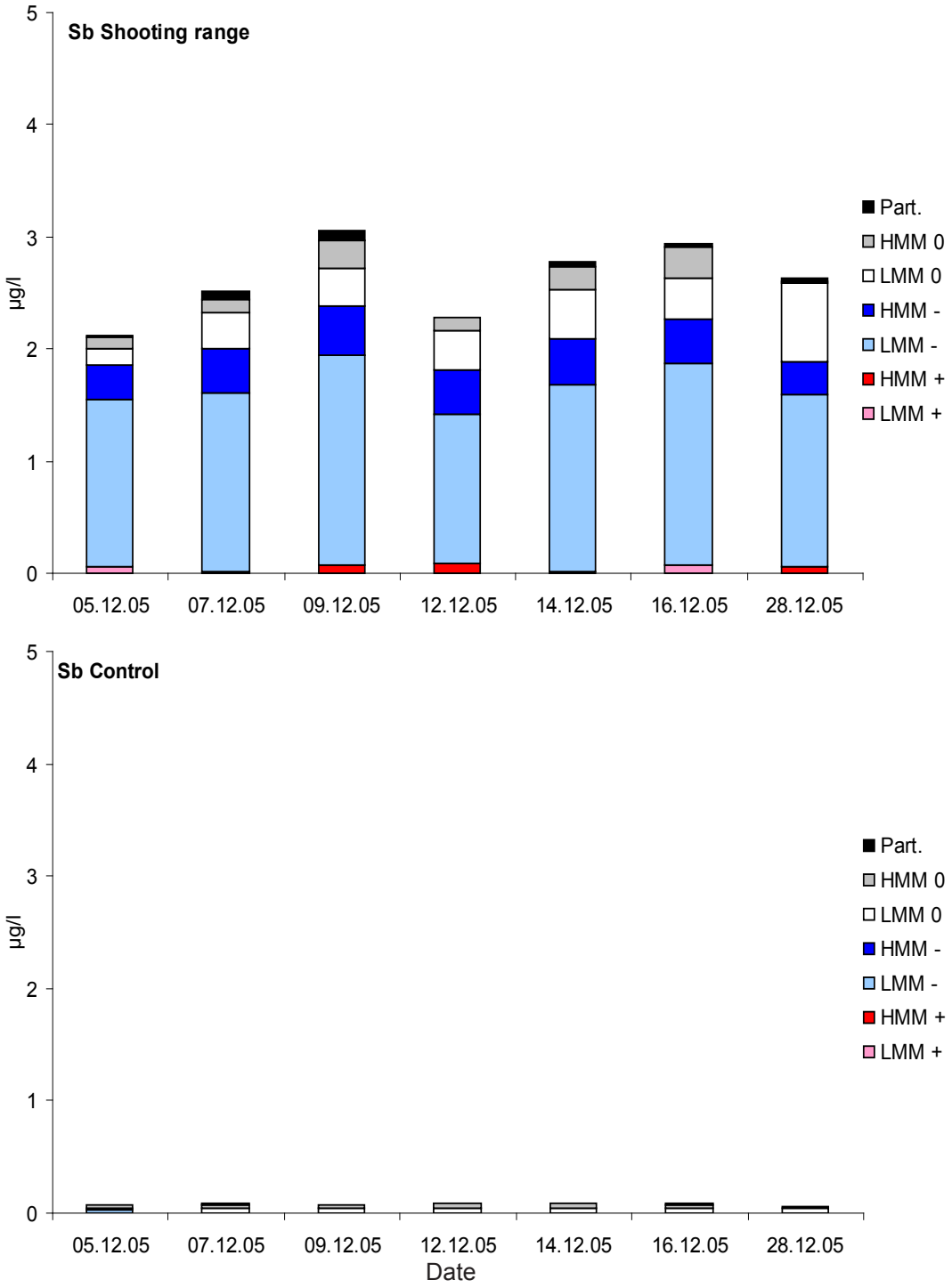


Figure 3.2b. *Concentration of Sb species found in the shooting range effluent and the uncontaminated stream (control) at each sampling. Sb speciation is presented as cationic (+), anionic (-) and non-labile (0) ions found in the low molecular mass fraction (LMM<10 KDa), in the high molecular mass fraction (10 KDa<HMM<0,45µm), or assoicated to particles (Part.>0,45µm).*

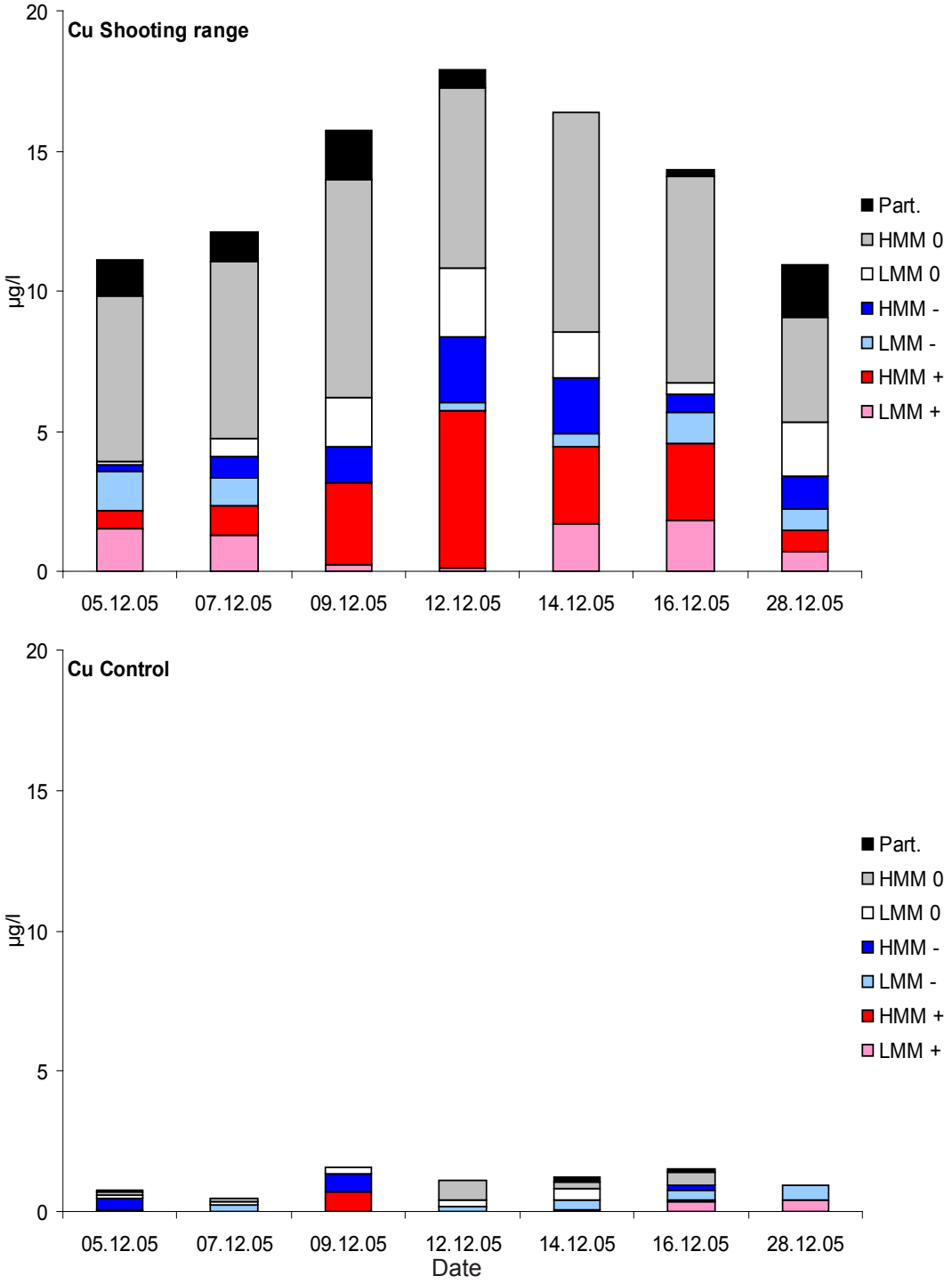


Figure 3.2c. *Concentration of Cu species found in the shooting range effluent and the uncontaminated stream (control) at each sampling. Cu speciation is presented as cationic (+), anionic (-) and non-labile (0) ions found in the low molecular mass fraction (LMM<10 KDa), in the high molecular mass fraction (10 KDa<HMM<0,45µm), or assoicated to particles (Part.>0,45µm).*

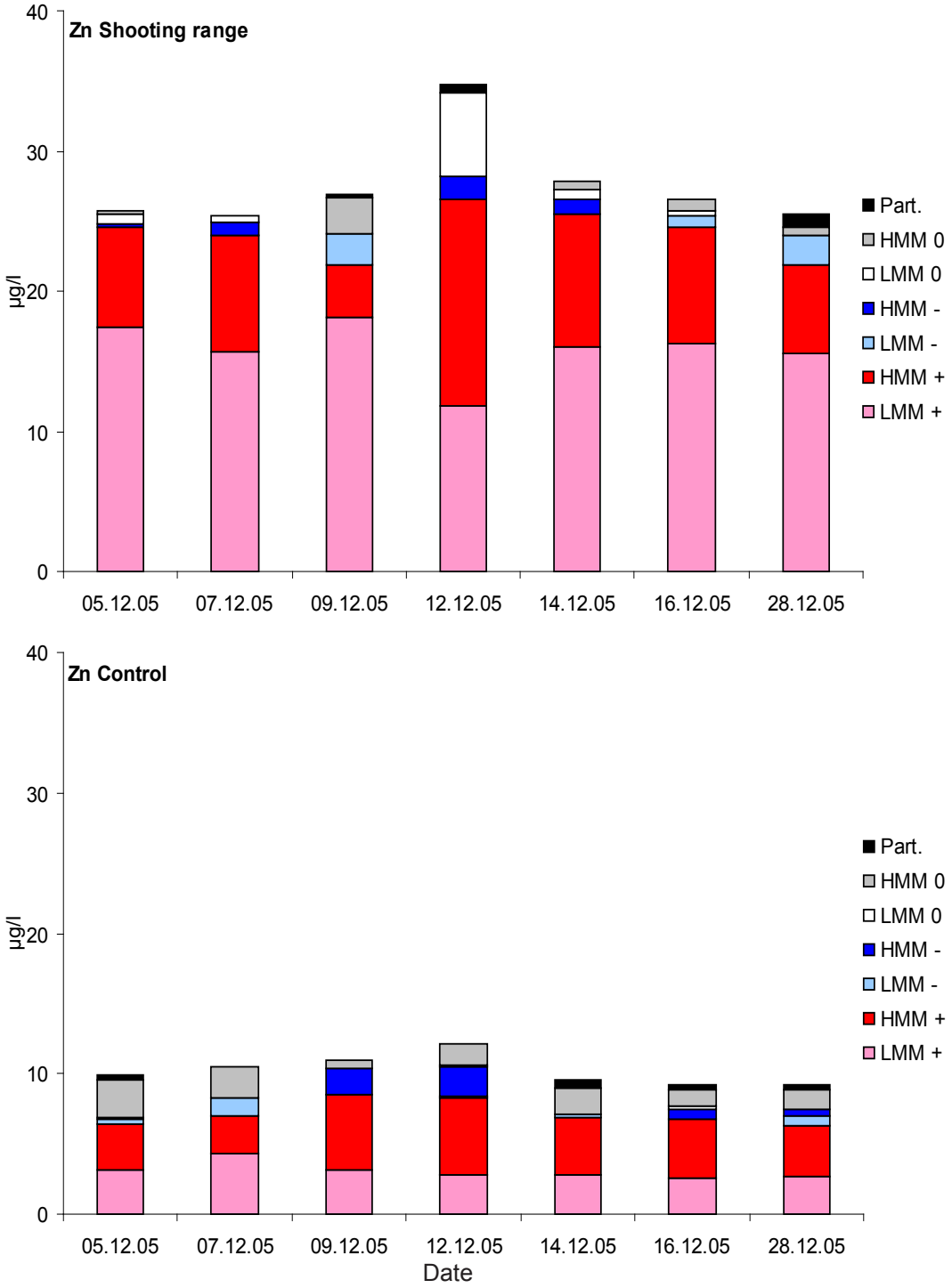
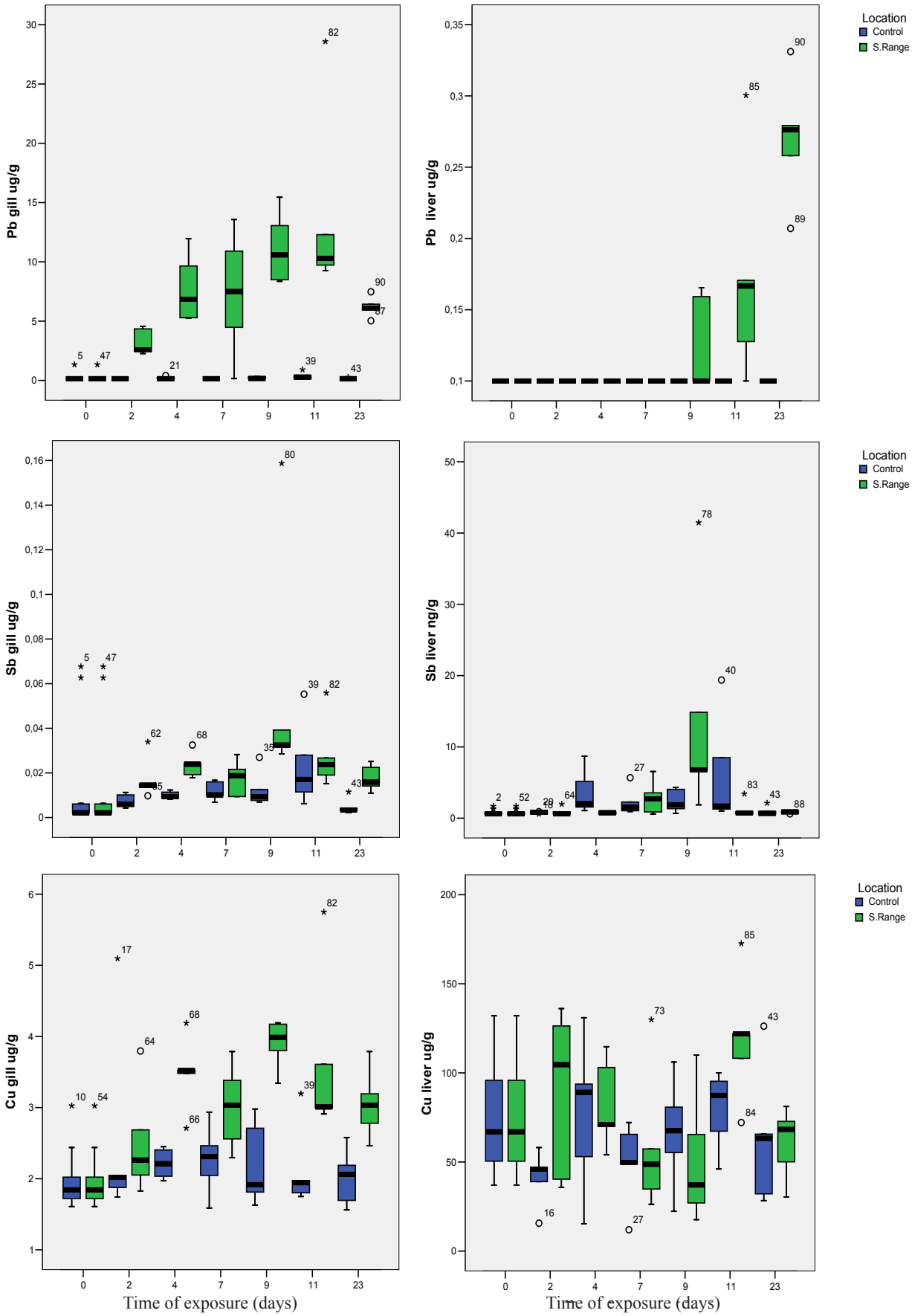


Figure 3.2d. *Concentration of Zn species found in the shooting range effluent and the uncontaminated stream (control) at each sampling. Zn speciation is presented as cationic (+), anionic (-) and non-labile (0) ions found in the low molecular mass fraction (LMM<10 KDa), in the high molecular mass fraction (10 KDa<HMM<0,45µm), or assoicated to particles (Part.>0,45µm).*


Pb gill ug/g
Pb liver ug/g
Sb gill ug/g
Sb liver ng/g
Cu gill ug/g
Cu liver ug/g
Location
Control
S.Range
Time of exposure (days)
Time of exposure (days)

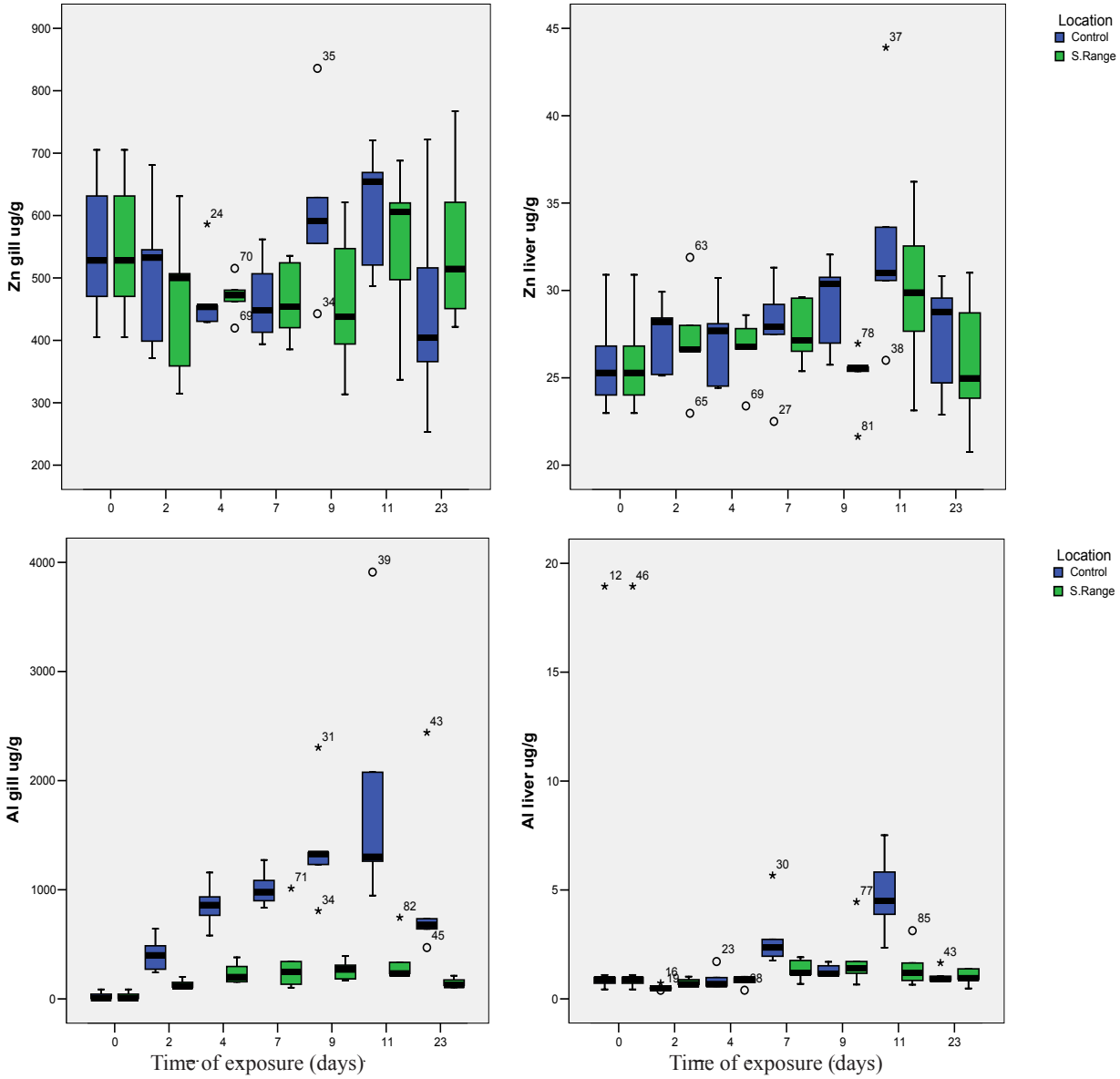


Figure 3.3. *Concentration of Pb, Sb, Cu, Zn and Al in gill (dry weight) and liver (wet weight) from brown trout after 0-23 days of exposure in the shooting range effluent (green boxes) and the control stream (blue boxes).*

3.3 Accumulation of heavy metals in fish

Fish exposed to the shooting range effluent showed significant gill-precipitation and liver-accumulation of Pb, Sb and Cu, but not Zn (Fig. 3.3 and Tab. 3.1). No accumulation of heavy metals was detected in fish exposed to the control stream. However, Al significantly accumulated in gills and liver in the control group (Fig 3.4). In the shooting range effluent, precipitation of Pb on gills was increasing the first 9 days (significant from day 4, see Tab. 3.2), and showed great individual variation during snow melting. After 9 days the concentrations of Pb in gills stabilized at ~10 µg/g, declining to ~6 µg/g after 23 days.

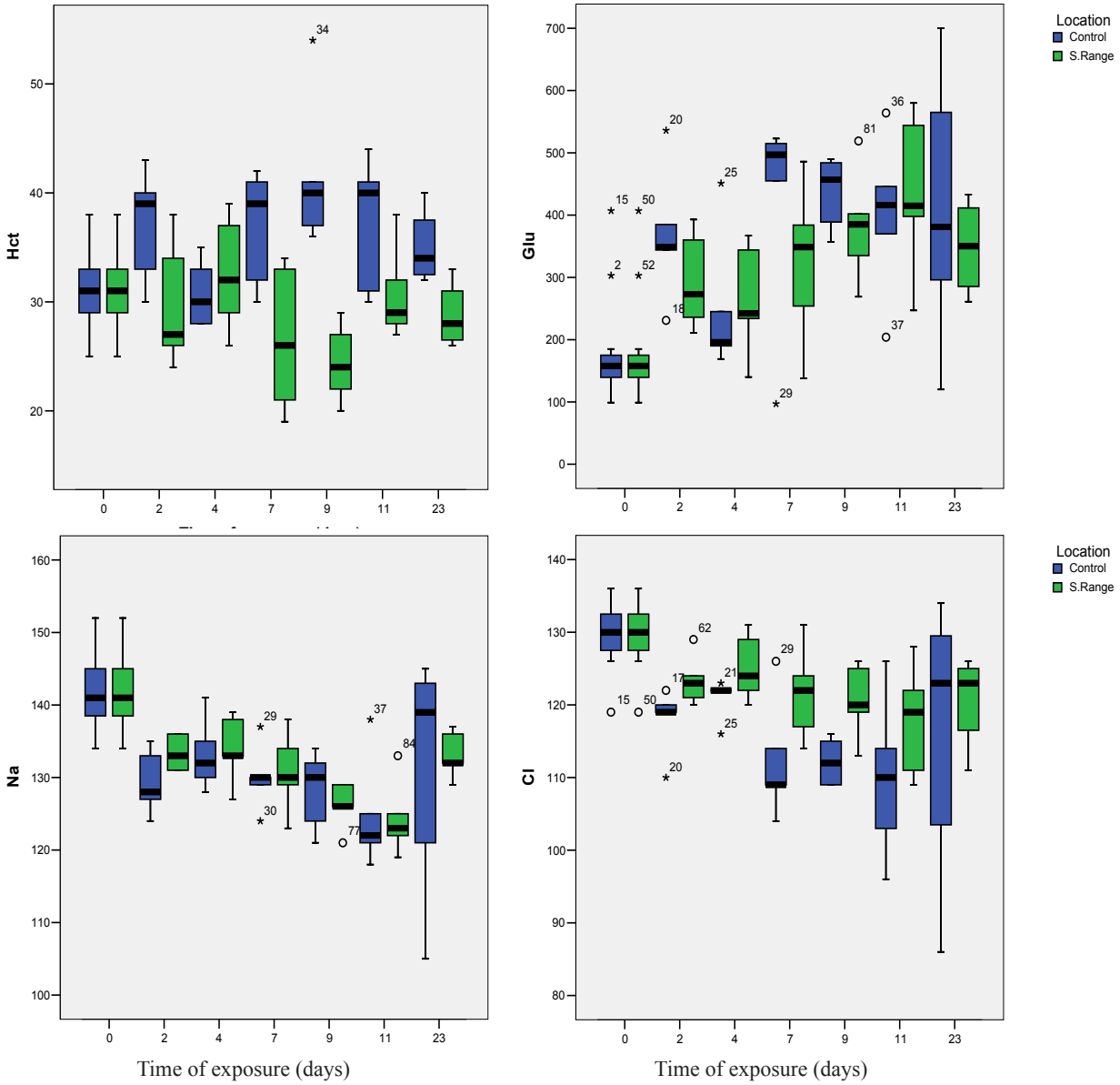


Figure 3.4. *Hematocrit (% red blood cells) and concentrations of glucose, Na and Cl in whole blood from brown trout after 0-23 days of exposure in the shooting range effluent (green boxes) and the control stream (blue boxes).*

Accumulation of Pb in liver was detected from day 9, was significant from day 11, and increased (to 0.28 µg/g) at the end of experiment (Fig 3.3). Accumulation of Sb in gills and liver showed great individual variations in fish from both streams. In the shooting range effluent this accumulation was significantly at day 9 (compared with start of experiment), and only at this sampling date.

Table 3.1 *One-way ANOVA analysis of fish parameters by time of exposure to the shooting range effluent. Tested variables are metal levels (Pb, Cu, Zn and Sb in gill and liver), and blood parameters associated with function of respiration and osmoregulation (Hct, glucose, Na, Cl).*

		Sum of Sq.	df	Mean Square	F	Sig.
Pb gill	Between Groups	1017	6	169.4	14.12	.000
	Within Groups	468.1	39	12.00		
	Total	1485	45			
Cu gill	Between Groups	23.35	6	3.891	10.61	.000
	Within Groups	14.30	39	.367		
	Total	37.65	45			
Zn gill	Between Groups	83270	6	13878	1.283	n.s.
	Within Groups	421794	39	10815		
	Total	505063	45			
Sb gill	Between Groups	.009	6	.001	2.637	.030
	Within Groups	.021	39	.001		
	Total	.030	45			
Pb liver	Between Groups	.134	6	.022	22.75	.000
	Within Groups	.036	37	.001		
	Total	.170	43			
Cu liver	Between Groups	16670	6	2778	2.350	.049
	Within Groups	46113	39	1182		
	Total	62784	45			
Zn liver	Between Groups	91.83	6	15.31	1.871	n.s.
	Within Groups	310.8	38	8.179		
	Total	402.6	44			
Sb liver	Between Groups	787.3	6	131.2	4.668	.001
	Within Groups	1040	37	28.11		
	Total	1828	43			
Na blood	Between Groups	1765	6	294.1	13.94	.000
	Within Groups	823.1	39	21.10		
	Total	2588	45			
Cl blood	Between Groups	844.0	6	140.7	4.944	.001
	Within Groups	1081	38	28.47		
	Total	1926	44			
Glucose blood	Between Groups	359824	6	59971	6.795	.000
	Within Groups	335365	38	8825		
	Total	695190	44			
Hct blood	Between Groups	274.0	6	45.67	2.166	n.s.
	Within Groups	801.2	38	21.08		
	Total	1075	44			

Table 3.2 ANOVA Post Hoc LSD test (p<0,05) of differences in parameters measured in fish from start of experiment to a given time of exposure in the shooting range effluent.

Variable	Time of exposure (days)					
	2	4	7	9	11	23
Na	x	x	x	x	x	x
Cl	x		x	x	x	x
Glu	x		x	x	x	x
Hct				x		
Pb gill		x	x	x	x	x
Pb liver					x	x
Cu gill		x	x	x	x	x
Cu liver					x	
Zn liver					x	
Sb gill				x		
Sb liver				x		
Al gill		x	x	x	x	
Fe gill	x				x	x
Fe liver				x	x	
Mn gill	x	x				
Mn liver						x
As liver			x		x	
Se liver			x		x	
ALA-D					x	x

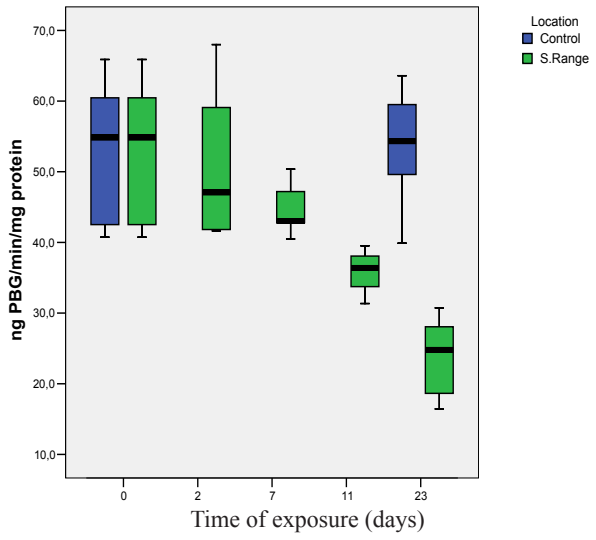


Figure 3.5. Activity of ALA-D (presented as ng porphobilinogen per min. and mg protein) after 0-23 days of exposure in the shooting range effluent (green boxes) and the control stream (blue boxes).

Accumulation of Cu on gills was significant from day 4 (Fig 3.3 and Tab. 3.2). In liver, Cu-concentration was significantly higher than the start concentration only at day 11 (~120 µg/g). Zn was not found in higher concentrations in fish from the shooting range effluent than the control group. In the shooting range effluent, however, Zn was detected in higher liver-concentrations at day 11 than at start of exposure (Tab. 3.2).

In the shooting range effluent precipitation of Pb on gills was positively correlated (r=0.58 p<0,01) to the concentration of Pb in unfiltered water (Pb total, Tab. 3.3a). Contrary to our expectations, precipitation of Pb was neither significantly correlated to the LMM^+ fraction nor the entire LMM fraction. The same pattern was found for Cu (r=0.62, p<0.01), but the LMM^+-fraction had a weak positive correlation (r=0,33, p<0,05) to gill concentrations. For Sb weak positive correlations were found for the total, LMM, and LMM^+ fractions (r=0.31, 0.32, and 0.35, respectively, p<0.05).

At each sampling, bioaccumulation factors (BAFs) were calculated for the total, LMM and LMM^+ fractions. BAFs by start and end are presented in Tab. 3.3b (for each BAF it is premised that this fraction alone is the only one to accumulate). To find causality between bioaccumulation, Ca-concentration in water and water pH, the BAFs were then analyzed for covariation with Ca and pH. For Pb and Cu, bioaccumulation of the LMM and LMM^+ fractions were significant when Ca and pH were included as dependent variables, though little variation was explained (Tab. 3.3c). BAFs of Sb-fractions were relatively

constant and neither affected by Ca-concentration in water nor pH.

3.4 Toxicity

Fish exposed to both streams had a significant decline in blood-Na and blood-Cl, and a significant increase in blood-glucose (Fig 3.4 and Tab. 3.1).

Hematocrit increased in control fish, but was not affected in fish exposed in the shooting range effluent. Exposure to Pb in the shooting range effluent caused inhibition of ALA-D in red blood cells after 11 days of exposure (Fig 3.5, Tab. 3.2 and Tab. 3.4). Depletion in ALA-D continued the following 2 weeks, following the accumulation of Pb in liver. ALA-D activity was not affected in fish from the control stream.

Fish from the control stream developed respiratory dysfunction (mucus coagulation and secondary infections) after 23 days (Fig 3.4a), and for this reason the experiment was terminated. Gills that were stained *in situ* with an Al-complexing agent (solochrome azurine) indicated that precipitation of Al on gills (Fig. 3.6) was the reason to the observed stress response. This indication was confirmed when the gills were analyzed for Al accumulation. The average accumulation of Al on fish gills exceeded 1 mg/g (Fig. 3.3). Teien *et al*. (2006) studied Atlantic salmon during a sea salt event and found that ~500 µg Al/g precipitated on fish gills caused 50% mortality within a month.

Table 3.3 A. Correlations between concentrations of Me-species in the shooting range effluent and precipitation of Me on fish gills. B. Average bioaccumulation factors of Me by start and end of experiment in the shooting range effluent. BAFs are calulated for Me in total water (Me total), species with low molecular mass species (LMM<10KDa) and cationic species with low molecular mass species (LMM^+<10KDa). C. Results from linear regression analysis of BAFs and the independent variables pH and Ca (mg/L).

	A	**B**		**C**				
	Corr. coeff.	BAF_{start}	BAF_{end}	[Ca]	pH	Constant	R^2	Sig.[a]
Pb total	.580**	15	274	-656	721	-2159	.436	.000
Pb LMM	n.s.	85	2765	-12981	24283	-99961	.515	.000
Pb LMM^+	n.s.	411	4194	-29351	37885	-129615	.283	.001
Cu total	.616**	177	237	n.s.	144	n.s.	.137	.042
Cu LMM	n.s.	656	1021	-1413	1941	-6263	.568	.000
Cu LMM^+	.330*	1341	1282	-970	1 279	n.s.	.131	.048
Zn total	n.s.	21799	22352	10379	n.s.	n.s.	.201	.008
Zn LMM	n.s.	30094	32414	n.s.	n.s.	n.s.	.056	n.s.
Zn LMM^+	n.s.	760414	287613	959346	n.s.	n.s.	.334	.000
Sb total	.314*	5.4	6.6	n.s.	n.s.	n.s.	.086	n.s.
Sb LMM	.315*	6.8	8.7	n.s.	n.s.	n.s.	.094	n.s.
Sb LMM^+	.348*	7.0	8.7	n.s.	n.s.	n.s.	.088	n.s.

a. ANOVA (regression vs. residual)
*. Pearson correlation is significant at the 0.05 level (2-tailed), n=46.
**. Pearson correlation is significant at the 0.01 level (2-tailed), n=46.

Table 3.4 One-way ANOVA of ALA-D activity (PBG/min/mg protein) by time of exposure in shooting range effluent and control stream.

		Sum of Sq.	df	Mean Sq.	F	Sig.
Shooting range	Between Groups	2904	4	725,9	10.86	.000
	Within Groups	1270	19	66.85		
	Total	4174	23			
Control	Between Groups	,603	1	0.603	.006	n.s.
	Within Groups	823,9	8	103.0		
	Total	824,5	9			

Figure 3.6. Solochrome Azurine staining of gills sampled from the control stream (left) and the shooting range affluent. Dark blue colour indicate precipitation of Al. Method as described by Denton et al. (1984). Photo: A E Strømseng.

4 DISCUSSION

Earlier investigations of Pb speciation during runoff events have found that an increase of the Pb concentration is mainly caused by flushing of Pb combined to particles (Heier *et al*. 2004). In this study we found that the particular fraction of Pb (and Cu) was almost absent, probably because a flushing of particles by a rainstorm prior to the experiment or that flushing of particles was reduced by the snow cover.

Dissolved Pb accumulated in gills and liver, as expected. However, the LMM^+ fraction of Pb was not significantly correlated to the precipitation of Pb on fish gills. Even when Ca and pH were included as variables, the LMM^+ fraction of Pb explained less variation in precipitation on gills than all of the size- and charge-fractions of Pb (pooled). It can therefore not be ruled out that Pb in the HMM fraction precipitated on fish gills (i.e. Pb species had stronger binding affinity to fish gills than the chelating molecules of high molecular mass). Hence, the accumulation of Pb may have been caused by flushing of Pb in the HMM fraction. These findings should however be interpreted with caution, because we do not know if the observed accumulation pattern occurred as a function of acclimatization, rather than a response to the flushing event. Anyway, the inhibition of ALA-D activity in red blood cells shows that a concentration of 15-45 μg Pb(Total)/L (6-25 μg Pb (dissolved cationic)/L) caused acute toxic effects in juvenile trout.

The hypothesis that Sb does not accumulate in fish gills was deduced as a result of search in literature. However, we found that Sb actually did concentrate in gills and liver, though it only was detected temporarily. Even though the uptake of Sb was correlated to the LMM^+ fraction of Sb, the uptake was not correlated with pH or Ca. This may indicate that Sb does not take part in the same transport mechanisms as Pb, Cu and Zn. We also found indications that excretion of Sb occurs immediately: When the gill concentration of Sb was reduced, the concentration in liver also declined. Hence, it is not likely that Sb will accumulate in gills or liver over time. On the other hand, it cannot be ruled out that Sb may have contributed to the acute dysfunction in osmoregulation that could be observed in fish exposed to the shooting range effluent.

Cu precipitated on gills, as expected. Accumulation of Cu in liver occured however only temporarly. This indicates that unacclimatized juvenile trout, even under low temperatures, is capable to handle (i.e. sequester or excrete) an acute liver burden of 170 μg Cu/g (wet weight). This may be confirmed by analyzing the enzymatic activity (e.g. methallothionein) in liver. As for Pb, the precipitation of Cu on fish gills was less correlated to the LMM^+ fraction of Cu than total-Cu in the water. Even when Ca and pH were included as variables, the LMM^+ fraction of Cu could not explain the variation in precipitation on gills. It can therefore not be excluded that Cu in the HMM^+ fraction is weakly combined in the HMM fraction, and hence may be bioavailable. This is earlier proved by Marr *et al*. (1999), who showed that Cu bound to organic complexes was available for uptake in rainbow trout.

Water concentrations of 26-35 μg Zn (Total)/L (12-22 μg Zn (LMM+)/L did not give any precipitation of Zn on gills of the juvenile trout, and only a slight increase of concentration in liver (from 25 to 30 μg/g). Still we were not able to connect the dysfunction in osmoregulation to a distinct stressor, and Zn can not be excluded as a potential stressor in the “cocktail” of heavy metals

5 CONCLUSION

Dissolved heavy metals caused acute and evident osmoregulatoric dysfunction in juvenile trout exposed to the shooting range effluent. The hypothesis that heavy metals in the low molecular mass fraction are the most bioavailable could not be retained, even when the water pH and Ca-content were included in the models. We found two possible explanations for this; either that the heavy metals associated to molecules > 10 KDa are bioavailable; or the observed accumulation pattern occurred as function of acclimation. A finding of special interest was the demonstration of Sb precipitation in fish gills and accumulation in fish livers. Among the heavy metals, the fish seemed more capable to handle the internal doses of Cu, Zn and Sb, than Pb. Pb was proved to inhibit the heme-synthesis in red blood cells, but it was not possible to connect accumulation of Cu, Zn and Sb to any specific toxic interaction. However, the patterns of accumulation and acute stress responses indicate that Pb probably was the main stressor. We also found indications that flushing events are more likely to cause chronic accumulation of Pb than Cu, Zn and Sb. In further investigations of how runoff events affect toxicity to fish, it is therefore important to focus on adaptation and potential chronic effects induced by Pb flushing.

Other findings of interest were the lethal dysfunction in respiration caused by precipitation of Al on gills in fish exposed for the control stream, and the acute mortality caused by ferrous iron that we detected in the pre-survey. In Norway, many rifle ranges are established on peat, where these metals may have high natural background levels. In aquatic risk assessment of pollution from rifle ranges, it is therefore necessary to incorporate focus on these metals as well.

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Bio-terminology

Bioaccumulation is a general term for the accumulation of substances in an organism or part of an organism, see also the more specified terms bioconcentration and biomagnification.

Bioaccumulation factor (BAF) describes bioaccumulation as the ratio of the concentration of a chemical observed inside an organism to the concentration observed in the surrounding environment:

$$BAF = \frac{C_{(obs)Biota}}{C_{(obs)Water}}$$

Bioactivity refers to the effect of a given agent upon a living organism or on living tissue.

Bioavailability describes the proportion of a chemical in the environment which is available for uptake by biota under environmental conditions.

Bioconcentration considers uptake from the non-living environment (e.g. water) in an organism or trophic level. The term includes the effect on an organism's internal concentration as a result of uptake via the respiratory surface and skin, internal distribution, metabolism and elimination.

Bioconcentration factor (BCF) of a chemical is defined by Opperhuizen (1991) as the ratio of its concentrations in the lipid phases in an organism to the water concentration during steady state or equilibrium. For each chemical it may be established a steady state BCF by partition coefficients as follows:

$$BCF = \frac{k_{Water}}{k_{Biota}} = \frac{C_{Biota}}{C_{Water}}$$

Biomagnification describes uptake through the food chain, or concentration from a trophic level to another.

Biomarkers are defined as a change in biological response (molecular, cellular, physiological or behavioral changes) which can be related to either exposure or toxic effects of environmental chemicals.