Effects of heavy metals from outdoor shooting ranges on aquatic organisms

Øyvind Voie and Espen Mariussen

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Emneord

Skytebaner

Metaller

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Kjetil S. Longva	Prosjektleder
Jan Ivar Botnan	Avdelingssjef

English summary

Metals deposited in shooting ranges (Pb, Zn, Cu and Sb) can affect fish health, fish populations and fish communities through a wide spectrum of mechanisms. In the current report the literature on the mechanisms of metal toxicity and their effects on fish are reviewed.

Some effects on fish are especially interesting since they are likely to affect the fish at a population or community level;

1) Effects on reproduction such as sexual maturation and larval growth and survival
2) Behavioural effects on spawning that again affect reproduction
3) Chronic stress

Effects on reproduction are interesting due to the sensitivity of the endpoints and because these effects are of ecological relevance. In addition to indirect effects on reproduction, the effects on behaviour can affect social structures that are important for the population as a whole. The stress response is interesting, since many adverse effects that show up in chronic studies are assumed to be due to chronic stress. A practical issue is that there are many biomarkers available for studying the stress response in fish.

Metal discharge from shooting ranges has a certain signature of relative concentrations of Pb, Cu, Zn and Sb. There is limited information on how this mixture affects fish. The fact that these pollutants may have additive, or even synergistic effects, is of concern. Additivity seems to be a frequent reported effect of these metals in mixture. It has been shown that metals in a mixture can give adverse effects by additivity even though the individual concentrations of the metals are below their ecotoxicological benchmark levels. This indicates that additivity is an important property of metals and should be taken into account in ecological risk assessments.

Two main strategies are currently recognized valid to predict the toxicity of a mixture: Concentration addition, also called Loewe additivity, and effect addition also called Bliss model of independent action. In order to predict a mixture effect, both models requires information on dose-response relationships of the single metals. We propose that in order to predict the mixture effects on fish of metals from shooting ranges, laboratory studies should be performed with concentrations of metals based on the signature reported from shooting ranges. Cage-exposure and field studies should then be performed in order to validate the model.

Sammendrag

Metaller deponert på skytebaner (Pb, Zn, Cu og Sb) kan påvirke fiskens helse, fiskepopulasjoner, og akvatiske samfunn gjennom et stort spekter av mekanismer. I denne rapporten er det gjort en litteraturstudie på metallers toksiske effekter på fisk.

Noen effekter på fisk er særlig interessante ettersom de kan innvirke på populasjons- eller samfunnsnivå;

1) effekter på reproduksjon slik som kjønnsmodning og vekst og overlevelse av fiskelarver
2) effekter på gyteadferd som indirekte har konsekvenser for reproduksjon
3) kronisk stress

Effekter på reproduksjon er interessant ettersom effektene inntreffer ved lave konsentrasjoner og fordi effekter på reproduksjonen kan ha konsekvenser på populasjonsnivå. I tillegg til indirekte konsekvenser for reproduksjon kan endret adferd påvirke sosiale strukturer som er viktige for populasjonen som helhet. Indusering av stressresponsen er interessant ettersom dette inntreffer ved lave konsentrasjoner og siden mange av de mer alvorlige effektene som fremkommer i kroniske studier, slik som effekter på immunsystemet, er antatt å ha bakgrunn i kronisk stress.

Avrenning av metaller fra skytebaner har en egen signatur når det gjelder relative konsentrasjoner av Pb, Cu, Zn and Sb. Det er lite kunnskap om hvordan denne blandingen av metaller virker på fisk. Potensialet for at disse stoffene har additive, eventuelt synergistiske effekter er av bekymring. Additiv effekt er hyppig rapportert for enkelte av disse metallene i blanding. Det er vist at metaller i blandning kan gi skadelige effekter på akvatiske organismer selv om konsentrasjonen av de enkelte metallene i blanding er under grensen for effekt. Dette indikerer at en kan forvente additiv effekt av metaller i blanding, noe som det bør tas hensyn til ved miljø-risikovurderinger.

To hovedstrategier er per i dag anerkjent for å kunne predikere giftigheten av stoffer i blanding: Konsentrasjonsaddisjon, også kalt Loewe additivitet, og Bliss modell for uavhengige mekanismer. For å predikere en effekt av stoffer i blanding, er begge modellene avhengig av dose-respons sammenhenger for enkeltstoffene. Det anbefales å benytte laboratoriestudier med en blanding av metaller basert på den signaturen man finner i feltstudier, for å predikere effekter på fisk i forbindelse med avrenning fra skytebaner. Burforsøk i bekker som mottar vann fra skytebaner, eventuelt andre feltstudier på stedegen fisk, kan så gjennomføres for å validere modellen.

Abbreviations

As	arsenic
Ca	calcium
Cd	cadmium
Cl	chloride
Cr	chromium
Cu	copper
Hg	mercury
Na	sodium
Ni	nickel
Mg	magnesium
Mo	molybdenum
Pb	lead
Sb	antimony
W	tungsten
Zn	zinc
AChE	acetylcholine esterase
ALA-D	δ-aminolevulinic acid dehydratase
ATP	adenosine triphosphate
APT	antimony (III) potassium tartrate
BCF	bioconcentration factor
BLM	biotic ligand model
CAT	catalase
CF	concentration factor
CO2	carbon dioxide
DNA	deoxyribonucleic acid
Eco SL	ecotoxicological screening levels
EROD	7-ethoxyresorufin-O-deethylase
GABA	gamma amino butyric acid
GPx	glutathione peroxidase
GR	glutathione reductase
GSH	glutathione S-transferase
Hb	hemoglobin concentration
Ht	hematocrit value
IC	inhibition concentration
LC	lethal concentration
LD	lethal dose
LDH	lactate dehydrogenase
LPO	lipid peroxidation
LPS	lipopolysaccharide
MATC	maximum acceptable toxicant concentration
MT	Metallothionein
NOEC	no observable effect concentration

RBC	red blood cells, erythrocyte count
ROS	reactive oxygen species
ODC	ornithine decarboxylase
SD	standard deviation
SH	sulfhydryl
SOD	superoxide dismutase
TOC	total organic carbon
US-EPA	United State Environmental Protection Agency

Innhold

1 Introduction

1.1 Background

In Norway there are approximately 65 military areas for small arms training where more than 500 shooting ranges are distributed. In addition to the military shooting ranges, there are even more civilian ranges. Outdoor shooting ranges can be seriously contaminated by heavy metals, and metalloids. The composition of the small arms ammunition may vary, but consists typically of lead (Pb), copper (Cu), zinc (Zn), and antimony (Sb). The bullets are fragmented by hitting other bullets in the berms, or by hitting rocks. Once in the soil, the bullets and the bullet fragments gradually oxidise through the weathering actions of air, water, organic acids and microbial activity (Lin *et al*., 1995; Johnson *et al*., 2005; Labare *et al*., 2004). Metals from the ammunition residues may then leach into the soil and surrounding watercourses where they pose a threat to exposed wildlife. There is reason to believe that discharge of heavy metals into the watershed may influence organisms living there. Several studies have reported effects on the terrestrial organisms and ecosystem at outdoor shooting ranges. These effects include changes in the community of soil arthropods and litter decomposers (Migliorini *et al*., 2004; Tuomela, 2005; Rantalainen *et al*., 2006; Kähkönen *et al*., 2008), poisoning of local songbirds (Vyas *et al*., 2000; Johnson *et al*., 2007; Bennet *et al*., 2007) and calves pasturing in the target area of the shooting range (Braun *et al*., 1997). Few field studies have been performed on effects on aquatic biota from shooting ranges. The method frequently used by ecotoxicologists is to use the range of chronic toxicity data, as shown in Table 1.1, to derive toxicity screening levels or water quality criteria. When a measured value in a recipient exceeds the ecotoxicological screening level there might be aquatic organisms at risk in the recipient. A screening of 26 military training areas containing a varying number of shooting ranges revealed that concentrations of heavy metals and antimony in streams exceeded ecotoxicological screening levels (Eco SL) in about half of the surveyed areas (Mørch *et al*., 2007). In 35 % of the surveyed streams the concentrations of Cu exceeds the Eco SL and in 16 % of the streams the concentrations of Pb exceeds the Eco SL. In addition there are a few streams that exceed the Eco SL for Sb, nickel (Ni), chromium (Cr) and cadmium (Cd). The elevated levels of heavy metals are not found in the water system as a whole, but are confined to some areas in the vicinity of the shooting ranges.

The sensitivity of organisms to metals varies substantially, even within same species depending on specific conditions. Laboratory conditions, of which the water quality criteria is derived, are generally different from field conditions where all the factors affecting toxicity of metals such as water hardness, pH, organic content, episodic events, and genetic diversity come into play. An example of genetic diversity is variation in gill binding capacity of metals in different populations (Birceanu *et al*., 2008). In addition, aquatic organisms are exposed to a mixture of contaminants in the environment, making risk assessment even more complicated.

Table 1.1 Norwegian ecotoxicological screening levels (Eco SL) for protection of aquatic organisms (Lydersen, 2000), and the water directive (EU, 2000).

Metal	*Pb*	*Cu*	*Zn*	*Sb*
Eco SL (μg/l)	*7.2*	*3.0*	*50*	*No value*

1.2 Main Objectives

The great numbers of recipients with potential hazardous concentrations of metals from shooting ranges, makes it important to have proper tools to perform risk assessment in order to reduce the uncertainty that arise when laboratory conditions are extrapolated to field conditions. In spite of numerous studies on the effects of metals on aquatic organisms it has been a challenge to make a synthesis of the findings. Still, risk assessment is generally based on the most sensitive organisms in laboratory conditions and site specific conditions in the field are not taken into account. In order to develop a tool with the purpose of characterizing the risk from metal exposure more exactly, we aim with this report to review the literature on the mechanisms of metal toxicity and their effects on aquatic organisms. In this respect it is crucial to gain knowledge about how the organisms have been exposed in the studies in addition to concentration levels and type of endpoints.

Metal discharge from shooting ranges has a certain signature of Pb, Cu and Sb, and there is limited knowledge on how they interact. The possibility that these pollutants have additive, or even synergistic effects, is of concern. In the last section we will therefore give an overview of the available literature on effects of mixtures of metals and discuss how one can approach to assess the effects of mixtures. In relation to effects of mixtures we will discuss other factor that influence the toxicity, such as water hardness, in order to achieve a better understanding of risk at site specific conditions. Although we will cover relevant information on different aquatic species, emphasis will be on fish communities. Fish is regarded as one of the most sensitive aquatic organisms (Table 1.2), and is of importance, not only in recreational fishing, but also due to their commercial value in some areas. Fish communities are in addition shown to be sensitive biomarkers of external influences, such as pollution and climate changes.

Table 1.2 Overview of chronic NOECs (µg Pb/L) for freshwater and saltwater organisms (Data summarised by Tukker et al., 2001; Janus, 2000.)

Taxonomic group	NOEC (µg Pb/L)		Toxicological endpoints
Freshwater	Mean ± SD (n)	Range	
Bacteria	1,183 ± 683 (3)	450-1,800	Growth
Unicellular algae	10,005 ± 55,744 (15)	10-200,000	Growth
Multicellular algae	1,033 ± 945 (3)	300-2,100	Growth
Protozoas (fresh water)	403 ± 604 (4)	20-1,300	Growth, reproduction
Molluscs	204 ± 317 (3)	12-570	Hatching, survival
Crustaceans	502 ± 913 (8)	1-2,500	Reproduction, survival, growth
Fish	77 ± 74 (17)	7-250	Reproduction, survival, growth, abnormalities, development, hatching

2 Effects of metals on aquatic organisms – mechanisms of toxicity

2.1 How to measure effects of metals on fish

Risk assessment is defined as the systematic characterization of potential adverse effects resulting from exposures to hazardous agents or situations. In order to characterize risk one has to identify the hazard and gain knowledge about dose-response, exposure and emission characteristics. Usually, intensive research is required to achieve good estimates of risk. Numerous studies are performed on aquatic organisms in order to reveal metal toxicity. However, relatively few studies can be regarded as relevant in order to do proper risk assessment. Often the study designs cannot be extrapolated to the field due to use of unrealistically high concentrations to reveal lethal dose, both from acute and chronic exposure. In a recent study by Vieira *et al*., (2009) LD_{50} for Cu and Hg were found to be 568 and 62 µg/L respectively in a 96h study, whereas effects on swimming behaviour were observed at 50 and 3 µg/L of Cu and Hg respectively. Swimming performance is obviously crucial for fish survival. Lethality tests, therefore, in general only give information about relative toxicity between different compounds. Usually aquatic organisms are exposed to sub-lethal concentrations, which can reduce the viability of a population, but less often lead to obvious visible effects in a short time frame. Another approach to investigate effects is with use of *in vitro* tests. Such studies are cost- and time effective to perform and suitable for mechanistic research, but often have small predictive power and cannot easily be extrapolated to field situations. An alternative approach is therefore to combine *in vivo* and *ex vivo* studies in laboratory of which fishes are exposed to a toxicant and subjected to e.g. behavioural or reproductive tests followed by analysis of a range of biomarkers for effects, which might have consequences for the fish condition. Such biomarkers can be extrapolated for use in the field, both as early markers for certain effects and as well as for exposure.

A range of different effects on aquatic organisms have been reported following metal exposure. These include effects on the endocrine, nervous and immune system, reproduction, growth and

condition and detrimental effects on the gills influencing ion balance and respiration. All of which may influence both individuals as well as populations. The most sensitive effect parameters at sub lethal concentrations appear to be reproduction, behaviour and immune effects, whereas at higher concentrations effects on gills are more pronounced. Effects on reproduction, behaviour and immune system may be due to a range of factors that might be interconnected, such as effects on parameters in the endocrine and nervous system, induction of oxidative stress and other stress related factors e.g. in blood, metabolism and regulation of enzymes. Effects on the gills are usually due to direct effects on ion regulating proteins, such as sodium and calcium exchange proteins or obstruction of respiration.

To reveal potential effects it is developed a set of biomarkers of effect and exposure, which are supposed to be sensitive tools to reveal potential risk following exposure. For example ALA-D (δ-aminolevulinic acid dehydratase), which is an enzyme in the heme synthesis, is a sensitive biomarker for lead exposure. ALA-D activity in fish blood is inhibited by low lead concentrations in the water. However, whereas inhibition of heme synthesis in mammals is detrimental, the significance of ALA-D inhibition in fish is still not clear, but clearly indicates that the fish are at risk. A challenge is therefore to link biomarkers to effects on a higher level, such as behavior or reproduction.

Copper is an element that has been subjected to intensive research. A range of responses have been reported from Cu exposure of which elevation of the hormone cortisol and an increase in adrenergic response, such as release of catecholamines, can explain many of the observed effects such as effects on behavior and reproduction (Handy, 2003). Vieira *et al*., (2009) made an effort to link biomarkers of effects to behavior effects following Cu and Hg exposure. They found that sub-lethal doses influenced swimming behavior of an estuarine fish (*Pomatoschistus microps*). In addition they analyzed effects on several biomarkers such as inhibition of acetylcholine esterase (AChE), lipid peroxidation (LPO), lactate dehydrogenase (LDH), glutathione S-transferase (GSH), 7-ethoxyresorufin-O-deethylase (EROD), superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and glutathione peroxidase (GPx). Positive correlations between behavioral effects and biomarkers were found on AChE and EROD activity and negative correlations were found for LPO and the anti oxidant enzymes. AChE activity may be directly involved in behavior, whereas effects on the anti-oxidant system may be response on stress. For Cu the effects on the biomarkers appeared at lower concentrations than the effect on behavior, indicating that these can be used as early markers for potential effects on swimming behavior.

For application in environmental risk assessment we recommend that endpoints should be selected according to some criteria. 1) The endpoint should be sensitive enough to reflect events that can occur at environmental relevant concentrations. 2) More weight should be given to endpoints that cause more severe effects. A weight of evidence approach has been suggested by Sanchez *et al*., (2008), where endpoints are ranked from low to high weight of evidence in the following order; chemical level < biochemical level < histological level < population level < community level. Further, if there is an established plausible mechanism that relates the endpoints to population or community effects they should be given more weight of evidence. This appears

difficult, but has been demonstrated in three spined sticklebacks (*Gasterosteus aculatus*) in the Rhonelle river, where effects on a set of biomarkers and physiological parameters were clearly affected by water pollution and fish assemblage was moderately disturbed and characterized by a clear decline in the number of young stickleback (Sanchez *et al*., 2008). According to these criteria endpoints on reproduction are interesting since they are both sensitive and have ecological relevance as shown in Figure 2.1 (adapted from Sanchez and Pocher, 2009). In the following chapters different effect endpoints are reviewed in respect to their ecological relevance and sensitivity with emphasis on effects of metals used in ammunition.

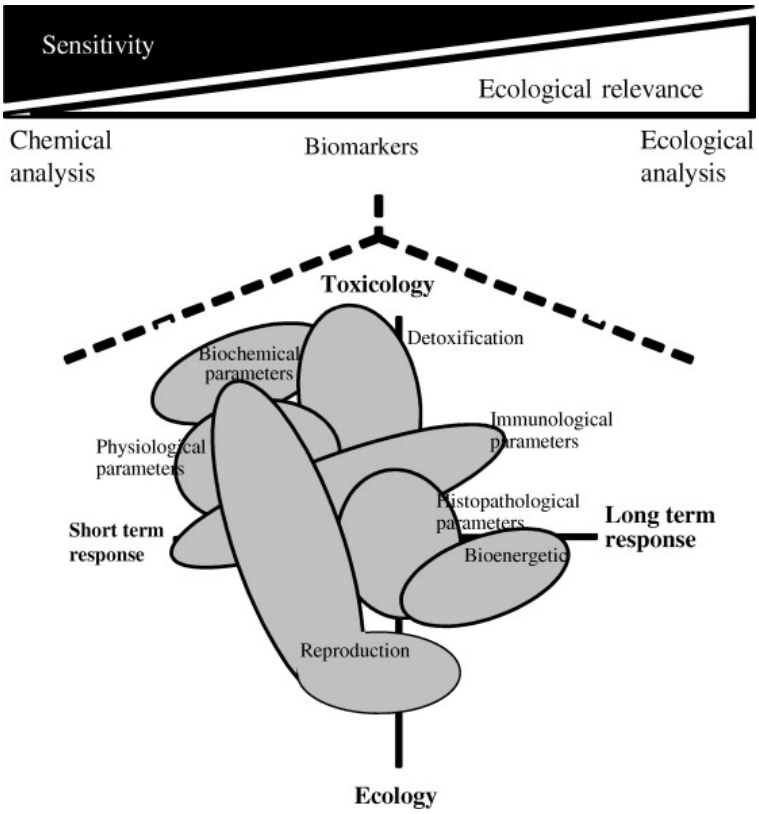

Figure 2.1 Position of biomarkers among other environmental monitoring methods according to their specificity, ecological relevance and temporal sensitivity (from Sanchez and Procher, 2009).

2.2 Toxicity screening levels and bioaccumulation

2.2.1 Ecotoxicological screening levels (EcoSL)

Measuring the concentration of metal contaminants in water and the subsequent comparison with established ecotoxicological screening levels (EcoSL) is often the initial method used to access the potentially risk in a contaminated aquatic system. EcoSLs are based upon the most sensitive laboratory studies and do not necessarily mean that effects occur above these levels. Factors such as organic content, pH, and water hardness can reduce the sensitivity of the endpoints as reviewed below in this report. EcoSLs are therefore used to screen areas and select the localities where the concentration of metals exceeds the EcoSLs for further studies. EcoSLs for Pb, Cu, and Zn are listed in Table 1.1.

2.2.2 Accumulation of metals in fish

Bioaccumulation of the metal are measured in whole body or selected organs. Background concentrations from the region can be used to reveal bioaccumulation that impart from naturally occurring concentrations. In Norway background levels of Pb, Cu, and Zn in freshwater fish are 0.1, 0.8 and 10 mg/kg respectively for muscle, and 0.2, 40, and 80 mg/kg for liver (Grande, 1987). Some authors also calculate the accumulation factor (CF) or bioconcentration factor (BCF). Cu shows the greatest affinity for liver, but sometimes high concentrations may be present in the gills. Also in unpolluted water fish have certain amounts of Cu in the liver since it is an essential metal. Lower concentrations of Cu are found in the spleen, kidneys, and digestive tract (Jezierska and Witeska, 2001). Lead deposits mainly in the liver, kidney and gills, but also spleen and bones may contain high levels. In some cases lead can be found in brain (Jezierska and Witeska, 2001). The highest concentrations of Zn are observed in the gills, but are also found in the liver, spleen, kidneys and digestive tract.

2.3 Molecular and cellular endpoints

Effects on the molecular and cellular level are often related to adverse effects in the fish, and should be interpreted as an early warning of potential damage and are often sensitive biomarkers. Molecular and cellular effects where the causal connection with an eventual adverse effect is understood should be given more weight. Beyond that, effects on the molecular and cellular level are useful in order to elucidate the mechanism behind the adverse effects. Some of the observed effects on the molecular level, such as changes in pH, ion, glucose and protein content in blood plasma, are secondary effects of metal-induced toxicity other places in the organism.

2.3.1 Stress response

Stress response has been defined as a condition in which the dynamic equilibrium of animal organisms called homeostasis is threatened or disturbed as a result of the actions of intrinsic or extrinsic stimuli, commonly defined as stressors (Wendelaar-Bonga, 1997). The actions of stressors are twofold: they produce effects that threaten or disturb the homeostatic equilibrium, and they elicit a coordinated set of behavioural and physiological responses thought to be

compensatory and/or adaptive, enabling the animal to overcome the threat. If an animal is experiencing chronic stress, the stress response may lose its adaptive value and become dysfunctional, which may result in inhibition of growth, reproductive failure, and reduced resistance to pathogens (Wendelaar-Bonga, 1997). Stress changes are non-specific and are similar for various metals. They often involve compensatory reactions as increased glucose level, erythrocyte count (RBC), hemoglobin concentration (Hb), and hematocrit value (Ht) as well as alteration of cortisol level in fish blood. An increase of cortisol concentration in plasma has been observed in fish exposed to Cu and Pb (Jezierska and Witeska, 2001). Hematological disturbances often occur earlier than other symptoms of intoxication. The reason behind the stress response is partly due to the effect of the metals on branchial structure and, as a result, hydromineral balance. However, the most important reason is the great variety and exquisite sensitivity of the sensor systems of the integument. Fish respond to metals at intensity levels frequently far below those that can be perceived by terrestrial mammals (Wendelaar-Bonga, 1997). The stress response includes the principal messengers of the brain-sympathetic-chromaffin cell axis and the brain-pituitary-interrenal axis, as well as their functions, involving stimulation of oxygen uptake and transfer, mobilization of energy substrates, reallocation of energy away from growth and reproduction, and mainly suppressive effects on immune functions. Stressors increase the permeability of the surface epithelia, including the gills, to water and ions, and thus induce systemic hydromineral disturbances. High circulating catecholamine levels as well as structural damage to the gills are prime causal factors. This is associated with increased cellular turnover in these organs (Wendelaar-Bonga, 1997).The chronic effects of Cu exposure have been suggested to be caused by interference of neuro-endocrine functions in fish. Chronic Cu exposure involves complex physiological adjustments in many body systems, including increased oxygen consumption, reduced mean swimming speed, up-regulation of ionic regulation, decreasing lymphocyte levels and increasing neutrophils, altered immunity, modulation of Cu-dependent and independent enzyme activities, and proliferation of epithelial cells in gills or intestine. These responses can occur with exposure via the food or the water and can be rationalised into three major categories: (1) up-regulation of enzymes/metabolism, (2) altered haematopoietic responses and, (3) altered cellularity (cell type, turnover or size) in tissues (Handy, 2003). As an example of up-regulation of metabolism, the metabolic rate of *Pimephales promelas* increased after exposure to Cu (96 h) (Pistole *et al*., 2008). Recent studies measures up-regulation of stress related genes and proteins as biomarkers for the stress response (Hansen *et al*., 2007).

2.3.2 Metabolism

After entering the cells, metals may interfere with the cellular metabolism of proteins, carbohydrates and lipids, and change the transformation of these compounds in various ways. The effect of metals on cellular metabolism is related to metal affinity to various proteins and their function. Several metals are known to affect the Na^+, K^+-ATPase which is of great physiological importance (Jezierska and Witeska, 2001). Cu and lead have been shown to inhibit the ATPase, while Zn has been shown to stimulate ATPase activity (Jezierska and Witeska, 2001). Other metabolic enzymes known to be affected by metals are transaminases, phosphatases, oxidoreductases, ribonucleases, mixed function oxidases and glutathione hydrogenase (Jezierska and Witeska, 2001). Metals are shown to affect the level of glucose in blood. Different

mechanisms for this effect have been proposed; it is related to the stress response and increased levels of cortisol and catecholamines (Jezierska and Witeska, 2001), it is related to reduced insulin secretion (van Vuren *et al*., 1994), or metal-induced changes are related to gas-exchange processes (Banerjee, 1980). Lipid metabolism seems also to be affected by metals in a study by Tulasi *et al*. (1992), where it was found that exposure of *Anabas testudineus* to sublethal concentrations of lead reduced the total lipids, phospholipids, and cholesterol levels in liver, and ovary tissues, while the free fatty acid levels, and lipase activity increased. Effects on metabolic capacities of fish are thought to be related to several primary disorders. The observed up-regulation of liver protein metabolism can be due to the need for detoxification (Couture and Kumar, 2003). Impaired aerobic capacities in the muscle and whole fish suggest that mitochondria are primary targets for Cu (Coture and Kumar, 1996). The stress response and hyperactivity as accounted for in chapter 2.3.8 will also affect the metabolic capacity. Swimming capacity and oxygen uptake during swimming has been suggested as biomarkers of metal pollutant (McKenzie *et al*., 2007).

2.3.3 Free radicals and reactive oxygen species (ROS)

Metals are able to cause the generation of ROS through various mechanisms. Among these mechanisms, Fenton- and Haber-Weiss type reactions are most common (Leonard *et al*., 2004). Through ROS-mediated reactions, metals cause DNA damage, lipid peroxidation, and protein modification. Metals also cause activation of nuclear transcription factors, activation of various signalling proteins, cell cycle arrest and apoptosis (Leonard *et al*., 2004). Pb has been shown to induce the nuclear transcription factors NF-kB, and HIF-1 (Leonard *et al*., 2004). Lead is also known to have toxic effects on membrane structure and functions (Ahamed and Siddiqui, 2007). Often antioxidants such as catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) are used as biomarkers for oxidative stress (Ferreira-Cravo *et al*., 2008; Ruas *et al*., 2008). Another characteristic is the depletion of intracellular antioxidants (GSH), free-radical scavengers (vitamin E and C) and levels of lipid peroxidation (Deviller *et al*., 2005; Ruas *et al*., 2008). An overall increased production of ROS (superoxide anion radical, hydrogen peroxide, peroxyl radical, hydroxyl radical, nitric oxide, peroxynitrite radical, etc.) will be a result of exposure to metals (Formigari *et al*. 2007). Damage to tissue will eventually occur and protein carbonyls and lipid peroxidation in the liver are demonstrated useful as biomarkers for this (Carney Almroth *et al*., 2008a; 2008b). Zn has been shown to reduce oxidative stress probably due to its ability to displace redox active metal ions from site-specific loci (Stohs and Bagchi, 1994). Several studies have shown that also Sb may induce oxidative stress, reduce cell viability and induce DNA damage (Mann *et al.* 2006; Tirmenstein *et al*., 1995; Huang *et al*., 1996; Lecureur *et al*., 2002;).

2.3.4 Metallothionein

A biochemical biomarker is usually a level of an enzyme, protein or substance that reflects normal biological processes. As such, they can be used as markers of effects on these processes caused by xenobiotics. Effects are interpreted as levels that are statistical significantly different from normal levels in control groups. Metallothionein (MT) plays an important part in the natural regulation of essential metals in an organism, as well as the detoxification of non-essential metals.

They are normally present in the cells in trace amounts, and sub-lethal concentrations of metal may induce their synthesis. For this reason, MT concentrations are used as biomarkers for exposure to metals (Reynders *et al.* 2008). Several field studies with *feral* (escaped from domesticated) fish have supported the experimental data which show that MTs sequester heavy metals and that MT levels correlate with tissue levels of heavy metals (van der Oost *et al.* 2003). The induction of MT by one metal could impact the effects of another metal. This could occur through both toxicokinetics (transportation and accumulation), and toxicodynamics (sequestration) of metals (Wang and Fowler, 2008). Zn is an essential mineral and deficiency may cause reproductive dysfunction in mammals. Since MT regulates Zn homeostasis, its induction due to metal exposure could affect reproduction. This relationship has not been established by scientific evidence.

2.4 Physiological endpoints

Effects at the molecular level may be followed by effects on the physiological level, e.g. effects on the functions of an organism's organs such as gills and liver. Any disorder caused by metals in the gills, liver, kidneys or other organs is reflected in the changes of blood parameters, such as alterations of ionic composition of plasma blood, pH, protein content, and enzymatic functions (Jezierska and Witeska, 2001). Below is an overview of effects of metals on different physiological endpoints. The effects are usually not independent of each other. E.g. effects on the endocrine system may influence behaviour and reproduction, and effects on behaviour may be a consequence of neurotoxic events.

2.4.1 Ionoregulatory disruption

Fish gills are the primary target of most environmental pollutants. The gills comprise over 50 % of external body surface; therefore they play an important role in the exchange of various substances between the organism and environment. Due to their structure and function, the gills are susceptible to injury by waterborne metals that are absorbed through the gill epithelium. Metals do also accumulate in the gills (Jezierska and Witeska, 2001). Although effects on gills can be detrimental for a population, it appears that effects on gills occur at relatively high metal concentrations compared to sublethal effects on the endocrine system and on behaviour. According to the literature some metals can cause pathological changes in the gills and reduce oxygen uptake in fish, however, this effect is poorly documented (Jezierska and Witeska, 2001). Fish gills, besides their respiratory function, are also involved in maintaining the water-electrolyte balance. Metal-induced impairment of ion-transport often results in osmoregulatory disorders. Several metals are shown to compete directly with Ca^{2+} for uptake at calcium binding sites (Verbost *et al.*, 1987, 1989; Spry and Wood, 1985). Hence, by this mechanism hypocalcemia can occur and be lethal for the fish. Such a relationship has been proven for Zn and Cd, but not directly for Pb. Pb is thought to be a disruptor of the ion balance of Na^+ and Cl^- as exhibited by Cu (Lauren and McDonald, 1985) and silver (Wood *et al.*, 1996; Morgan *et al.*, 1997). A disruption of Na^+ balance and a 40% inhibition of Na^+/K^+ ATPase activity were observed in crayfish chronically exposed to 0.5 mg Pb l^{-1} (Ahern and Morris, 1998). The mechanism for acute toxicity of Pb in rainbow trout (*Oncorhynchus mykiss*) has been investigated at Pb concentrations

close to the 96 h LC_{50} of 10 /Lmg/L Pb (Rogers *et al*., 2003). Tissue Pb accumulation associated with death was highest in the gill, followed by kidney and liver.

Metal-induced disturbances of ion uptake and excretion often result in alterations of plasma ion content. Significant ionoregulatory effects were observed in adult rainbow trout (200-300 g) fitted with indwelling dorsal aortic catheters and exposed to 1.1 ± 0.04 mg/L dissolved Pb (Rogers *et al*., 2003). Decreased plasma [Ca^{2+}], [Na^{+}] and [Cl^{-}] occurred after 48 h of exposure followed after120 h, with increases in plasma [Mg^{2+}], ammonia, and cortisol. Branchial Na^{+}/K^{+} ATPase activity in juvenile trout exposed to concentrations close to the 96 h LC_{50} was inhibited by approximately 40% after 48 h of Pb exposure. Calcium ion flux measurements using ^{45}Ca as a radiotracer showed 65% inhibition of Ca^{2+} influx after 0, 12, 24 or 48 h exposure to the 96 h LC_{50} concentration of Pb. There was also significant inhibition (40-50%) of both Na^{+} and Cl^{-} uptake, measured with ^{22}Na and ^{36}Cl simultaneously. This leads to the conclusion that the mechanism of acute toxicity for Pb in rainbow trout occurs by ionoregulatory disruption rather than respiratory or acid/base distress at Pb concentrations close to the 96 h LC_{50} in moderately hard water (Rogers *et al*., 2003). Another study argue that the Pb induced ionoregulatory toxicity in rainbow trout, particularly the disturbance of Ca^{2+} homeostasis, is not exclusively a branchial phenomenon, but is in part a result of disruption of ionoregulatory mechanisms of the kidney (Patel *et al*., 2006).

In freshwater, Cu is primarily an ionoregulatory toxicant, exerting substantial pathophysiologic effects at concentrations well below 100 µg/L. Cu exposure induces large net losses of Na^{+} and Cl^{-} across the gills, with internal sequelae (declining osmolality, fluid volume disturbance, cardiovascular collapse) similar to those caused by low pH and silver. There is a strong correlation between gill total Cu burden and acute toxicity (Playle *et al*., 1993). Most, but not all studies agree that both hardness and alkalinity are strongly protective against Cu burden and acute toxicity, whereas the reported influence of pH is variable, ranging from antagonism to synergism at very low pH (<5.0) and from no effect to protection in the circumneutral and alkaline pH range. There are two mechanism of action by which Cu induces ionoregulatory dysfunction. One is a mixed competitive (i.e. increased K_m) and non-competitive (reduced J_{max}) inhibition of Na^{+} and Cl^{-} influx, which occurs at much lower threshold than the other which is a stimulation of passive effluxes of these ions, e.g. 12.5 versus 100 µg/L in the rainbow trout and at much higher levels in the more resistant tilapia. The inhibition of influx is insensitive to [H^{+}] and develops progressively over the first 24 h, reminiscent of the actions of silver and aluminium. Like silver, Cu has a strong affinity for sulfhydryl groups which explains its well-documented ability to inhibit branchial Na^{+}, K^{+} -ATPase activity both *in vitro* and *in vivo*. This accounts for the influx blockade. The efflux stimulation at higher levels is more rapid and occurs because Cu is quite potent in weakening the junctions between the branchial epithelial cells, thereby increasing paracellular permeability. Effects of Cu on Ca^{2+} uptake processes appear to be minor or non-existent.

Zn is an essential metal for fish and other aquatic organisms (Watanabe *et al*., 1997). Dietborne Zn is relatively non-toxic to fish (Clearwater *et al*., 2002) whereas excess exposure of Zn in water may influence the regulation of calcium transport through gills. It is hypothesized that Zn^{2+} and

Ca^{2+} compete for the same ion channel (Santore *et al*., 2002). Therefore, elevated Ca^{2+} concentrations can be protective against Zn toxicity. Magnesium (Mg) is also shown protective for the invertebrate *Daphnia magna* (Heijerick *et al*., 2002). Exposure to sub-lethal concentrations of Zn can disturb the acid-base regulation due to inhibition of carbonic acid anhydrase activity, leading to an impact on CO_2 excretion (Spry and Wood, 1985). Both Zn and Cu exhibit similar properties, suggesting that metals from shooting ranges can have additive acute effects.

2.4.2 Endocrine disruption

According to the Environmental Protection Agency, US, an environmental endocrine disruptor may be defined as an exogenous agent that interferes with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development and/or behaviour (Kavlock, 1996). The fact that metals are indeed endocrine disruptors is discussed in chapter 2.3.1. The mechanisms behind endocrine disruption have not been conclusive. The question is whether 1) it involves a random series of coincidental events in individual body systems that fortuitously allow the fish to survive; that the increased concentrations of the circulating hormones are due to the natural ability of the fish to sense metals in its environment (Wenelar-Bonga, 1997), or 2) whether they are part of an ordered neuro-endocrine response leading to inevitable physiological change (Handy, 2003). As an example of the latter, Cu is thought to interfere directly with the adrenergic response leading to release of intracellular stored adrenaline and noradrenaline, and with the stimulation of the cortisol release (Handy, 2003). Besides that, a possible account of estrogenicity has been documented. In an evaluation of estrogenicity of metals, antimony chloride was found to exhibit estrogenic activity by proliferation of MCF-7 breast cancer cells, an endpoint that is estrogen dependent (Choe *et al*., 2003).

2.4.3 Anemia

In lead-induced anemia, the red blood cells are microcytic (abnormal small) and hypochromic (pale). As in iron deficiency there are usually increased numbers of reticulocytes (young blood cells in the bone marrow) with stippling after staining. Anaemia results from two basic defects: shortened erythrocyte life and impairment of heme-synthesis. Shortened life span of the red blood cell is thought to be due to increased mechanical fragility of the cell membrane. The biochemical basis for this effect is not known, but the effect is accompanied by inhibition of sodium- and potassium-dependent ATPases. Because of its affinity for -SH groups, lead is known to inhibit δ-aminolevulinic acid dehydratase (ALA-D), an enzyme involved in heme biosynthesis. Over 99% of the lead present in the blood accumulates in erythrocytes. Of this, over 80% is bound to ALA-D (ATSDR, 1999). Inhibition of ALA-D indicates that Pb is both bioavailable and biochemically active. It is generally believed that ALA-D is not a rate limiting step in heme synthesis in fish and that most Pb concentrations in the environment are lower than those necessary to produce anemia and other haematological effects, but it might be that the use of more sensitive indicators could demonstrate a relationship between levels of ALA-D and heme status (Schmitt *et al*., 2007).

There is also depression of coproporphyrinogen oxidase, resulting in increased coproporphyrin activity. Lead also decreases ferrochelatase activity. This enzyme catalyzes the incorporation of the ferrous ion into the protoporphyrin ring structure. Failure to insert iron into the protoporphyrin ring structure, results in depressed heme formation. Depressed heme synthesis is thought to be the stimulus for increasing the rate of activity of the first step in the heme synthetic pathway (ATSDR, 1999; Goyer and Thomas, 2001). Cu and Zn can also induce anemia probably through haemolysis (Jezierska and Witeska, 2001).

2.4.4 Immunosuppression

Intoxication of fish with metals may result in impairment of the non-specific and the specific immune response which may result in an increased susceptibility to infections. Cu-related immunotoxicity has been reported in a number of experimental studies. Results prior to 1994 provided evidence that exposure to Cu suppressed specific antibody responses and phagocyte-mediated ROS production, in addition to increasing the incidence of infectious fish disease. More recent studies, not only support earlier findings, but also add to the body of knowledge about Cu-induced effects on innate and acquired immunity. In a study by Dethloff and Bailey (1998), rainbow trout (*Oncorhynchus mykiss*) were exposed to 6, 16, and 27 µg/L (in soft water) for 3, 7, 14, and 21 days and effects on circulating blood cell profiles, respiratory burst activity, and B- and T-lymphocyte proliferation were examined. Cu tended to reduce both LPS-stimulated B-lymphocyte proliferation (primarily at that concentration which also reduced cell survival) and head kidney O_2- production (for all doses at all time points examined). Percentages of circulating lymphocytes were consistently 9 – 13 % below control values, while monocytes were consistently elevated in fish exposed to 27 µg/L. Metals affect fish immune system, usually by causing a decrease of circulating leukocytes, mainly lymphocytes, often accompanied by high levels of phagocytic cells. The results of many *in vitro* studies indicate inhibition of antibody production and inactivation of phagocytic cells. This may result in increased susceptibility to infections (Jezierska and Witeska, 2001). Zn suppresses phagocytosis by macrophages and was shown cytotoxic to lymphocytes *in vitro*. Suppression of antibody production by Zn was reported by Cenini (1985) and by Pb (O'Neill, 1981). A battery of immunological assays is suggested to predict the immunotoxicity of xenobiotics. The battery constitutes assays that measure antibody-forming cell response to T-dependent antigens, T-dependent antigens, T- and B-cell lymphoproliferation, macrophage function, and host resistance against infectious bacteria (Zelikoff, 1998).

2.4.5 Behaviour and neurotoxicity

Changes in fish behaviour are often first observable symptoms of metal contamination. The changes such as avoidance might be a direct reaction to the presence of metal in the water perceived by receptors. This is part of a complex adaptive response to environmental changes and should not always be perceived as a toxic effect. However, more serious anti predator behaviour has been shown to be affected at 2 µg Cu/L (Sandahl *et al.*, 2007) stressing the importance of this endpoint. Exposure to sub-lethal concentrations of metals often causes changes in locomotor activity in fish. Locomotor activity is measured using various parameters such as duration of spontaneous activity, range of movements, swimming velocity, ability to swim against water

current, or number of turns (Jezierska and Witeska, 2001). An early response to waterborne metals is hyperactivity. Initial hyperactivity allows the fish to escape from the contaminated area, but since the fish may swim erratically, changing direction, it often remains in the contaminated area (Jezierska and Witeska, 2001). Lead exposure caused a general increase in the locomotor activity in the mirror carp (*Cyprinus carpio*) (Shafiq-ur-Rehman, 2003). Avoidance is observed in some species where e.g. rainbow trout (*Oncorhynchus mykiss*) avoided copper concentrations above 5 µg/L (Svecevicius, 1999). Alteration in avoidance or attraction has been observed in response to Cu. In rainbow trout (*Oncorhynchus mykiss*), lake whitefish (*Coregonus clupeaformis*), Atlantic salmon (*Salmo salar*) and goldfish (*Carassius auratus*), Cu induces avoidance behaviour (Atchison *et al*., 1987). This avoidance behaviour is attributed to the effects of Cu on the olfactory bulb. Cu attenuates electrical responses of the olfactory bulb and receptor cells to excitatory compounds (Hara *et al*., 1976; Sutterli and Sutterli, 1970; Winberg *et al*., 1992). Furthermore, Cu exposure causes degeneration of specific olfactory receptor cells, likely through oxidative-stress-mediated apoptosis (Julliard *et al*., 1993).

Higher concentrations and longer exposure have been shown to cause a reduction in swimming activity, orientation disorders, and impaired reaction to different stimuli. These disturbances often make the fish more susceptible to predation (Jezierska and Witeska, 2001). Waterborne metals may injure receptors in fish causing impaired perception – especially olfaction and sight, which eventually lead to impaired prey search and capture. Lead exposure induces apoptosis in rod photoreceptor cells, neuronal cells, hepatocytes and macrophages by Bax translocation to the mitochondria (Pulido and Parish, 2003). In zebrafish (*Danio rerio*) and fathead minnows (*Pimephales promelas*), lead exposure reduced feeding ability, as evidenced by feeding miscues and increased prey-handling times (Di Gulio and Hinton, 2008). The reduction in feeding ability in walking catfish (*Clarias batrachus*) was attributed to psychomotor coordination, and decreases in GABA concentrations (Katti and Sathyanesan, 1986).

Metal induced impairment of olfactory perception may interfere with spawning behaviour in fish. Bloom *et al*. (1978) observed that Zn exposed *Brachydanio rerio* females did not react to sexual pheromone present at concentrations that attracted control individuals. Disturbances in spawning behaviour were reported in *Pimephales promelas* exposed to Pb (Weber, 1993). The fish spent less time on mating, the males were less active and the females laid eggs less frequently than under control conditions. Fish exhibit complex behaviour that provides the foundation for fish population structure and aquatic communities. Changes in social behaviour that affects reproduction may result in a decrease or even extinction of fish populations or communities in polluted waters (Jezierska and Witeska, 2001).

One of the mechanisms behind neurotoxicity of Pb is the ability of Pb to mimic biologically relevant metals and cations, particularly calcium (e.g. Bressler *et al*., 1999; Marchetti, 2003). This ability leads to neurotoxicity by interaction with calcium regulated proteins such as calmodulin, synaptotagmin, and cadherin, disrupting gene expression, the synaptic machinery, and neuronal migration (Bressler *et al*., 1999; De Gennaro, 2002; Marchetti, 2003; Prozialeck *et al*., 2003). Binding site competition displaces calcium ions, subsequently exposing cells to higher levels of

free calcium and potentially leading to oxidative stress (De Gennaro, 2002), and impaired myelination (Brubaker *et al*., 2009). Pb also blocks calcium ion flux across dopamine receptors, glutamate receptors, and voltage-sensitivity calcium channels. This blockage effect can affect neurotransmitter synthesis and release, ultimately leading to neuron hyperexcitability and damage (Chang and Verity, 1995; Cory-Slechta, 1995; De Gennaro, 2002; Marchetti, 2003).

2.5 Mortality and population effects

2.5.1 Growth and condition

Fish have a highly variable growth rate, and exposure to metals has been associated with decreased growth. Any metal-induced disturbance of energy production, allocation, or consumption is reflected in fish growth rate. Hence, effects on the growth of fish are related to the stress response. However, the effect of metals on fish growth rate first becomes clearly visible during long-term exposure. Statistical significant reduction of growth was observed already at 0,47 µg/L in *Salmo salar* alevins exposed to Cd (Rombough and Garside,1982). Field studies in recipients contaminated by metals from mining in Norway revealed that effects on fish condition were observed down to 20 µg Cu/L (total values). Fultons condition factor is a much used measure of the fish health condition and is based on the relationship between the length of the fish and body mass (e.g. Pyle *et al*., 2005). Growth, together with density, has been suggested as quantitative indicators of essential fish habitat quality in respect to contaminants (Gilliers *et al*., 2006). Growth and exercise performance were used to assess the effects from metal mining effluent on the fish species slimy sculpin (*Cottus cognatus*) and Atlantic salmon (*Salmo salar*) at a Zn, Pb, and Cu mine in New Brunswick, Canada. The growth promoting enzyme, ornithine decarboxylase (ODC) has been proposed as a biomarker for assessing chronic toxicity of metals to naturally reproducing fish populations (Norris *et al*., 2000).

2.5.2 Reproduction and early life stages

Metals seem to accumulate in the gonads. Higher concentrations of Pb, Cu and Zn were found in both testes and ovaries in fish from contaminated sites (Jezierska and Witeska, 2001). Metals can inhibit both oogenesis and spermatogenesis. According to Pierson (1981) maturation is the most sensitive period to metal intoxication in fish. Zn was found to inhibit maturation in both males and females in *Poecilia reticulate* (Pierson, 1981). Ovarian development was less advanced in Pb-exposed *Pimephales promelas* than in controls, and Pb suppressed also spermatocyte production (Weber, 1993). It is not certain that the effects on maturation are solely caused by a direct toxic action of the metals in the gonads. Some results indicate that the toxic effect is induced at the level of the hypothalamus and pituitary gland, disturbing hormonal control of reproductive cycle. An effect of Zn on GABAergic regulation of gonadotropin in *Cyprinus carpio* was reported by Sokolowska-Mikolajczyk *et al*. (2000). As mentioned for in Chapter 2.3.8 metals may adversely affect spawning behaviour in fish. In addition metals are shown to reduce egg production, and sperm motility (Jezierska and Witeska, 2001). Proper egg swelling is important for embryo development since it allows the embryo to change its position within the egg. Pb and Zn have been shown to reduce egg swelling in a concentration dependent manner (Jezierska and Witeska,

2001). Metals present in the aquatic environment may affect the rate of fish development. Retarded development has been observed in various fish embryos after Pb and Cu exposure as well as disturbances of cleavage and organogenesis (Jezierska and Witeska, 2001). Further, Zn and Cu have been shown to extend the hatching, and Zn also induced premature hatching. Pb shortened the hatching process. These metals also decrease the hatchability and induce body malformation in fish larvae. Larvae from metal exposed eggs are also smaller (Jezierska and Witeska, 2001). Effects on hatchability was observed at 1 µg/L for Cu, at 295 µg/L for Zn and 500 µg/L for Pb. LeBlanc and Dean (1984), could not find any effects on survival or hatching of fathead minnow embryos exposed to antimony trioxide for 30 days at a concentration of 7.5 µg/L. Larval stages are the most susceptible to metal toxicity and in Japanese anchovy (*Engraulis japonicus*), and LC_{50} was found at 5 µg/L for Pb and 149 µg/L for Zn (Cherkashin *et al*., 2004). In a recent study by Nam *et al*. (2009), larva and embryos of Japanese medaka (*Oryzias latipes*), and the crustaceans *Moina macrocopa* and *Simeocephalus mixtus*, were exposed to antimony (III) potassium tartrate (APT), which is regarded as one of the most toxic Sb-compounds. The crustaceans appeared to be the most sensitive organisms with 24-h LC_{50} values of 4.92 mg/L and 12.83 mg/L respectively, whereas the 24-h LC_{50} of the larvae of Japanese medaka was 261 mg/L. Effects on reproduction and early life stages are very sensitive and are endpoints of high ecological relevance. Fish produce a great number of gametes that are easy to count and investigate, and fertilization and hatching are easy to study.

3 Effects of biotic and abiotic parameters on toxicity and the biotic ligand model

The method frequently used by ecotoxicologists to calculate ecotoxicological screening levels (Eco SL) and water quality criteria is to use a range of chronic toxicity data of the most sensitive organisms and endpoints. Usually factors affecting toxicity, such as chemical speciation, bioavailability and field conditions have not been taking into account, making estimated risk levels at best highly qualitative and often conservative. Since field conditions can impart much from laboratory conditions it is important to have information about the range of endpoint variability due to biotic and abiotic factors. Biotic factors that are known to affect the range of endpoints are individual genetic variability, age and the reproductive stage. Abiotic parameters primarily include hardness of water, water pH and content of organic matters (Birceanu *et al.*, 2008). The relationship between metal toxicity and water hardness was shown early. Water hardness can be generalized as the sum of concentration of the divalent cations Ca^{2+} and Mg^{2+} of which Ca^{2+} exert a more protective effect than Mg^{2+} (Wood, 2001). Loyd (1961) showed that lethal concentration of Ca^{2+} in hard water in a 3 day exposure was 1.1 mg/L, whereas in soft water in a 7 day exposure was 0.044mg/L. A similar protective effect is shown for Pb in hard water (Davies *et al*., 1976). The protective effect of Ca/Mg is ascribed to actions on gill and several mechanisms are suggested (Wood, 2001):

1) In gills there are a range of binding sites for ionic transport such as Ca, Na, K and H which are essential for the fish physiology. Several toxic metals exist as cations and compete for the same sites as Ca/Mg in the gills, and high Ca-concentrations will prevent binding of toxic metals.
2) The divalent Ca and Mg exert a stabilizing effect of the junctional complexes between gill cells, which limits permeability to ions and water and reduce overall osmoregulatory costs.
3) In soft water it is observed increased proliferation of mucus and chloride cells, which might result in increased uptake and binding of toxicants transported via chloride cells. The gill epithelium will also increase in thickness, which may increase respiratory toxicity.
4) Hardness of water is influenced of other aspect of water chemistry such as pH and solubility of other metals. For example, increased calcium concentration in water may reduce lead bioavailability due to a concomitant increase in pH, reduction in dissolved Pb and an increase in lead complexes and precipitation of lead carbonate.

In a thorough survival study by Grosell *et al* (2006) the influence of Ca, humic acid and pH on lead accumulation and toxicity on fish (Fathead minnow) were performed. In control water with pH 7 the mean 4 day LC_{50} value was 52µg/L Pb. A reduction in pH by approximately one unit to pH 6.3 or increasing the pH to 8.3 increased the Pb sensitivity approximately five-fold to 4-days LC_{50} values of 7.9 µg/L and 15 µg/L respectively. Addition of 0.5 and 2 mM Ca lead to 4-days LC_{50} values of 206 µg/L and 524 µg/L, whereas addition of 2 mg and 16mg humic acid lead to 4-days LC_{50} values of 372 µg/L and 1656 µg/L. Humic acid is mainly phenolic and acidic

compounds with strong cation exchange capacity with a high capacity for metal adsorption and will presumably make Pb and other metals less bioavailable and therefore less toxic. These results strongly suggest that water chemistry must be taken into account when considering water quality criteria. Lead is as most other metals less toxic in hard water where lead readily complexes to form $Pb(CO_3)$ which are less available for uptake (Davies *et al*., 1976; Holcombe *et al*., 1976). In addition calcium, a prominent component of hard water, is believed to compete with lead for uptake, contributing to the protective effects of water hardness. In soft acidic water lead species such as Pb^{2+} and $Pb(OH)^{+}$ are more available and toxic (Davies *et al*., 1976; Hodson *et al*., 1978). Metal uptake seems also to depend upon seasonal variations in feeding intensity and metabolic rate (Dragun *et al*., 2007).

The biotic ligand model (BLM) has been developed to make a more quantitative estimate of risk to allow for site specific conditions., where the site specific water chemistry and its influence on metal bioavailability is included in the calculation of risk (Figure 3.1). The site of action of toxicity corresponds to the biotic ligand, and BLM is used to predict metal binding at this site and in turn related to a toxicological response (Paquin *et al*., 2002). The water quality input parameters for BLM calculations are temperature, pH, dissolved organic carbon, cations such as calcium, magnesium, sodium, and potassium, dissolved inorganic carbon, dissolved oxygen, major anions such as chloride, sulphate and alkalinity ($CaCO_3$) (e.g. USEPA-2007, MacDonald *et al*., 2002; van Genderen *et al*., 2007; Paquin *et al*., 2002). The model assumes that the metal and its complexes are in chemical equilibrium with each other and the site of action (Slaveykova and Wilkinson, 2004). Environmental systems are, however, not in equilibrium and the model is therefore an approximation of reality. For example metal complexation with natural organic matter is in general thought to reduce metal bioavailability and toxicity. However, in some studies it has been shown that humic substances can absorb to biological surfaces and increase the metal uptake flux followed by an increased susceptibility (Slaveykova and Wilkinson, 2004). Further, the model does not account for seasonal variation in water chemistry, and a thorough examination of the watershed through seasons is essential to achieve good predictions of risk (Paquine *et al*., 2002). According to US-EPA (USEPA-2007) the BLM approach is now considered appropriate for use to derive freshwater acute water quality criteria, whereas further development is required for deriving chronic water quality criteria (freshwater or saltwater). For a more detailed description of the use and limitations of BLM we recommend further readings such as Slaveykova and Wilkinson (2004), Paquin *et al*. (2002), Niyogi and Wood (2004) and Reiley (2007).

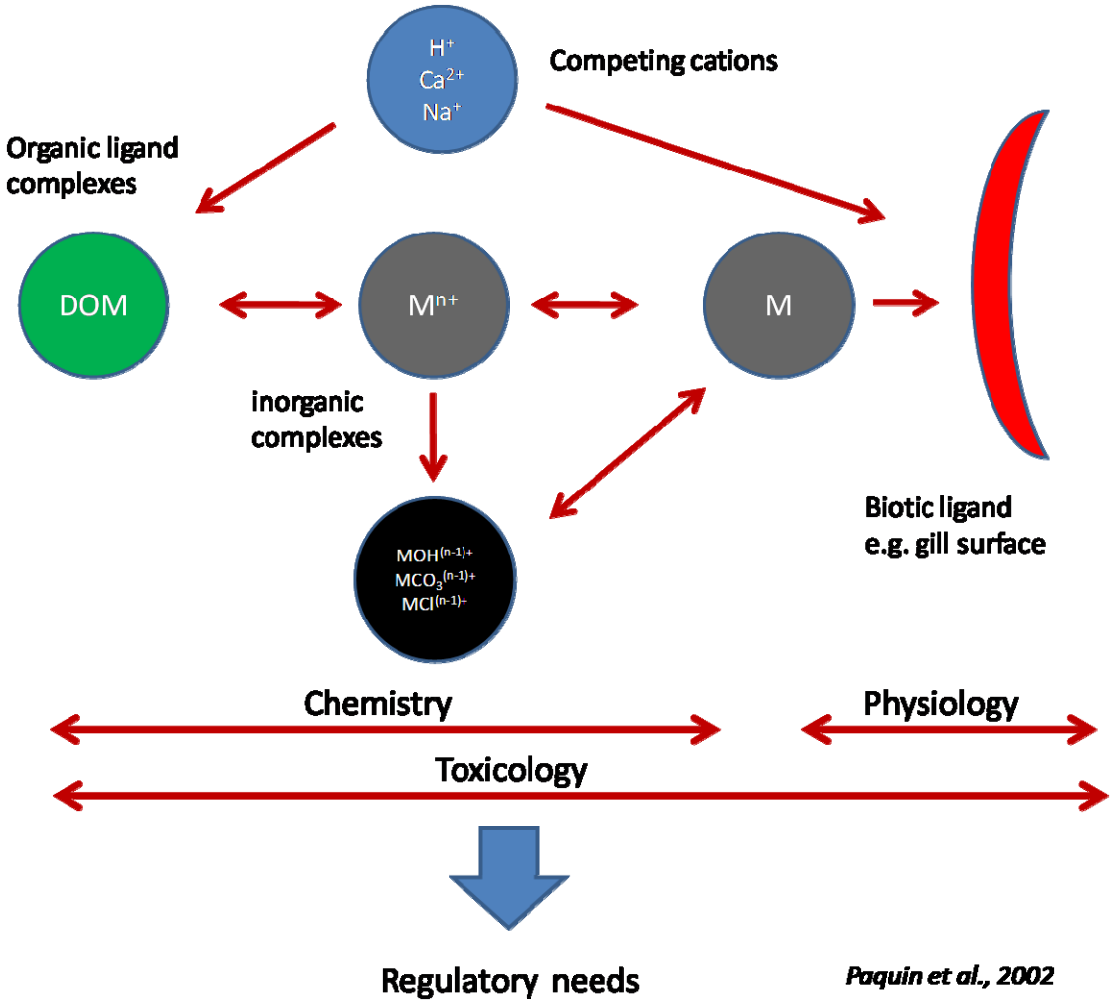

Figure 3.1 An overview of the biotic ligand model (BLM) according to Pequin et al (2002).

4 Effects on aquatic organisms from mixtures of metals

4.1 Assessing mixture effects

Traditionally, risk assessments have focused on the toxicity of single compounds. However, in the environment, an organism will never be exposed to only one toxic compound at a time. They will generally be exposed to a myriad of different compounds, with similar or different modes of action in their toxicity. Some studies have shown that some compounds, mixed in doses where they do not exhibit an effect, together cause an effect. In risk assessments, it might therefore be important to look at the toxicity of the mixture as a whole, instead of the components of the mixture (Fairbrother *et al.* 2007).

Interactions between chemicals are defined as a deviation from an expected additive outcome. Additivity can be defined as the obtained effect if two doses of same chemical are mixed (Sühnel, 1990). Synergy is defined as a stronger response than expected by additivity, whereas antagonism is defined as a weaker response than expected by additivity. Different models have been proposed

to investigate potential interactions between chemicals. The one, perhaps most intuitive and mostly used, is simple effect summation, which is based on an expectation that the effect of a mixture is the sum of the effects of the individual compounds. However, this method is only valid for chemicals that have a linear dose-response relationship, which is rare.

There are few guidelines for investigation of mixture effects. Nelson (1994) reported that more than 90% of the terminology used in studies on interactive effects was not according to recognized toxicological definitions. Further, Kortenkamp and Altenburg (1998) also reported risks for both underestimation and overestimation of mixture effects by erroneous use of models and interpretations of the results. Two main strategies, however, are currently recognized valid to predict toxicity of a mixture: Concentration addition, also called Loewe additivity, and effect addition also called Bliss model of independent action (Baas *et al*., 2010; Goldoni and Johansson, 2007). In order to predict a mixture effect, both models requires information on dose-response relationships of the single chemicals.

4.1.1 Concentration addition

The concentration of each metal is compared adjusted for its toxic potential. This strategy can be used for mixtures where the individual components have a similar/the same mode of action. If a mixture contains many different metals, this method tends to overestimate the toxicity of the mixture (causing an upward bias) (Fairbrother *et al.,* 2007). The Loewe additivity model was proposed by the pharmacologists Loewe and Muischnek (1926). The model is built upon an assumption that the substances tested act on the same cellular target, through the same molecular mechanism, only with differences in potency. Loewe additivity for binary mixtures can be defined as shown in Eq 2.

Eq 2
$$\frac{d_1}{D_1} + \frac{d_2}{D_2} = 1$$

In Eq 2, d_1 and d_2 are the concentration of substance 1 and substance 2, which in combination exert a certain effect. D_1 and D_2 are the doses of the same substances, which exert similar effect tested alone. If the substances in combination are more potent than expected from the effect of the substances individual effects, the equation will be <1, which indicates Loewe synergy. And opposite, if the substances in combination are less potent than expected from the effects of the substances individual effects the equation will be >1, indicating Loewe antagonism. So-called isobole diagrams are graphic descriptions of Loewe additivity model for binary mixtures. This model is also valid for multiple mixtures of n substances, which are defined in Eq 3 (Berenbaum, 1985; Rajapakse *et al*., 2001). C_{di} are the concentrations of substances di in a mixture that elicit a given effect, and ECx_{Di} are the concentrations of each substance which elicit the same effect if individually applied.

Eq 3
$$\sum_{i=1}^{n} \frac{c_{di}}{\mathrm{ECx_{Di}}} = 1$$

4.1.2 Effects (response) addition

The second strategy is effects addition, where the toxic potential of each metal is ignored. Instead, each of the metals effects is used to calculate the toxicity of the whole mixture. Effects addition is often used when the metals have different modes of action, i.e. cause different effects (Fairbrother *et al.,* 2007). The Bliss independence model was proposed by the biometrician Bliss (1939). This model uses theory from the field of probability, describing the independent situation where the combined additive effect equals the contribution of the effect of the single compounds, minus the product of these. This independence can be defined by Eq 4.

Eq 4 $$fa_{1,2} = fa_1 + fa_2 - fa_1 fa_2 \qquad (0 \leq f \leq 1)$$

In Eq 3 fa_1, fa_2 and $fa_{1,2}$ are the fractions of effect of substance 1 and 2, and a combination of these, respectively. If IC25-value is found for the two compounds fa_1 and fa_2 to be 0.25, the calculated combined effect will then be 0.4375. This model is also valid for multiple mixtures of n substances are defined as Eq 5 (Rajapakse *et al*., 2001). $E(c_{mixture})$ is the total effect of the mixture and $E(c_{Di})$ is the effects of the individual components. Effects E are defined as fractions of maximum effects.

Eq 5 $$E(c_{mixture}) = 1 - \prod_{i=1}^{n} (1 - E(c_{Di}))$$

4.2 Practical issues, how to make the mixtures used in experiments

Investigations of joint effects are a challenging approach with an infinite possible number of combinations of chemicals. Important factors, other than type of chemicals, are the relative contribution of each chemical in the mixture and target organ, and the complexity of the test system to be investigated. Further, interactions cannot be revealed without knowledge about the effect of the single compounds. In order to make good predictions of joint effects, studies of mixtures must be performed under identical conditions as done for the single compounds.

An important issue that should be addressed is the complexity of the test system. Interaction between toxicants that is revealed in one test system may disappear or be disguised in another more complex test system. For example, toxicants that are shown to interact as endocrine disruptor in a simple in vitro system may not interact in an in vivo system. One can also imagine the opposite; toxicants that do not interact in a simple in vitro system may interact in a more complex in vivo system. The reasons for this are that a living organism has a complex machinery, which has developed compensatory and protective mechanisms from adverse external influences. In addition, toxicants may also act through different mechanisms of action, which is not discovered if the test system is too simple.

There are several ways to approach study of joint effects. Due to the complex nature of mixtures one has to take into account site specific conditions. For example, metal runoff from shooting ranges has a certain signature of Pb, Cu, Sb and Zn, and potential interactive effects should be predicted according to this signature. When the intention is to study site-specific conditions, EPA

(the US Environmental Protection Agency), recommends using water or sediments from the site (Fairbrother *et al*. 2007). However, by taking water from the site, into a lab, entails the loss of additional stressors the fish are exposed to in their natural environment. Therefore, several experiments use cage-exposures instead. Often, controlled laboratory fish are used for these experiments, to protect the natural population from sampling (Reynders *et al*. 2008).

4.3 Reported effects

Studies of effects of mixtures of metals on fish were addressed as early as 1946 by Bandt (1946) and Doudroff (1952) who indicated synergistic effects between Cu and Zn. In a later study by Loyd *et al*., (1961) lethal effects of mixtures of Zn and Cu in hard and soft water on fish were tested, showing additive effects at low concentrations and more than additive effects at the higher concentrations independently of water quality. Two technical report published by the Food and agriculture organization of the United Nations in 1980 and 1987 (EIFAC, 1980; 1987) summarize published studies performed on joint action of mixtures on aquatic organisms (Table 4.1). The reports include studies on both metals and other chemicals, such as pesticides, and most studies use mortality as endpoints. The findings are in general inconclusive and no combinations of metals appear to induce a predominantly synergistic or antagonistic effect, albeit some reports show trends towards the one or another. Although the design of many of the older studies appears adequate, they are mostly mortality studies of which have several limitations. First, the concentrations needed to induce mortality are unrealistically high compared to real life and second, mortality studies can reveal joint effects, but cannot exclude the occurrence of joint effects. In order to do more conclusive studies it is necessary to do investigations with sublethal concentrations.

Few studies have focused on the combined toxic action of metals, and interactions between. Additive effects on the estuarine mysid *Neomysis integer* (Crustacea: Mysidacea) were reported for a mixture of metals comprised of Hg, Cd, Cu, Zn, Ni and Pb (Verslycke *et al*., 2003). However, the mixture effect was assessed by concentration addition which is not a method recommended by the current study. A mixture of As, Cd, Cr, Cu, Hg and Pb was tested on fathead minnows (*Pimephales promelas*) and daphnids. Adverse effects at mixture concentrations of one-half to one-third the MATC (maximum acceptable toxicant concentration) was found for fathead minnows and daphnids, respectively, suggesting that components of mixtures at or below no effect concentrations may contribute significantly to the toxicity of a mixture on a chronic basis (Spehar and Fiandt, 1986). Kamo and Nagai (2008) studied the toxicity of Zn, Cu and cadmium toxicity to rainbow trout. It was found that the toxic effects of metal mixtures are the sum of the toxicities of each metal (additive effect), corresponding to the bioavailable form of the metals (Kamo, 2008).

Effects of mixtures of heavy metals on the filtration rate and survival of the freshwater mussel *Dreissena polymorpha* were studied during chronic exposure. In laboratory experiments, mussels were exposed to mixtures of Cu + Zn, Cu + Cd, Zn + Cd, and Cu + Zn + Cd in concentrations causing a 50% decrease in filtration rate in 48 hr experiments. The effects on mortality and filtration rate were not related. In short-term experiments Cu + Cd were more than concentration

additive, whereas in chronic experiments Cu + Cd were strongly less than additive. This indicates a loss of potential for additivity during prolonged exposure. In general, Cu, Zn, and Cd did not affect each others uptake. It was concluded that the chronic effects of mixtures could not be predicted from their short-term effects or from the chronic effects of the metals tested individually (Kraak *et al.* 1993). Birceanu *et al.* (2008) exposed rainbow trout to metal mixtures with Cd and Pb. It was found that Pb and Cd bound to gills in a less than additive manner. This was probably due to competition for binding sites. However, ionic disturbances by the mixture were found to be more than additive (Cd < 100 nmol/Ll; Pb < 500 nmol/Ll). Roch and McCarter, (1984ab) exposed rainbow trout to both water from contaminated lakes and to an artificial mixture in the laboratory. In both experiments, the fish exposed to Zn, Cu and Cd showed an elevated MT level in their livers. In the field experiment, they found the level of MT in the liver to be correlated with Zn levels. For the laboratory experiment, they found that the combined toxicity of the mixture was additive, and that changes in the parameters measured were due to Cu exposure, with no interactive effect of Zn (Roch and McCarter, 1984ab).

Lock and Janssen made an attempt to predict the chronic toxicity of mixtures of Zn, Cd, Cu and Pb to a potworm (*Enchytraeus albidus).* The effect prediction based on the concentration addition model was always higher than those gained with the independent action model. For all the mixtures, the experimental results showed an effect that was always lower than the effect that had been predicted by the concentration addition model. They thereby conclude that the concentration addition approach represents the worst case scenario for the risk assessment of metal mixtures in terrestrial environments. (Lock and Janssen 2002)

Some studies have reported on the ability of Zn to interact with Cu and Fe, decreasing the metal content in tissues and retarding oxidative processes. In both in vitro and in vivo models, Zn supplementation prevents apoptosis induced by a variety of agents (by increasing the ratio of Bcl-2 to Bax). Zn may also have a more indirect protective role by maintaining the level of MT. However, its protective role depends upon concentration, as it can contribute to the induction of apoptosis at higher concentration (Formigari *et al.*, 2007).

	Additive	More than additive	Less than additive
Cu + Zn	3	1	
Cu + Cd		2	1
Cu + Zn + Ni	2		
Cu + Zn + Cd	1	3	1
Cu + Mn	1		
Cu + Hg	1		1
Cu + Ni	1		
Zn+ Ni	1		
Cr + Ni	1	2	
Cr + Ni + Cd			1

Table 4.1 Studies of mixture effects on the mortality of fish in the years from 1960-1980.

5 Emerging contaminants in shooting ranges

Effects of metals on aquatic organisms have been subject to intensive research for decades of which knowledge on elements such as Cu, Pb, Hg, and Zn are well documented. US-EPA recently released ambient freshwater quality criteria on Cu, based on the BLM (USEPA, 2007) and other metals-BLM, such as Zn, Ni, Ag and Pb, are under development (Reiley, 2007). Some elements, relevant for the Norwegian shooting ranges, such as antimony and tungsten, are however less studied. Antimony is used as an alloy in lead bullets and W is used in so-called green ammunition. Recent investigations have shown that these elements are readily mobilized from berms and released into the aquatic system. Therefore, there is a need to increase knowledge of these elements on the toxicity of aquatic organisms, in order to develop adequate water quality criteria. Below is an overview of some of the existing knowledge on Sb and W on aquatic organisms.

5.1 Antimony (Sb)

Antimony (Sb) is one of the least studied elements. Sb is a metalloid between arsenic and bismuth in group 15 of the periodic table. Its resemblance to As has made it a so-called pollutant of interest by the environmental authorities in U.S. and E.U. (Filella *et al*., 2002a). Sb can exist in several oxidation states, but is primarily found as Sb (III) and Sb (V), the trivalent and pentavalent oxidation states respectively. The toxicity of Sb depends both on its oxidation state and type of antimony compound. In natural oxic waters Sb (V) predominates, primarily as $[Sb(OH)_6]^-$ (Filella *et al*., 2002b). Sb is used for treatment of certain parasitic infections, such as

Leishmaniasis, as flame retardants, as alloys in semi conductors and even as alloy in lead containing small arm ammunition (Filella *et al*., 2002a).

Similar to As, the trivalent forms of Sb are regarded as most toxic. Most concerns are raised with regards to the potential genotoxic and carcinogenic effects of Sb. Given that antimony and arsenic belong to the same periodic group and have the same oxidation states, it is possible that the DNA damage induced by antimony follows similar pathways as arsenic. In general, there are the trivalent forms of Sb that have tested positive in mammalian genotoxicity tests (Boeck *et al*., 2003 Pulido and Parrish 2003). The International Agency on Research of Cancer has concluded that there are sufficient evidences to regard antimony trioxide (Sb_2O_3) as a carcinogen following inhalation. Sb, however, usually co-occur with the carcinogenic As, and it is therefore difficult to evaluate carcinogenicity data (e.g. De Boeck *et al*., 2003). A study by McCarty *et al*, (2004) could not support the hypothesis that Sb contributed significantly to the health effects in relationship with As in drinking water from wells in Bangladesh.

Sb can be found in substantial concentrations in aquatic environment, especially from anthropogenic activities such as mining (Migon *et al*., 1999, Migon and Mori, 1999) and from deposition of small arm ammunition (Johnson *et al*., 2005, Strømseng *et al*., 2009). Antimony does not bioaccumulate in the food chain (Veenstra *et al.* 1998; Culioli *et al*., 2009). Very few toxicological studies have been performed on aquatic organisms. A few acute and sub-chronic studies on aquatic organisms have, however, shown that Sb is not particularly toxic compared to other heavy metals, such as Cu, Pb and Cd. Estimated LC_{50} of $SbCl_3$ for 3-day tilapia larvae in a 96-h study is approximately 35.5 mg/Ll(Lin and Hwang, 1998). Estimated LC_{50} of $SbCl_3$ for juvenile common carp (C. carpio) in a 96-h study was shown to be 14.05 mg/L (Chen and Yang, 2007). Takayanagi (2001) exposed juvenile red seabream (*Pargus major*) for $SbCl_3$, $SbCl_5$ and $K[Sb(OH)_6]$ with 24-h LC_{50} values of 15.5 mg/L, 0.93 mg/L and 6.9 mg/L respectively. Interestingly it was the pentavalent Sb-compounds that were the most toxic in this study.

5.2 Tungsten (W)

Tungsten is a transition metal in the periodic system along with chromium and molybdenum. Tungsten is an element with high boiling and melting point, high density, high thermal and electrical conductivity and is used in a range of different applications, including ammunition as a substitute for lead and as high kinetic energy penetrators (Koutsospyros *et al*., 2006). Tungsten has a very complex chemistry and can exist in oxidation states ranging from -2 to +6 and is capable of forming a large number of soluble complexes. Under alkaline and neutral conditions dissolved W exist primarily as WO_4^{-2}, whereas under acidic conditions it tend to polymerize into isopolytungstates (Seiler *et al*., 2005; Koutsospyros *et al*., 2006). Mobility of tungsten in different soil types in a columns study showed that neutral to alkaline soil increased mobility (> 40mg/L in effluent), whereas acidic conditions reduced mobility (<1-3mg/L in effluent). Addition of phosphate in acidic conditions increased tungsten mobility, whereas the addition of calcium reduced mobility. The mobilization of tungsten with phosphate where attributed to the anionic character of W (Bednar *et al*., 2009).

Tungsten is regarded as an element with low toxicity and lethal oral dose for rodents (rats and mice) is reported to approximately 1900 mg/kg (Koutsospyros *et al*., 2006). The occurrence of a childhood leukaemia cluster in Fallon Nevada, US, raised concerns about tungsten as a carcinogen. This area is known for W mines and there is an active W mine in the Fallon area. It was found elevated levels of airborne tungsten (Sheppard *et al*., 2006), in urine of residents (CDC, 2003; Seiler *et al*., 2005) and in ground- and tap water (CDC, 2003; Seiler *et al*., 2005) in the vicinity of Fallon. A thorough study by US Centers for Disease Control concluded that residents were subjected to increased exposure to Tungsten, but was not able to identify Tungsten as the cause of the leukaemia (CDC, 2003). A later study by Sheppard *et al*. (2007) with use of dendrochemistry (measurement of element concentrations in tree rings) showed a temporally increase in tungsten and cobalt from the early 1990, which co-occurred with the excessive childhood leukaemia. The authors warranted further research on the linkage between cobalt and tungsten and leukaemia.

There are limited amounts of studies performed on aquatic organisms. A few acute studies on aquatic organisms have been performed with Na_2WO_4 on rainbow trout, goldfish, narrow-mouthed toad and an invertebrate (*Daphnia magna*) showing lethal concentrations ranging from approximately 2.5 mg/L to 90mg/L (Strigul *et al*., 2010). Strigul *et al*. (2010) exposed two different species of tungsten (mono- and polytungstate) to guppies in a 96-h acute test followed by a 14-day prolonged toxicity test with fish that survived the 96-h test. It was found that the polytungstate species was 5-6 times more toxic than monotungstate with 96-h and 14-day LD_{50} values of 850mg/L and 130 mg/L respectively. These finding shows that tungsten has a relatively low toxicity to aquatic organisms, but also that tungsten toxicity differs between different anionic species.

6 Conclusion

In respect to the criteria for selecting the most relevant endpoints discussed in chapter 2.1, some endpoints are noteworthy;

1) Sensitive endpoints on reproduction such as sexual maturation, larval growth and survival
2) Behavioural effects on spawning
3) Chronic stress

The endpoints on reproduction are important due to the sensitivity of the endpoints and the ecological relevance. The effects on behaviour are also among the most sensitive endpoints, and since they are related to reproduction by disturbed spawning, they have ecological relevance. Behaviour can also affect social structures that are important for population viability. Many important adverse effects observable in chronic studies are thought to be rooted in the stress response. This, in addition to the sensitivity of this endpoint, makes the stress response a relevant endpoint. There are many biomarkers available for studying the stress response in fish. In general, Pb, Cu and Zn all seem to affect the same molecular, cellular, and physiological endpoints. This suggests that additive effects should be anticipated.

Additivity seems to be a frequent observed effect of metals in mixture (Table 3.1). However, Zn is a metal that at some circumstances show protective properties when administrated together with other metals. Regarding runoff from shooting ranges Zn is present at lower concentrations, thus not expected to interfere with the toxicity of the other metals. By additivity metals in a mixture can give adverse effects even though the individual concentrations of the metals are below ecotoxicological benchmark levels. This indicates that additivity is an important property of metals and should be a major consideration in ecological risk assessments. Two main strategies are currently recognized valid to predict toxicity of a mixture: Concentration addition, also called Loewe additivity, and effect addition also called Bliss model of independent action. In order to predict a mixture effect, both models requires information on dose-response relationships of the single chemicals. In order to predict the mixture effects on fish of metals from shooting ranges laboratory studies should be performed with mixture concentrations based on the signature reported from shooting ranges. By using the Bliss or the Loewe models predictions of additivity could be made. Cage-exposure and field studies could then be performed in order to validate the models. Other strategies for the prediction of the toxicity of mixtures have been suggested. One of these approaches, the Biotic Ligand Model (BLM), also take into account environmental factors that could affect toxicity, such as pH, TOC as discussed in chapter 3. According to US-EPA (USEPA-2007) the BLM approach is now considered appropriate for use to derive freshwater acute water quality criteria, whereas further development is required for deriving chronic water quality criteria (freshwater or saltwater).

References

Ahamed, M., Siddiqui, M. K. J. (2007) Low level lead exposure and oxidative stress: Current opinions. *Clinica Chimica Acta* **383**(1-2), 57-64.

Ahern, M.D., Morris, S. (1998) Accumulation of lead and its effects on Na balance in the freshwater crayfish *Cherax destructor*. *Journal of Experimental Zoology* **281**(4), 270-279.

Atchison, G.J., Henry, M.G., Sandheinrich, M.B. (1987) Effects on metals on fish behavior: a review. *Environmental Biology of Fishes* **18**, 11-25.

ATSDR (1999). Toxicological profile for lead. U.S. Department of health and human services. Agency for Toxic Substances and Disease Registry. Atlanta, Georgia.

Baas, J., Jager, T., Kooijman, B. (2010) A review of DEB theory in assessing toxic effects of mixtures. Science of The Total Environment. In press.

Bednar AJ, Boyd RE, Jones WT, McGrath CJ, Johnson DR, Chappell MA, Ringelberg DB. (2009) Investigations of tungsten mobility in soil using column tests. *Chemosphere* 75, 1049-1056.

Bennet, J.R., Kaufman, C.A., Koch, I., Sova, J., Reimer, K.J. (2007) Ecological risk of lead contamination at rifle and pistol ranges using techniques to account for site characteristics. *Science of the Total Environment* **374**, 91-101.

Berenbaum, M.C. (1989). What is synergy? *Pharmacological Reviews* **41**, 93-141.

Birceanu, O., Chowdhury, M.J., Gillis, P.L., McGeer, J.C., Wood, C.M., Wilkie, M.P. (2008) Modes of metal toxicity and impaired branchial ionoregulation in rainbow trout exposed to mixtures of Pb and Cd in soft water. *Aquatic Toxicology* **89,** 222-231.

Bliss, C.I. (1939) The toxicity of poisons applied jointly. *Annals of Applied Biology* **26**, 585-615.

Bloom, H.D., Perlmutter, A., Seeley, R.J. (1978) Effect of a sublethal concentration of zinc on an aggregating pheromone system in the zebrafish, *Brachydanio rerio* (Hamilton-Buchanan). *Environmental Pollution* **17**(2), 127-131.

Braun, U., Puterla, N., Ossent, P. (1997) Lead poisoning of calves pastured in the target area of a military shooting range. *Schweizer Archiv für Tierheilkunde* **139**(9), 403-407.

Bressler, J., Kim, K., Chakraborti, T., Goldstein G (1999) Molecular mechanisms of lead neurotoxicity. *Neurochemical Research* **24**(4), 595-600.

Brubaker, C.J., Schmithorst V.J., Haynes, E.N., Dietrich, K.N., Egelhoff, J.C., Lindquist, D.M., Lanphear, B.P., Cecil, K.M. (2009) Altered myelination and axonal integrity in adults with childhood lead exposure: A diffusion tensor imaging study. *NeuroToxicology* **30**(6), 867-875.

Carney Almroth, B., Albertsson, E., Sturve, J., Förlin , L. (2008) Oxidative stress, evident in antioxidant defences and damage products, in rainbow trout caged outside a sewage treatment plant. *Ecotoxicology and Environmental Safety* **70**, 370–378.

Carney Almroth, B., Sturve, J., Berglund, Å., Förlin, L. (2005) Oxidative damage in eelpout (*Zoarces viviparus*), measured as protein carbonyls and TBARS, as biomarkers. *Toxicology* **73**, 171–180.

CDC (2003) Cross-Sectional Exposure Assessment of Environmental Contaminants in Churchill County, Nevada. Final report. Centers for Disease Control and Prevention Available at: http://www.cdc.gov/nceh/clusters/Fallon/

Cenini, P. (1985) Induction of metal-binding proteins by zinc is accompanied by reduction of other proteins including antibodies. *Comparative Biochemistry and Physiology Part C: Comparative Pharmacology* **81**(1), 213-217.

Chang, L.W., Verity, M.A. (1995) Mercury neurotoxicity: Effects and mechanisms. In: Handbook of Neurotoxicology, Chang L.W., Dyer, R.S. (eds). Marcel Dekker, New York, pp 31-59.

Cherkashin, S.A. Nikiforov, M.V. Shelekhov V.A. (2004) The use of the mortality rate of marine fish prelarvae for the estimation of Zn and lead toxicity. *Russian Journal of Marine Biology* **30**(3), 217–222.

Choe, Y.S., Kim, S.J., Lee, J.H., Choi, Y., Lee, H., Kim, Y. (2003) Evaluation of estrogenicity of major heavy metals. *Science of the Total Environment* **312**,15-21.

Clearwater, S.J., Farag, A.M., Meyer J.S. (2002) Bioavailability and toxicity of dietborne copper and zinc to fish. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* **132**(3), 269-313.

Cory-Slechta, D.A. (1995) Relationships between lead-induced learning impairments and changes in dopaminergic, cholinergic, and glutamatergic neurotransmitter system functions. *Annual Review of Pharmacology and Toxicology* **35**, 391-415.

Couture, P., Kumar, P.R. (2003) Impairment of metabolic capacities in copper and cadmium contaminated wild yellow perch (*Perca flavescens*). *Aquatic Toxicology* **64**(1), 107-120.

Culioli J.L., Fouquoire, A., Calendini, S., Mori, C., Orsini, A. (2009) Trophic transfer of arsenic and antimony in a freshwater ecosystem: a field study. *Aquatic Toxicology* **94**, 286-293.

Davies PH, Goettl JP, Sinley JR, Smith NF (1976). Acute and chronic toxicity of lead to rainbow-trout salmo-gairdneri, in hard and soft-water. *Water Research* **10**, 199-206.

De Boeck, M., Kirsch-Volders, M., Lison, D. (2003) Cobalt and antimony: genotoxicity and carcinogenicity. *Mutation Research* **533**, 135-152.

De Gennaro L.D. (2002) Lead and the developing nervous system. *Growth, Development & Aging* **66**(1), 43-50.

Dethloff, G.M., Bailey, H.C. (1998) Effects of copper on immune system parameters of rainbow trout (*Oncorhynchus mykiss*). *Environmental Toxicology & Chemistry* **17**, 1807–1814.

Deviller, G., Palluel, O., Aliaume, C., Asanthi, H., Sanchez, W., Franco Nava, M. A., Blancheton, J. P., Casellas, C. (2005) Impact assessment of various rearing systems on fish health using multibiomarker response and metal accumulation. *Ecotoxicology and Environmental Safety* **61**(1), 89-97.

Di Gulio R.T., Hinton, D.E. (2008) The toxicology of fishes. CRC Press, ISBN: 978-0415248686, 1096 pp.

Dragun, Z., Raspor, B., Podrug, M. (2007) The influence of the season and the biotic factors on the cytosolic metal concentrations in the gills of the European chub (*Leuciscus cephalus* L.). *Chemosphere* **69**(6), 911-919.

Dube¸.M.G., MacLatchy, D. L., Kieffer, J. D., Glozier, N. E., Culp, J. M., Cash, K. J. (2005). Effects of metal mining effluent on Atlantic salmon (*Salmo salar*) and slimy sculpin (*Cottus cognatus*): using artificial streams to assess existing effects and predict future consequences. *Science of the Total Environment* **343**(1-3), 135-154.

EIFAC (1980). Water quality criteria for European freshwater fish – report on combined effects on freshwater fish and other aquatic life of mixtures of toxicants in water. EIFAC techn. Paper No. 37.

EIFAC (1987). Water quality criteria for European freshwater fish – report on combined effects on freshwater fish and other aquatic life of mixtures of toxicants in water. EIFAC techn. Paper No. 37 Revision 1.

EU (2000). Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000 establishing a framework for Community action in the field of water policy. Official Journal of the European Union. L327/1, 72.

Fairbrother, A., Wenstel, R., Sappington, K., Wood, W. (2007) Framework for Metals Risk Assessment. *Ecotoxicology and Environmental Safety* **68**(2), 145-227.

Ferreira-Cravo, M., Ventura-Lima, J., Sandrini, J.Z., Amado, L.L., Geracitano, L.A., Rebelo, M., Bianchini, A., Monserrat, J.M. (2008) Antioxidant responses in different body regions of the polychaeta *Laeonereis acuta* (Nereididae) exposed to copper. *Ecotoxicology & Environmental Safety* **72**(2), 388-393.

Filella, M. Belzile N. Chen, Y. W. (2002a) Antimony in the environment: a review focused on natural waters I. Occurrence. *Earth-Science Reviews* **57**, 125-176.

Filella, M. Belzile N. Chen, Y. W. (2002b) Antimony in the environment: a review focused on natural waters II. Relevant solution chemistry, *Earth-Science Reviews* **59**, 265-285.

Formigari, A., Irato, P., and Santon, A. (2007). Zn, antioxidant systems and metallothionein in metal mediated-apoptosis: Biochemical and cytochemical aspects. *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology* **146**(4), 443-459.

Gilliers, C., Le Pape, O., Désaunay, Y., Morin, J., Guérault, D., Amara, R. (2006) Are growth and density quantitative indicators of essential fish habitat quality? An application to the common sole Solea solea nursery grounds. *Estuarine, Coastal and Shelf Science* **69**(1-2), 96-106.

Goldoni, M., Johansson, C. (2007) A mathematical approach to study combined effects of toxicants *in vitro*: Evaluation of the Bliss independence criterion and the Loewe additivity model. *Toxicology in Vitro* **21**, 759–769

Goyer, R.A., Clarkson, T.W. (2001). Toxic effects of metals. In: Cassaret and Doull's Toxicology, 6th ed., Klaassen, C.D., Amdur, M.O., Doull, J. (eds). Macmillan, New York, pp. 811-867.

Grande, M. (1987). Background levels of metals in freshwater fish. NIVA-report, O-85167, l, 1979, pp 34.

Grande, M. (1991) Biological effects of metal pollution from mining. N effekter av gruveindustriens metallforurensninger. Norwegian Institute for Water Research. pp 136.

Grosell M, Gerdes R, Brix KV. (2006). Influence of Ca, humic acid and pH on lead accumulation and toxicity in the fathead minnow during prolonged water-borne lead exposure. *Comparative Biochemistry and Physiology - Part C: Toxicology & Pharmacology* **143**, 473-483.

Handy, R.D. (2003) Chronic effects of copper exposure versus endocrine toxicity: two sides of the same toxicological process? *Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology* **135**(1), 25-38.

Hansen, B.H., Rømma, S., Garmo, Ø. A., Pedersen, S.A., Olsvik, P. A., Andersen, R. A. (2007) Induction and activity of oxidative stress-related proteins during waterborne Cd/Zn-exposure in brown trout (*Salmo trutta*). *Chemosphere* **67**(11), 2241-2249.

Hara, T.J., Law Y.M.C., MacDonald, S. (1976). Effects of mercury and copper on the olfactory response in rainbow trout, Salmo gairdneri. *Journal of the Fisheries Research Board of Canada* **33**, 1568–1573.

Heijerick, D.G., De Schamphelaere, K.A.C., Janssen C.R. (2002). Biotic ligand model development predicting Zn toxicity to the alga *Pseudokirchneriella subcapitata*: possibilities and limitations. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, **133**(1-2), 207-218.

Hodson, P.V., Blunt, B.R., Spry, D.J. (1978) Chronic toxicity of waterborne and dietary lead to rainbow trout (*Salmo gairdneri*) in Lake Ontario water. *Water Research* **12**, 869-878.

Holcombe, G.W., Benoit, D.A., Leonard, E.N., Mckim, J.M. (1976) Long-term effects of lead-exposure on 3 generations of brook trout (*Salvelinus fontinalis*). *Journal of the Fisheries Research Board of Canada* **33**, 1731-1741

Huang, H., Shu, S.C., Shih, J.H., Kuo, C.J., Chiu, I.D. (1998) Antimony trichloride induces DNA damage and apoptosis in mammalian cells. *Toxicology* **129**, 113–123.

Janus, J.A. (2000). Addendum to RIVM report 6010140 003 (Integrated Criteria Document Lead). Ecotoxicity of lead. Aquatic and terrestrial data, RIVM.

Jezierska, B. and Witeska, M. (2001). Metal Toxicity to Fish, University of Podlasie, Siedlce, Poland. pp. 318.

Johnson, C.A., Moench, H., Wersin, P., Kugler, P., Wenger, C. (2005) Solubility of antimony and other elements in samples taken from shooting ranges. *Journal of Environmental Quality* **34**, 248-254.

Johnson, M.S., Wickwire, W.T., Quinn Jr., M.J., Ziolkowski, Jr., D.J., Burmistrov, D., Menzie, C.A., Geraghty, C., Minnic, M., Parson, P. (2007) Are songbirds at risk from lead at small arms ranges? An application of the spatial explicit exposure model. *Environmental Toxicology and Chemistry* **26**(10), 2215-2225.

Julliard, A.K., Saucier, D., Astic, L. (1993) Effects of chronic low-level copper exposure on ultrastructure of the olfactory system in rainbow trout (*Oncorhynchus mykiss*). *Histology and Histopathology* **8**(4), 655-672.

Kähkönen, M.A., Lankinen, P., Hatakka, A. (2008) Hydrolytic and ligninolytic enzyme activities in the Pb contaminated soil inoculated with litter-decomposing fungi. *Chemosphere* **72**, 708-714.

Kamo, M. (2008) An application of the biotic ligand model to predict the toxic effects of metal mixtures. *Environmental Toxicology and Chemistry* **27**(7), 1479-1487.

Katti, S.R., Sathyanesan A.G. (1987) Lead nitrate induced changes in the thyroid physiology of the catfish *Clarias batrachus* (L). *Ecotoxicology and Environmental Safety* **13**(1), 1-6.

Kavlock, R.J. (1996) Research needs for risk assessment of health and environmental effects of endocrine disruptors: a review of the US EPA-sponsored workshop, *Environmental Health Perspectives* **104**, 715–740.

Kobayashi, N., Okamura, H. (2005) Effects of heavy metals on sea urchin embryo development. Part 2. Interactive toxic effects of heavy metals in synthetic mine effluents. *Chemosphere* **61**(8), 1198-1203.

Kortenkamp. A., Altenburger, R. (1998) Synergisms with mixtures of xenoestrogens: A reevaluation using the method of isoboles. *Science of the Total Environment* **221**, 59-73.

Koutsospyros A, Braida W, Christodoulatos C, Dermatas D, Strigul N. (2006). A review of tungsten: from environmental obscurity to scrutiny. *Journal of Hazardous Matererials* **136**, 1-19.

Kraak, M.H.S., Schoon, H., Peeters, W.H.M., Vanstraalen, N.M. (1993). Chronic Ecotoxicity of Mixtures of Cu, Zn, and Cd to the Zebra Mussel *Dreissena polymorpha*. *Ecotoxicology and Environmental Safety* **25**(3), 315-327.

Labare, M.P., Butkus, M.A., Riegner, D., Schommer, N., Atkinson, J. (2004). Evaluation of lead movement from the abiotic to biotic at a small-arms firing range. *Environmental Geology* **46**,(6-7), 750-754.

Lauren, D.J., McDonald, D.G. (1985). Effects of Cu on branchial ionoregulation in the rainbow trout, *Salmo gairdneri* Richardson. *Journal of Comparative Physiology* **155**, 635-644.

Le Blanc, G., Dean, J. (1984) Antimony and thallium toxicity of embryos and larvae of fathead minnows (*Pimephales promelas*). *Bulletin of Environmental Contamination and Toxicology* **32**, 565-569.

Lecureur , V., Lagadic-Gossmann, D., Fardel, O. (2002) Potassium antimonyl tartrate induces reactive oxygen species-related apoptosis in human myeloid leukemic HL60 cells. *International Journal of Oncology* **20**, 1071–1076.

Leonard, S.S., Harris, G.K., Shi, X. (2004). Metal-induced oxidative stress and signal transduction. *Free Radical Biology & Medicine* **37**(12), 1921-1942.

Lin, H.C., Hwang, P.P. (1998) Acute and chronic effects of antimony chloride ($SbCl_3$) on tilapia (*Oreochromis mossambicus*) larvae. *Bulletin of Environmental Contamination and Toxicology* **61**, 129-134.

Lin, Z., Comet, B., Qvarfort, U., Herbert, R. (1995) The chemical and mineralogical behaviour of Pb in shooting range soils from central Sweden. *Environmental Pollution*, **89**(3), 303-309.

Lloyd, R. (1961) The toxicity of mixtures of zinc and copper sulphates to rainbow trout (*Salmo gairdneri* Richardson). *Annals of Applied Biology* **49**, 535-538.

Lock, K., Janssen, C.R. (2002) Mixture Toxicity of Zinc, Cadmium, Copper, and Lead to the Potworm *Enchytraeus albidus*. *Ecotoxicology and Environmental Safety* **52**(1), 1-7.

Löewe, S., Muischnek, H. (1926) Über Kombinationswirkungen Mitteilung: Hilfsmittel der Fragestellung. *Naunyn Schmiedeberg's Archives of Pharmacology* **114**, 313-326.

Lydersen, E., Löfgren, S., Arnesen, R.T. (2002) Metals in Scandinavian surface waters: Effects of acidification, liming, and potential reacidification. *Critical Reviews in Environmental Science and Technology* **32**(2-3), 73-295.

Macdonald, A., Silk, L., Schwartz, M., Playle, R.C. (2002) A lead-gill binding model to predict acute lead toxicity to rainbow trout (*Oncorhynchus mykiss*). *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* **133**, 227-242.

Mann, K.K., Davison, K., Colombo, M., Colosimo, A.L., Diaz, Z., Padovani, A. M. S., Guo, Q., Scrivens, P.J., Gao, W., Mader, S., Miller, J. (2006) Antimony trioxide-induced apoptosis is dependent on SEK1/JNK signaling. *Toxicology Letters* **160**(2), 158-170.

Marchetti, C. (2003) Molecular targets of lead in brain neurotoxicity. *Neurotoxicity Research* **5**(3), 221-236.

McCarty, K.M., Senn, D.B., Kile, M.L., Quamruzzaman, Q., Rahman, M., Mahiuddin, G., Christiani, D.C. (2004). Antimony: an unlikely confounder in the relationship between well water arsenic and health outcomes in Bangladesh. *Environ Health Perspectives* **112**, 809-811.

McKenzie, D.J., Garofalo, E., Winter, M.J., Ceradini, S., Verweij, F., Day, N., Hayes, R., van der Oost, R., Butler, P.J., Chipman, J.K., Taylor, E.W. (2006) Complex physiological traits as biomarkers of the sub-lethal toxicological effects of pollutant exposure in fishes. *Philosophical Transactions of the Royal Society B: Biological Sciences* **362**(1487), 2043-2059.

Migliorini, M., Pigino, G., Bianchi, N., Bernini, F., Leonzio, C., (2004) The effects of heavy metal contamination on the soil arthropod community of a shooting range. *Environmental Pollution* **129**, 331-340.

Migon. C., Mori, C. (1999). Arsenic and antimony release from sediments in a Mediterranean estuary. *Hydrobiologia* **392**, 81-88.

Migon, C., Mori, C., Orsini A., Tian, R.C. (1995) Arsenic and antimony contamination in a riverine environment affected by and abandoned realgar mine. *Toxicology & Environmental Chemistry* **52**, 221–230.

Mørch, T., Pedersen, R., Sørli, S., Breyholtz, B., Lambertsen, E., Farestveit, T., Pegg, I., Været, L., Bismo, M. (2007) Runoff from shooting ranges in Norway. Monitoring of water contaminants. Program for Ground Pollution2006-2007. Sweco Grøner Rapport 152030.

Morgan, I.J., Henry, R.P., Wood, C.M. (1997) The mechanism of acute silver nitrate toxicity in freshwater rainbow trout (*Oncorhynchus mykiss*) is inhibition of gill Na^+ and Cl^- transport. *Aquatic Toxicology* **38**(1-3), 145-163.

Nam, S.H., Yang, C.Y., An, Y.J. (2009) Effects of antimony on aquatic organisms (Larva and embryo of *Oryzias latipes*, *Moina macrocopa*, *Simocephalus mixtus*, and *Pseudokirchneriella subcapitata*). *Chemosphere* **75**, 889–893.

Nelson, B.K. (1994). Interactions in developmental toxicology: a literature review and terminology proposal. *Teratology* **49**, 33-71.

Newman, M.C., Unger, M.A. (2003) Fundamentals of Ecotoxicology, second ed. Lewis Publishers, New York.

Niyogi S, Wood CM (2004) Biotic ligand model, a flexible tool for developing site-specific water quality guidelines for metals. *Environmental Science & Technology* **38**, 6177-6192.

Norris, D.O., Camp, J.M., Maldonado, T.A., Woodling, J.D. (2000) Some aspects of hepatic function in feral brown trout, *Salmo trutta*, living in metal contaminated water. *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology* **127**(1), 71-78.

O'Neill, J.G., (1981) The humoral immune response of *Salmo trutta* L. and *Cyprinus carpio* L. exposed to heavy metals. *Journal of Fish Biology* **19**(3), 297 – 306.

Paquin, P.R., Gorsuch, J.W., Apte, S., Batley, G.E., Bowles, K.C., Campbell, P.G., Delos, C.G., Di Toro, D.M., Dwyer, R.L., Galvez, F., Gensemer, R.W., Goss, G.G., Hostrand, C., Janssen, C.R., McGeer, J.C., Naddy, R.B., Playle, R.C., Santore, R.C., Schneider, U., Stubblefield, W.A., Wood, C.M., Wu, K.B. (2002) The biotic ligand model: a historical overview. *Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology* **133**, 3-35.

Patel, M., Rogers, J.T., Pane, E.F., Wood, C.M. (2006). Renal responses to acute lead waterborne exposure in the freshwater rainbow trout (*Oncorhynchus mykiss*). *Aquatic Toxicology* **80**(4), 362-371.

Pierson, K.B., (1981) Effects of chronic zinc exposure on the growth, sexual maturity, reproduction and bioaccumulation of the guppy, *Poecilia reticulate*. *Canadian Journal of Fisheries and Aquatic Sciences* **38**(1), 23-31.

Pistole, D.H., Peles, J.D., Taylor, K. (2008) Influence of metal concentrations, percent salinity, and length of exposure on the metabolic rate of fathead minnows (*Pimephales promelas*). *Comparative Biochemistry and Physiology, Part C Toxicology and Pharmacology* **148**, 48-52.

Playle, R.C., Dixon, D.G., Burnison, K. (1993) Copper and cadmium-binding to fish gills: estimates of metal gill stability-constants and modeling of metal accumulation. *Canadian Journal of Fisheries and Aquatic Sciences* **50**, 2678–2687.

Pulido, M.D., Parrish, A.R. (2003) Metal-induced apoptosis: mechanisms. *Mutation Research-Fundamental and Molecular Mechanisms of Mutagenesis* **533**(1-2), 227-241.

Pyle, G.G., Rajotte, J.W., Couture, P. (2005) Effects of industrial metals on wild fish populations along a metal contamination gradient. *Ecotoxicology and Environmental Safety* **61**(3), 287-312.

Rajapakse, N., Ong, D., Kortenkamp, A. (2001) Defining the impact of weakly estrogenic chemicals on the action of steroidal estrogens. *Toxicological Sciences* **60**, 296-304.

Rantalainen, M.L., Torkkeli, M., Strömmer, R., Setälä, H. (2006) Lead contamination of an old shooting range affecting the local ecosystem - a case study with a holistic approach. *Science of the Total Environment* **369**, 99-108.

Reiley MC. (2007) Science, policy, and trends of metals risk assessment at EPA: how understanding metals bioavailability has changed metals risk assessment at US EPA. *Aquatic Toxicology* **84**, 292-298.

Reynders, H., Bervoets, L., Gelders, M., De Coen, W. M., Blust, R. (2008) Accumulation and effects of metals in caged carp and resident roach along a metal pollution gradient. *Science of The Total Environment* **391**(1), 82-95.

Roch M, McCarter JA. (1984a). Hepatic metallothionein production and resistance to heavy-metals by rainbow-trout (*Salmo gairdneri*)--I. Exposed to an artificial mixture of zinc, copper and cadmium. *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology* **77**, 71-75.

Roch, M., McCarter, J.A. (1984b). Hepatic metallothionein production and resistance to heavy-metals by rainbow-trout (*Salmo gairdneri*)--II. Held in a series of contaminated lakes. *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology* **77**, 77-82.

Rogers, J.T., Richards, J.G., Wood, C.M. (2003) Ionoregulatory disruption as the acute toxic mechanism for lead in the rainbow trout (*Oncorhynchus mykiss*). *Aquatic Toxicology* **64**(2), 215-234.

Rombough, P. Garside, E. (1982) Cadmium toxicity and accumulation in eggs and alevins of the Atlantic salmon, *Salmon salar*. *Canadian Journal of Zoology* **60**(8), 2006–2014.

Ruas, C.B., Carvalho Cdos, S., de Araújo, H.S., Espíndola, E.L., Fernandes, M.N. (2008) Oxidative stress biomarkers of exposure in the blood of cichlid species from a metal-contaminated river. *Ecotoxicology and Environmental Safety* **71**(1), 86-93.

Sanchez, W., Katsiadaki, I., Piccini, B., Ditche, J.M., Porcher, J.M. (2008) Biomarker responses in wild three-spined stickleback (*Gasterosteus aculatus* L.) as a useful tool for freshwater biomonitoring: A multiparametric approach. *Environmental International* **34**, 490-498.

Sanchez, W., Porcher, J.M. (2009) Fish biomarkers for environmental monitoring within the Water Framework Directive of the European Union. *Trends in Analytical Chemistry* **28**(2), 150-158.

Sandahl, J.F., Baldwin, D.H., Jenkins, J.J., Scholz, N.L. (2007) A sensory system at the interface between urban stormwater runoff and salmon survival. *Environmental Science & Technology* **41** (8), 2998–3004.

Santore, R.C., Mathew, R., Paul R. Paquin, P.R., DiToro, D. (2002) Application of the biotic ligand model to predicting zinc toxicity to rainbow trout, fathead minnow, and *Daphnia magna*. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* **133**, (1-2), 271-285.

Schmitt, C.J., Whyte, J.J., Roberts, A.P., Annis, M.L., May, T.W., Tillit, D.E. (2007) Biomarkers of metals exposure in fish from lead-zinc mining areas of Southeastern Missouri, USA. *Ecotoxicology and Environmental Safety* **67**, 31-47.

Seiler, R.L., Stollenwerk, K.G., Garbarino, J.R. (2005) Factors controlling tungsten concentrations in ground water, Carson Desert, Nevada. *Applied Geochemistry* **20**, 423-441.

Shafiq-ur-Rehman (2003) Lead-exposed increase in movement behavior and brain lipid peroxidation in fish. *Journal of Environmental Science & Health* **38**A, 631–643.

Sheppard, P.R., Ridenour, G., Speakman, R.J., Witten, M.L. (2006) Elevated tungsten and cobalt in airborne particulates in Fallon, Nevada: Possible implications for the childhood leukemia cluster. *Applied Geochemistry* **21**, 152-165.

Sheppard, P.R., Speakman, R.J., Ridenour, G., Witten, M.L. (2007) Temporal variability of tungsten and cobalt in Fallon, Nevada. *Environmental Health Perspectives* **115**, 715-719.

Slaveykova, V.I., Wilkinson, K.J. (2005) Predicting the bioavailability of metals and metal complexes: Critical review of the biotic ligand model. *Environmental Chemistry* **2**, 9-24

Sokolowska-Mikolajczyk, M., Chyb, J., Socha, M., Epler, P. (2000) The effect of zinc on maturational gonadotropin (GtH2) secretion modulated by GABAergic system in common carp (*Cyprinus carpio* L). *Archives of Polish Fisheries* **8**(1), 129-143

Spehar, R.L., Fiandt, J.T. (1986) Acute and chronic effects of water quality criteria-based metal mixtures on three aquatic species. *Environmental Toxicology & Chemistry* **5**(10), 917-931.

Spry, D.J., Wood, C.M. (1985) Ion flux rates, acid-base status, and blood gases in rainbow trout, *Salmo gairdneri*, exposed to toxic zinc in natural soft-water. *Canadian Journal of Fisheries and Aquatic Sciences* **42**, 1332-1340.

Stohs S.J., Bagchi, D. (1994) Oxidative mechanisms in the toxicity of metal ions. *Free Radical Biology & Medicine* **18**, 321–336.

Strigul, N., Koutsospyros, A., Christodoulatos, C. (2010). Tungsten speciation and toxicity: acute toxicity of mono- and poly-tungstates to fish. *Ecotoxicology & Environmental Safety* **73**, 164-171.

Strømseng, A.E., Ljønes, M., Bakka, L., Mariussen, E. (2009) Episodic discharge of lead, copper and antimony from a Norwegian small arm shooting range. *Journal of Environmental Monitoring* **11**(6), 1259-1267.

Sühnel, J. (1990) Evaluation of synergism or antagonism for the combined action of antiviral agents. *Antiviral Research* **13**, 23-39.

Sutterlin, A.M., Sutterlin, N. (1970) Taste responses in Atlantic salmon (*Salmo salar*) parr. *Journal of the Fisheries Research Board of Canada*. **27**(11), 1927-1942.

Svecevicius, G. (1999). Fish avoidance response to heavy metals and their mixtures. *Acta Zoologica Lituanica. Hydrobiologia* **9**(2), 103-113.

Takayanagi K. (2001) Acute toxicity of waterborne Se(IV), Se(VI), Sb(III), and Sb(V) on red seabream (*Pargus major*). *Bulletin of Environmental Contamination and Toxicology* **66**, 808-813.

Tirmenstein, M.A., Plews, P.I., Walker, C.V., Woolery, M.D., Wey, H.E., Toraason, M.A. (1995) Antimony-induced oxidative stress and toxicity in cultured cardiac myocytes. *Toxicology and Applied Pharmacology* **130**(1), 41-47.

Tukker, A., Buist, H., van Oers, L., van der Voet, E. (2001) Risks to health and the environment related to the use of lead in products, Report STB-01-39, Delft, Netherlands.

Tulasi, S.J., Reddy, P.U.M., Ramana Rao, J.V. (1992) Accumulation of lead and effects on total lipids and lipid derivatives in the fresh-water fish Anabas-testudineus (Bloch). *Ecotoxicology & Environmental Safety* **23**, 33-38.

Tuomela, M., Steffen, K.T., Kerko, E., Hartikainen, H., Hofrichter, M., Hatakka, A. (2005). Influence of Pb contamination in boreal forest soil on the growth and ligninolytic activity of litter-decomposing fungi. *FEMS Microbiology Ecology* **53**, 179-186.

USEPA (2007) Aquatic life ambient freshwater quality criteria – copper. Report no EPA-822-R-07-001. http://www.epa.gov/waterscience/criteria/aqlife.html.

van der Oost, R., Beyer, J., Vermeulen, N.P.E. (2003) Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environmental Toxicology and Pharmacology* **13**(2), 57-149.

van Genderen, E., Gensemer, R., Smith, C., Santore, R., Ryan, A. (2007) Evaluation of the Biotic Ligand Model relative to other site-specific criteria derivation methods for copper in surface waters with elevated hardness. *Aquatic Toxicology* **84**, 279-291.

van Vuren J.H.J., van der Merwe, M., Du Preez, H.H. (1994) The effects of copper on the blood chemistry of *Clarias gariepinus* (Clariidae). *Ecotoxicology and Environmental Safety* **29**(2), 187-199.

Veenstra, G.E., Deyo, J., Penman, M. (1998) Risk assessment for the exposure to antimony compounds. *Toxicology Letters* **95** (Supplement 1), 136.

Verbost, P.M., Flik, G., Lock, R.A.C., Wendelaar-Bonga, S.E. (1987) Cadmium inhibition of Ca^{2+} uptake in rainbow trout gills. *American Journal of Physiology* **253**, R216-R221.

Verslycke, T., Vangheluwe, M., Heijerick, D., De Schamphelaere, K., Van Sprang, P., Janssen, C.R. (2003) The toxicity of metal mixtures to the estuaringe mysid *Neomysis integer* (Crustacea: Mysidacea) under changing salinity. *Aquatic Toxicology* **64**, 307-315.

Vieira, L.R., Gravato, C., Soares, A.M.V.M ., Morgado, F. Guilhermino, L. (2009) Acute effects of copper and mercury on the estuarine fish *Pomatoschistus microps*: Linking biomarkers to behavior. *Chemosphere*, **76**(10), 1416-1427.

Vyas, N.B., Spann, J.W., Heinz G.H., Beyer, W.N., Jaquette, J.A., Mengelkoch, J.M. (2000) Lead poisoning of passerines at a trap and skeet range. *Environmental Pollution* **107**, 159-166.

Wah Chu, K., Chow, K.L. (2002) Synergistic toxicity of multiple heavy metals is revealed by a biological assay using a nematode and its transgenic derivative. *Aquatic Toxicology* **61**(1-2), 53-64.

Wang, G., Fowler, B. A. (2008) Roles of biomarkers in evaluating interactions among mixtures of lead, cadmium and arsenic. *Toxicology and Applied Pharmacology* **233**(1), 92-99.

Watanabe, T., Kiron, V., Satoh, S. (1997) Trace minerals in fish nutrition. *Aquaculture* **151**(1-4), 185-207.

Weber, D.N. (1993) Exposure to sublethal levels of waterborne lead alters reproductive behavior patterns in fathead minnows (*Pimephales promelas*). *Neurotoxicology* **14**(2-3), 347-58.

Wendelar-Bonga, S.E. (1997) The stress response in fish. *Physiological Reviews* **77**(3); 591-625.

Winberg, S., Bjerselius, R., Baatrup, E., Doving, K.B. (1992) The effect of Cu(II) on the electro-olfactogram (EOG) of the Atlantic salmon (*Salmo salar* L) in artificial fresh-water of varying inorganic carbon concentrations. *Ecotoxicology and Environmental Safety* **24**(2), 167-178.

Wood, C.M. (2001) Toxic responses of gill. In Target organ toxicity in marine and freshwater teleost. Taylor and Francis, London, Editor Schlenk D. and Benson W.H. ISBN 0-415-24838-8

Wood, C.M., Hogstrand, C., Galvez, F., Munger, R.S. (1996) The physiology of waterborne silver toxicity in freshwater rainbow trout (*Oncorhynchus mykiss*) 1. The effects of ionic Ag^{+}. *Aquatic Toxicology* **35**, 111-125.

Zelikoff J.T. (1998). Biomarkers of immunotoxicity in fish and other non-mammalian sentinel species: predictive value for mammals? *Toxicology* **129**(1), 63-71.