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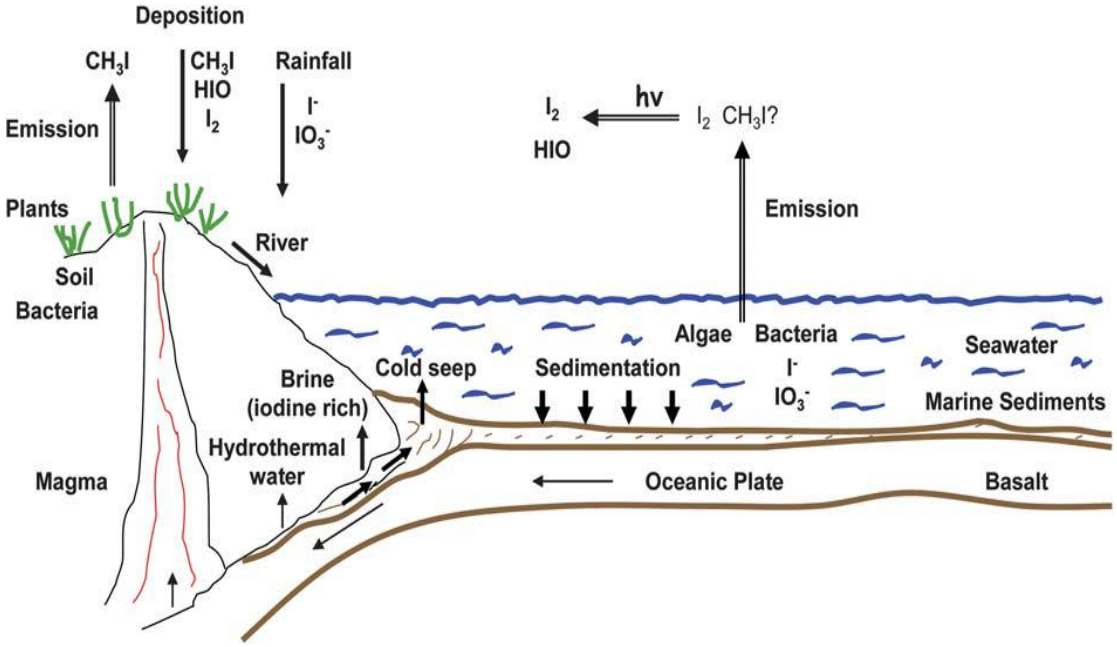

Author: Atabak M. Azad

THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR MASTER DEGREE IN NUTRITION OF AQUATIC ORGANISMS IN AQUACULTURE

June 2013

Front page figure: Cycle of iodine in the environment. From Moreda-Pineiro et al., 2011

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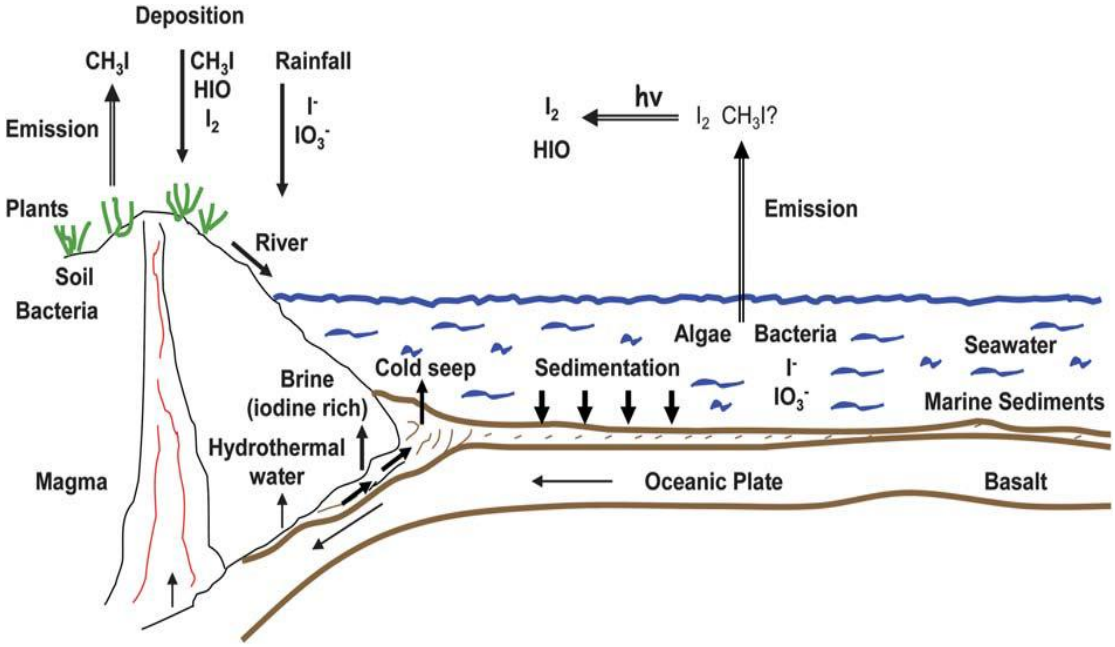

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This project was not possible to be accomplished without the help and support of my advisory committee, Eric-Jan Lock who was always positive and supportive. Thanks for your valuable assistance and guidance in all stages. It is really difficult to find more supportive supervisor than you, Daniel Fliegel for your helpful comments and advices and Rune Waagbø for your support and really constructive comments on the manuscript.

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CHAPTER 1.

General introduction

Iodine is an essential trace element and the biosynthesis of thyroid hormones in all vertebrates is deeply dependent on bioavailability of iodine (Lall, 2002).

Terrestrial animals absorb iodine from food but the main uptake route in fish is from the water (gills and intestine). Rainbow trout receives 80 percent of iodine from water, 19 percent from the diet and less than 1 percent from recycling iodine from thyroid hormone metabolites (Leloup, 1970 in Lall, 2002).

1.1. Thyroid gland anatomy and hormones synthesis

Contradictory to mammals, where thyroid is an encapsulate gland that is usually located in the neck area, in teleosts the thyroid gland is not encapsulated. Here the thyroid follicles are scattered in the ventral side to pharynx along the ventral aorta. In some fish species these follicles are not distributed only in the subpharyngeal region but also in heart, spleen, liver, esophagus, brain, choroid rete mirabile and head kidney. Heterotopic follicles are reported in several orders of teleost fish including anchovies and herrings (Clupeiformes, 1 species), catfish (Siluriformes, 4 species), killifish (Cyprinodontiformes, 3 species), swamp eels (Synbranchiformes, 1 species), perch-like fish (Perciformes, 3 species), rainbow fish and silversides (Atheriniformes, 1 species), minnows and suckers (Cypriniformes, 14 species) (Geven et al., 2007). Heterotopic follicles are involved in absorption of iodine and thyroid hormones synthesis.

Thyroid follicles are the functional unit of the gland and synthesize the thyroid hormones 3,5,3',5'-tetraiodothyronine (T4) and to a lesser extent 3,5,3'-triiodothyronine (T3). Each follicle has one layer of thyrocyte making extracellular lumen, which is filled with a colloid matrix. The Na^+/I^- symporter is responsible to carry iodine from plasma into the thyrocyte and the Cl^-/anion exchanger transfer the iodine into lumen. Subsequently, the thyroid peroxidase (TPO) oxidizes (I^-) to iodonium (I^+), whereafter iodonium replaces hydrogen in tyrosine in thyroglobin (Tg). This results in the formation of monoiodotyrosine (MIT) and diiodotyrosine (DIT). Next, TPO catalyses the coupling of two DIT molecules to produce T4, and one MIT with one DIT to produce T3 (Geven, 2009).

1.2. Thyroid hormones functions

T3 and T4 are the main hormones released from the thyroid gland into the bloodstream. Thyroid hormone receptors (THRs) are ubiquitously expressed in most tissues in vertebrates (Berry et al., 1998). THRs have a higher affinity for T3 than T4. Therefore removing one

iodine from T4 is very important for the activation of thyroid hormones (THs). This process is called deiodination and yields to the most biological active metabolite T3.

Several functions of THs have been reported in vertebrates but the three main roles are within development, growth and metabolism. THs are deeply involved in the regulation of metamorphosis and smoltification. Moreover, THs play an important role in the differentiation of tissues and proliferation of cells and thus play an important role in growth. The main and the best known role of THs however is the regulation of metabolic rate and heat production in endotherms. In this context it is reported that THs increase oxygen consumption, influence gluconeogenesis, increase lipolysis and protein synthesis in teleosts (Geven, 2009).

1.3. Regulation of thyroid hormones secretion

The hypothalamo-pituitary-thyroid axis is the main regulator of THs in vertebrates. In this process, the hypothalamus excretes thyrotropin releasing hormone (TRH) that itself stimulates the pituitary to release thyroid stimulating hormone (TSH). TSH stimulates the release of THs from the thyroid follicles. In this process, THs have a negative feedback loop on the release of TSH and TRH. In common carp (*Cyprinus carpio*) it has been demonstrated that TRH does not affect the release of TSH from pituitary but stimulates the excretion of both growth hormone (GH) and prolactin (Kagabu et al., 1998).

De Groef and colleagues (2006) found that corticotropin-releasing-hormone (CRH) can replace TRH and therefore stimulate the pituitary to excrete TSH.

1.4. Goitrogenic effect of bromine

Iodine deficiency is the main cause of thyroid dysfunction, but several natural and synthetic compounds have been reported as goitrogenic, that disrupt the function of thyroid hormone by affecting Na+/I- symporter, thyroid peroxidase or cytotoxic effects on thyrocytes (Thienpont et al., 2011).

Bromine (Br) is not an essential trace element and has no biological importance. Thus no specific regulatory mechanisms for Br exist in fish. Therefore once Br is introduced into the organism it will be distributed ubiquitously, and the excretion pathways are in general similar to other halogens (Hellerstein et al., 1960). Pavelka (2004) reviewed the effect of Br on metabolism of iodine. Br intake in rat could reduce iodine accumulation in the thyroid and skin and increases the excretion of iodine by kidney (Pavelka et al., 2001). In another study Br intake, even in high amounts, did not affect the iodine excretion rate in rat but decreased

the accumulated iodine in the thyroid gland by 20 percent, presumably via influencing the transportation process of iodine into the thyroid gland (Vobecky et al., 1996).

The goitrogenic effect of Br compounds becomes more severe in a condition of iodine deficiency (Buchberger et al., 1990). Administration of sodium bromine in Zebrafish eleutheroembryos significantly decreases the intrafollicular T4 concentration in compare with control condition (Thienpont et al., 2011). Baker (2004) reviewed the toxicity of iodine and concluded that long term over-exposure of iodine lowered the organic binding of iodine in the thyroid gland and resulted in hypothyroidism and goiter. Due to similar chemical properties, I atoms in both T3 and T4 could be replaced by Br without affecting the thyroid hormone function (Pavelka, 2004).

On the other hand, I deficiency will result in low TH levels. It is estimated that more than two billion people on earth have a deficient iodine intake (Zimmermann and Andersson, 2011) and face the risk of iodine deficiency and around 650 million people worldwide suffer from goiter disease (Pavelka, 2004).

1.5. Iodine species in the environment and their bioavailability

Iodine is a non-metallic element occurring mainly in the form of iodide or oxidized form iodate or in organic compounds such as methyl iodide or chloroiodomethane.

The main source of iodine in the human diet is marine fish, shellfish and seaweeds. Seawater aerosols containing the molecular iodine go to the air and through rainfall and human activity iodine can be distributed in the environment (Moreda-Pineiro et al., 2011).

Distribution of iodine in the soils and surface waters reduces with distance from the ocean. In some landlocked countries distant from the ocean with limited accesses to sea foods, iodine deficiency can develop into an epidemic. This problem can cause prenatal and postnatal growth and developmental disorders (Hollowell and Hannon, 1997). Iodine deficiency is mostly eradicated around the world by the easy method of iodine supplementation to salt. However it is still a serious problem in 54 countries of which several are found in Europe (Andersson et al., 2005).

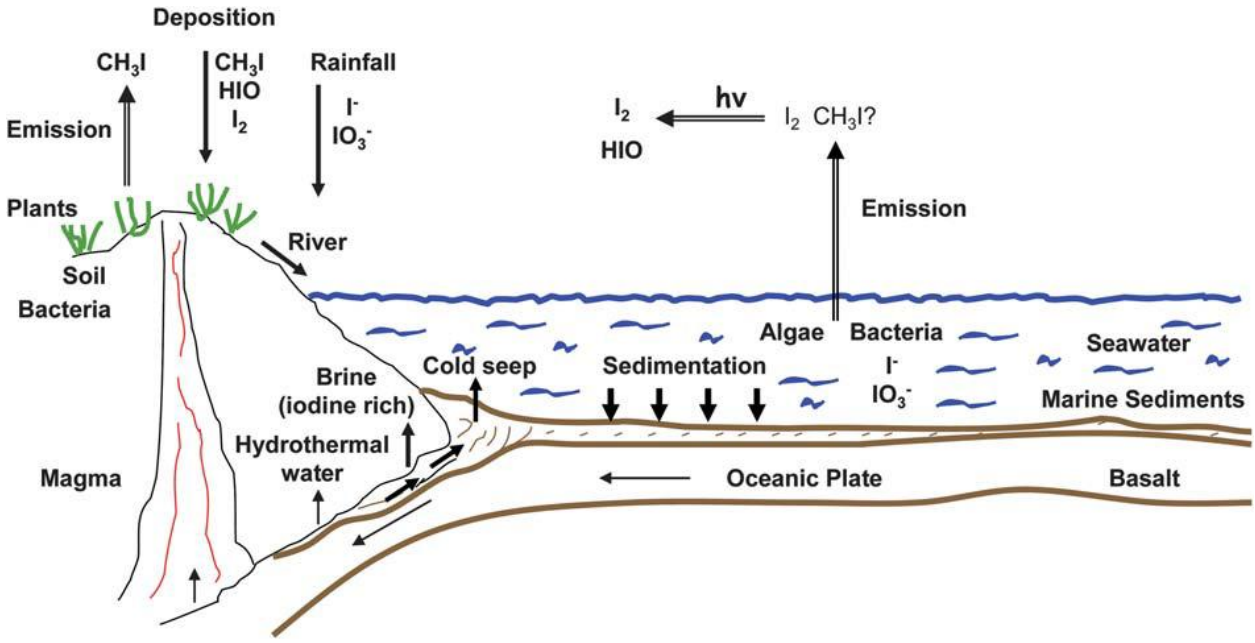

Fig. 1.5.1. Cycle of iodine in the environment. From Moreda-Pineiro et al., 2011

The iodine concentration in the sea varies between 0.44 and 0.49 µM (Kupper et al., 1998). Iodide and iodate are the main species of iodine present in the ocean (Truesdale et al. 1995). Both of these species are used as thyroid blocker or as a prophylaxis for people who may have been exposed to radioiodine. Pahuja and colleagues in 1993 demonstrated that potassium iodate and potassium iodide are equally available to rats and effective in blocking thyroid function. There is a general consensus that both iodide and iodate are the same in terms of the bioavailability to mammals.

Over the past decades increasing emissions of anthropogenic brominated compounds into the environment has occurred, which has triggered extensive research on the distribution of these compounds in the environment. However, the potential interference with iodine metabolism has been explored into a lesser extent, and information on fish is scant. In order to measure the various iodine and bromine species the need for compound separation and detection is inevitable. Liquid chromatography coupled with Inductively coupled Plasma Mass Spectrometry is one of the best method for this aim. In this regards, a new method is developed and described in this thesis using HPLC-ICP-MS to quantify iodine and bromine species in the same sample.

The thyroid endocrine system is similar in all vertebrate classes, demonstrating its vital function in basic metabolic processes. Zebrafish eleutheroembryos reacted similar to 15 out of 16 (93.75%) goitrogenic compounds (with a direct effect on thyroid gland function) as

mammals did (Thienpont et al., 2011). This demonstrates the similarity between the zebrafish and mammalian thyroid physiology. Because iodine is only involved in thyroid hormone synthesis and metabolites (Geven, 2007), by using radioiodine radioiodine in designed experiments, it is possible to investigate the uptake, deposition and clearance of Iodine.

1.6. Aims of the study

This study can provide basic knowledge for the analysis of halide effect on iodine metabolism as well as new insight to the metabolism and turnover of iodine in zebrafish.

There have been investigations that compare iodine species bioavailability in mammals; however to the best of our knowledge no data exist on fish today. In this study, the uptake and depuration curve for radioiodine in zebrafish was established. In this study we are going 1) to investigate the possible variable playing role in the uptake and depuration of iodine in zebrafish including Gender of the fish, pH and anesthesia 2) to Analyze the normal curves of uptake and depuration of iodine resulted in the identification of various phases and critical time points and to identify the active and passive processes as far as possible. 3) to compare the bioavailability of main iodine species to zebrafish and Finally 4) to investigate the effect of halogenic compounds on the kinetics of iodine in zebrafish.

CHAPTER 2.

Determination of inorganic iodine and bromine species in municipal and bottled waters using Anion Exchange Liquid Chromatography-coupled with Inductively coupled Plasma Mass Spectrometry

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keywords: iodine, bromine, drinking water

manuscript in preparation

Introduction

Bromine (Br) concentrations in water are known to vary widely and can be a significant source for Br exposure in humans. Adverse effects of Br on thyroid physiology have been described in mammals. Administration of elevated dietary doses of Br in rat reduced the thyroidal accumulation of iodine by 77 percent and enhanced renal iodine excretion (Pavelka et al., 2001). In another study Vobecky and colleagues (1996) demonstrated that one third of iodine in the thyroid of rat is replaced by Br after elevated ingestion of bromide. This change resulted in a decrease of iodinated thyroid hormones (TH) and an increase of brominated TH. Dietary exposure to Br resulted in a reduction in iodine level and an increase of the bromide concentration in the milk of lactating female rat. Ultimately, these changes reduced the body weight of the pups (Pavelka et al., 2002).

According to a recent investigation by NGU in 2012 in Norway, 71% of groundwater samples contain Br levels below the detection limit (0.1 mg L^{-1}). The maximum concentration reported was 9.04 mg L-1. In Norway no legislation exists on bromine (Br) levels in drinking water.

Disinfection of drinking water is important to eradicate pathogens and other disease vectors naturally found in most water bodies. However, depending on the method used, disinfection can have its own noxious by-products.

Chlorine is a widely applied water disinfectant, but now studies have demonstrated that trihalomethans, a carcinogen, can be produced when using chlorine as a disinfectant. Trihalomethans are the by-product of chlorination of drinking water in presence of organic compounds and halogens mainly chloride, bromide and iodide. Increased concentrations of iodide/bromide result in higher concentrations of iodinated/brominated trihalomethans. It has been demonstrated that iodo-trihalomethans are more toxic than brominated and chlorinated trihalomethans (Criquet et al., 2012). Chlorination of drinking water has often been replaced with other methods like ozonation (Geter et al., 2006).

Bromate (BrO_3^-) does not form naturally in the freshwater environment and human activities, like ozonation of bromide containing water, are the most likely routes to introduce this harmful compound to the environment. During ozonation, ozone (O_3) reacts with Br^- to produce hypobromus acid (HOBr). Subsequently HOBr in presence of O_3 changes into OBr^-, which on its turn is oxidized into bromate. This process is catalysed by increased ozone concentration, contact time and pH (EPA 2001; Haag and Holgne 1983). Lowering of pH and the addition of ammonia can reduce the formation of bromate from bromide by 50% and

these two measures are suggested to control bromate creation during ozonation of drinking waters (Von Gunten and Pinkernell 2000).The harmfull effect of bromate on biological systems is well documented. Long-term exposure to low concentrations of bromate causes renal tumor growth in laboratory animals (Geter et al., 2006; Pfaff and Brockhoff 1990; Kurokawa et al., 1990). In addition, many investigations showed that bromate has harmful effects on aquatic organisms. Hutchinson and colleagues (1997) reviewed the effect of bromate on the aquatic organisms and concluded that to avoid a chronic toxic effect in fish and invertebrates the concentration of bromate should be below 3.0 mg L^{-1}.

There are no data that suggest the accumulation of bromate in tissues of invertebrates, fishes and mammals (Hutchinson et al., 1997). In male rat, 24 hours after an oral dose potassium bromate (50 mg kg^{-1} body weight), bromate was detected in none of the eight analyzed tissues (less than 2.5 µg mL^{-1} in urine and plasma, less than 5 µg g^{-1} in other tissues). Ingested bromate is partly reduced into bromide and mainly excreted through urine in both forms of bromide and bromate (Fujii et al., 1984). Bromate is classified as a group 2B (possibly carcinogen to human) and the admissible upper limit set by WHO and The European Commission is 10 µg L^{-1} in drinking water (WHO, 2011; Fawell and Walker 2006).

In the past bromate and iodate were added to flour to enhance the oxidation of gluten and to create more elasticity of the dough. Whilst bromate and iodate are changed into bromide and iodide respectively during the rising and baking process, the use of bromate in bread making is banned in most countries.

Iodate is another by product of ozonation of drinking water. Ozone is oxidizing iodide into hypoiodus acid (HOI) and subsequently into iodate. Although iodate has similar physiochemical properties as bromate, it has a much lower oxidizing capacity. The carcinogenic and genotoxic capacities of bromate are well documented; however studies on harmful effects of iodate are scant (Pantsar-Kallio and Manninen 1998; Pfaff and Brockhoff 1990). Two case studies describe damage to retina and photoreceptors in human subjects of the ingestion of 10-20 mg iodate kg^{-1} body weight (Burgi et al., 2001). Andersen (1995) exposed the bactria *Escherichia coli* to sodium iodate and ionizing radiation while monitoring DNA damage. Iodate attenuated the damage caused by radiation and the author concluded that better documentation is needed to consider iodate a safe ingredient in cosmetic products (Andersen 1995). Currently there are not enough documents that support the toxicity of iodate but it should be taken into account as a potential toxic compound.

In this study a new method was described to measure iodine and bromine species in small water samples simultaneously. The chromatography was optimized to maintain adequate LODs for all species. Iodine and bromide species in bottled water as well as tap water and surface waters were measured and the data are presented.

Experimental set-up and sampling

Iodine (I^-), iodate ($IO3^-$) , bromine (Br^-) and bromate ($BrO3^-$) were measured using AE-HPLC-ICP-MS. The different species were separated using an anion exchange column (ICSep ION-120, 120×4.6 mm×10 μm particles, Transgenomics, San Jose, CA, USA). The HPLC set up consisted of a PE Series 200 metal-free HPLC pump (Perkin Elmer, Norwark, CT, USA), a Waters 717 autosampler (Waters, Milford, MA, USA) and a polymer-based strong anion-exchange column (ICSep ION-120, 120×4.6 mm×10 μm particles, Transgenomics, San Jose, CA, USA) operated under isocratic conditions. The HPLC was coupled to an ICP-MS (Agilent 7500ce, Yokogawa Analytical Systems Inc., Tokyo, Japan) using a V-groove nebulizer and a cooled scott double pass spray chamber. Isotopes monitored were ^{127}I, ^{79}Br and ^{81}Br. The ICP-MS settings were as follows: Rf power 1500 W, plasma gas flow-rate 15 L min^{-1}, auxiliary gas flow-rate 1 L min^{-1} and nebuliser gas flow-rate 0.95 L min^{-1}. External calibration with four levels of all species was used for the quantification of the samples.

Different concentrations of mobile phase Na-salicylate (Merck, Darmstadt, Germany), were tested in order to find the optimal condition for chromatography. Four different flow rates including 0.8, 1, 1.2 and 1.4 mL min^{-1} were tested in combination with mobile phase concentrations to adjust the peaks of iodine and bromine species separately. The experimental parameters are summarized in tables 2.1 and 2.2.

Standard solutions were made daily fresh by dissolving potassium iodide, potassium iodate, potassium bromide and potassium bromate (Sigma here give the type and the venodor for each compounds) in MilliQ water (Milli-Q, Milford, MA, USA).

Seawater sample was collected from NIFES, Bergen, Norway. Freshwater and tap water samples were collected from different areas in and around Bergen, Norway. The bottled waters were bought from supermarkets as samples (14 bottled waters including still and sparkling as different samples).

Calculating figures of merit

The peaks resulted from liquid chromatography conditions were assessed quantitatively. The peaks were exported to excel and then to Image Tool software to measure the peak lengths. Retention time was based on the maximum peak height. The number of theoretical plates (N) was calculated as $N=5.5\ \frac{Retention\ time^2}{Width\ at\ half\ oh\ peak\ height^2}$.

The tailing factor was measured by dividing the distance of front slope of the peak to back slope by twice the distance from the center line to the front slope at 5% of peak height.

Results and discussion

Separation and determination of iodine and bromine species

Seven different combinations of flow rate and mobile phase concentration were evaluated to find out the best LC condition of IO_3^- and I^- in terms of specificity and sensitivity. The tailing factor was between 1.80 and 2.54 and the number of theoretical plates ranged from 387 to 1226. The best separation and peak characteristics was obtained for a 32.5mM mobile phase at 1.4mL min^{-1} flow rate. This condition had lowest tailing factor and number of theoretical plates due to high flow rate and therefore lowest retention time of 57 second. The shape of both iodide and iodate peaks were approximately Gaussian.

The main intent for bromide and bromate chromatography was to find the best condition resulting in separate peaks with proper shape. For this setup, eight combinations of mobile phase and flow rate were examined. The best selectivity factor alpha (0.65) was obtained by 9.6mM concentration of mobile phase and flow rates of 0.8 and 1 mL min^{-1}. Among these two flow rates, 1mL min-1 flow rate resulted in lower tailing factor of 1.44 and 1.46 for bromate and bromide respectively. Peak characteristics were determined based on the ^{79}Br isotope, however ^{81}Br showed a similar behaviour.

Finally, two different set up were tested to determine the optimal chromatography condition in which it was possible to generate peaks for all four species during a single run. The optimal set up was determined as 11.9 mM concentration of mobile phase and 1.2 mL min^{-1} flow rate.

Table 2.1. Iodate peak figures of merit for different experimental conditions. The concentration of analyte is 72.6 nM.

Mobile phase concentration (mM)	Flow rate (mL min^{-1})	Tailing factor	Number of theoretical plates
25.6	1	2.16	489
7.3	1.2	2.53	1091
11.9	1.2	2.54	829
16.5	1.2	1.32	1226
30.2	1.2	2.25	643
25.6	1.4	2.00	433
32.5	1.4	1.80	387

Table 2.2. Bromate and bromide peak figures of merit for different experimental setups. The concentration of analytes are 200nM.

Mobile phase concentration (mM)	Flow rate (mL min^{-1})	Species	Retention time (s)	Tailing factor	Selectivity factor alpha	Number of theoretical plates
9.6	0.8	$BrO3^-$	168.18	1.75	0.65	1586
		Br^-	257.92	1.47		2518
16.5	0.8	$BrO3^-$	136	2.00	0.72	1073
		Br^-	189.34	2.05		1489
9.6	1	$BrO3^-$	134	1.44	0.65	1250
		Br^-	205.46	1.46		2490
16.5	1	$BrO3^-$	114.46	2.64	0.73	563
		Br^-	156.41	2.30		892
21	1	$BrO3^-$	105.45	2.20	0.75	1545
		Br^-	140	1.75		1340
23.3	1	$BrO3^-$	106.2	1.89	0.78	966
		Br^-	135.91	1.90		1183
23.3	1.2	$BrO3^-$	88.18	0.86	0.78	1156
		Br^-	112.73	0.73		1261

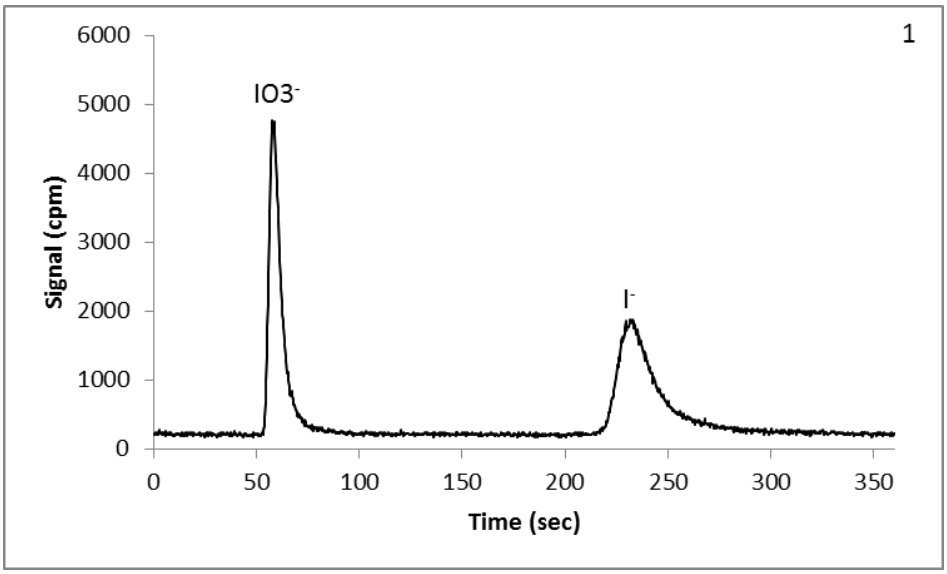

Fig. 2.1. Chromatogram of iodate and iodide using 32.5mM mobile phase and 1.4mL min^{-1} flow rate. Concentration of both species is 25 nM.

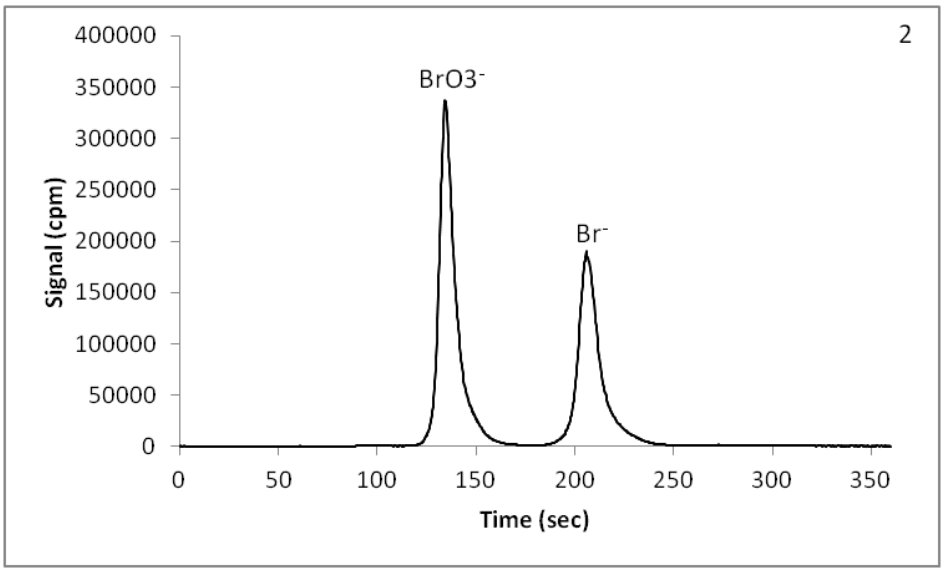

Fig. 2.2. Chromatogram of bromate and bromide using 9.6mM mobile phase and 1mL min^{-1} flow rate. Concentration of analytes is 500 nM for both species.

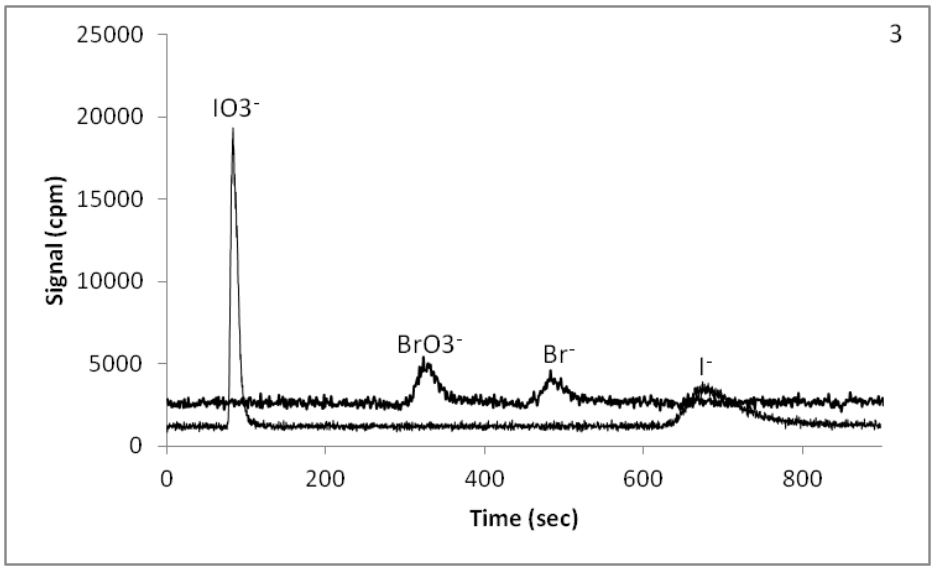

Fig. 2.3. Chromatogram of iodate, iodide, bromate and bromide using 11.9mM mobile phase and 1.2 ml min^{-1} flow rate. Concentration of iodine and bromine species are 50 nM and 100 nM respectively.

Method sensitivity

Limit of detection (LOD) and limit of quantification (LOQ) were calculated as three and 10 times the standard deviation plus the average of the blank, respectively. Table 2.3 presents the LOD and LOQ for iodine and bromine species. These LODs are consistent with routine methods for drinking water and the LOD for Bromate (0.51 µg L^{-1}) is lower than the recommended level by The European Commission of 2.5 µg L^{-1} (Diemer and Heumann 1997).

Table 2.3. The Limit of detection (LOD) and limit of quantification (LOQ) of the method based on 25µL of sample injection (µg L^{-1}).

	LOD	LOQ
Iodide (I^-)	0.87	2.90
Iodate ($IO3^-$)	0.40	1.33
Bromide (Br^-)	0.76	2.53
Bromate ($BrO3^-$)	0.51	1.69

Linear range and Repeatability

Four levels of standard solution for iodate and iodide (25, 50, 100 and 250nM) and six levels of standard solution of bromate and bromide (50, 100, 250, 800 and 2500nM) were measured to determine the linearity. Fig 2.4 shows the standard curves for iodine and bromine species. The regression coefficients (R^2) of all standard curves were better than 0.999 for all species. The RSD value for all four species, based on three replicates, was below 8%, which is considered a good repeatability of the method. The RSD for the three lowest concentrations of all four species are shown in Fig. 2.5.

According to The European Commission (1994) the accuracy of the method should be lower than 25% at the concentration of 10 µgL-1 (Diemer and Heumann 1997). In our method the first level (A) for all four analytes is less than 10 µg L^{-1} and is better than 5% which properly fits the recommended level by The European Commission of 2.5 µg L^{-1} (Diemer and Heumann 1997).

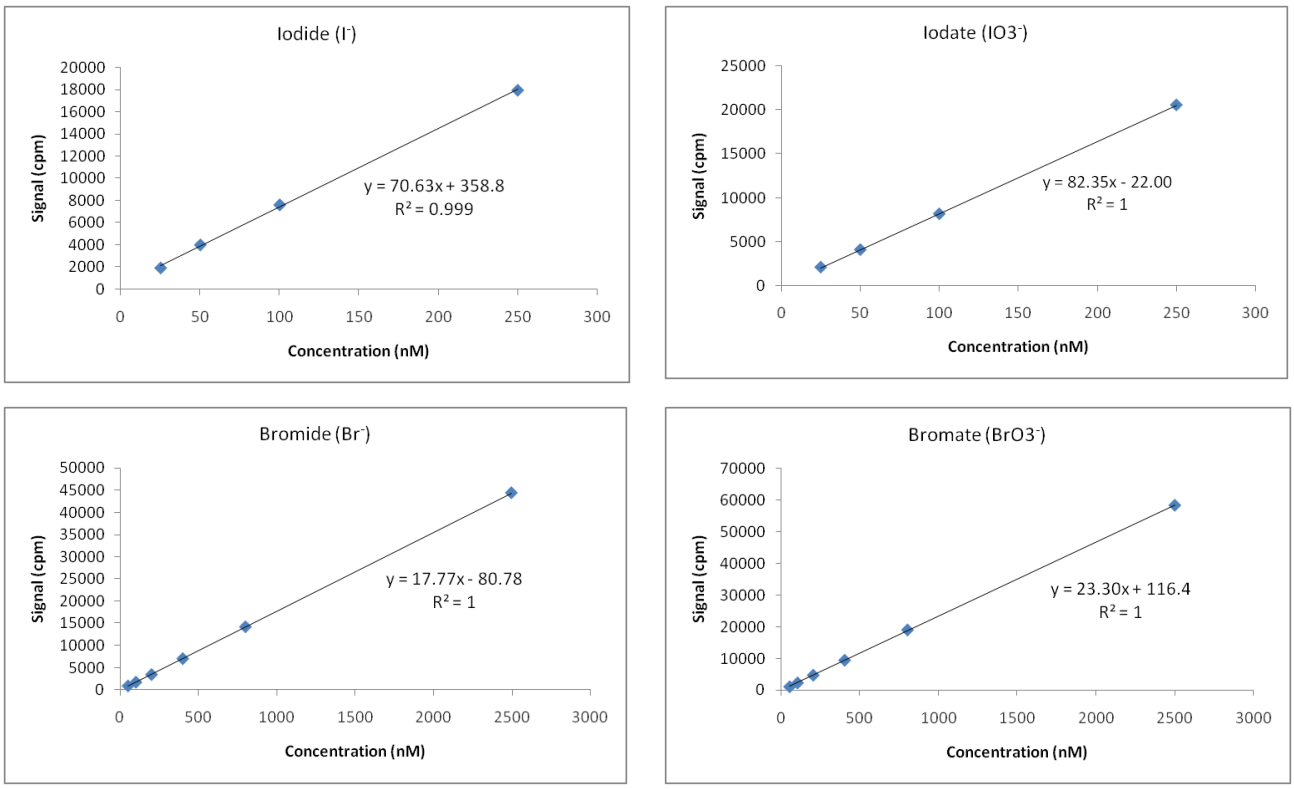

Fig. 2.4. Standard curves and related regression coefficients for iodine and bromine species

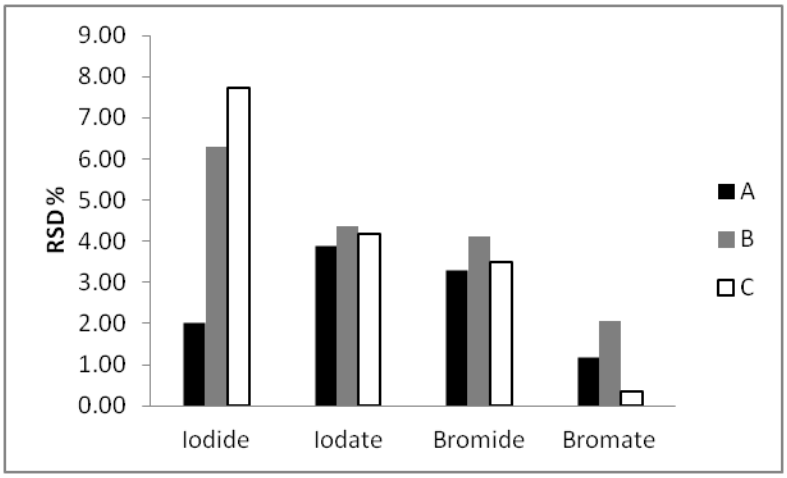

Fig. 2.5. Repeatability of measurements for iodine and bromine species in different concentration levels: A) 25nM for iodine species and 50nM for bromine species; B) 50nM for iodine species and 100nM for bromine species; C) 100nM for iodine species and 200nM for bromine species.

Iodine and bromine speciation measurements in the samples

In this study municipal waters contained lower levels of all anions in comparison to bottled mineral waters. In surface and municipal waters only low concentrations of bromide were detected within the range of 7 – 11 µg L^{-1}. About two-third of the bottled waters contained bromide, with concentrations ranging between 3 – 1923 µg L^{-1}, a much larger variation than found in surface and municipal waters. The concentration of bromide detected in this study in some bottled mineral waters was relatively high and if ozonation or chloration is used for disinfection in these cases, there can be a potential risk for the formation of bromate and trihalomethans. Due to high concentration of iodide (288 µg L^{-1}) in mineral water Farris, the formation of iodo-trihalomethan is also possible.

Iodide was not detected in surface and municipal water, and only found in two samples of bottled water (3 and 288 µg L^{-1}). The concentration of iodide in Farris is the highest (more than 30 times higher than in seawater). Concentration of iodine and bromine species in bottled, surface and municipal waters is shown in table 2.4.

Two samples including Voss and Pellegrino contained iodate concentration of 2 and 7.00 µg L^{-1} respectively. Bromate was detected in two bottled waters at the quantifiable concentration; Taffel (2 µg L^{-1}) and Farris (4 µg L^{-1}). Both values are under the maximum admissible concentration of 10 µg L^{-1}.

Currently no legislation exists for iodide, iodate and bromide in drinking water in European countries and North America. However there are concerns on the toxic properties of iodate.

Recently, Criquet and colleagues (2012) demonstrated that an increase in bromide concentration increases the conversion of iodide to iodate . In some countries like Russia (NGU 2012), the upper allowed concentration for total bromine in drinking water is 200 µg L^{-1}. According to that limit 3 of the 14 bottled waters tested in this study are above the admissible limit. Although the limit for bromine is determined as total bromine in Russia, it seems it is mainly due to the lack of a proper method for separation and quantification of bromine species. As mentioned before, bromate is the toxic species and there is not enough data on the potential harmful effects of bromide. According to current Norwegian law all municipal and bottled waters tested are considered safe.

Conclusion

A method based on IE-HPLC coupled with online ICP-MS and direct injection was developed for detection of iodine, iodate, bromine and bromide species in drinking water. This method fulfills the required conditions according to recommended values in standard methods with regard to the limit of detection and short time analysis.

The method was successfully applied to determine the concentrations of iodine, iodate, bromine and bromide in bottled and municipal waters. The municipal waters only contained measurable amounts of bromide in low concentrations that were similar between the different locations. The bottled waters showed a high variation in halogen content and concentration. Two of the bottled water samples contained bromate, however within the upper allowable level set under current Norwegian law.

Table 2.4. Concentration of iodide, iodate, bromide and bromate in bottled, surface and municipal tap water samples (µg L^{-1}). S= Still; Sp=Sparkling; <LOD= less than method's limit of detection; <LOQ= less than method's limit of quantification. Find LOD and LOQ values for different species in table 2.3

	I^-	$IO3^-$	Br^-	$BrO3^-$
Voss (S)	3	2	29	<LOD
Pellegrino (Sp)	<LOD	7	248	<LOQ
Acqua Panna (S)	<LOD	<LOD	21	<LOD
Isklar (S)	<LOD	<LOD	<LOQ	<LOD
Bonaqua (S)	<LOD	<LOD	3	<LOD
Bonaqua (S)	<LOD	<LOD	<LOQ	<LOD
Olden (S)	<LOD	<LOD	3	<LOD
Olden (Sp)	<LOD	<LOD	<LOQ	<LOD
Taffel (Sp)	<LOD	<LOD	58	2
Farris (Sp)	288	<LOD	1923	4
Bris (Sp)	<LOQ	<LOD	1001	<LOQ
Imsdal (S)	<LOD	<LOD	<LOD	<LOD
First Price (S)	<LOD	<LOD	<LOQ	<LOD
First Price (Sp)	<LOD	<LOD	3	<LOD
Fantoft tap	<LOD	<LOD	7	<LOD
Fana tap	<LOD	<LOD	11	<LOD
Nifes tap	<LOD	<LOD	11	<LOD
Tertnes tap	<LOD	<LOD	10	<LOD
Askoy tap	<LOD	<LOD	11	<LOD
Lake Langevatnet	<LOD	<LOD	9	<LOD
Lake Munkebotsvatnet	<LOD	<LOD	9	<LOD
Lake Garpetjernet	<LOD	<LOD	9	<LOD
Seawater Bergen harbour	9	48	40282	101

CHAPTER 3.

The main variables affecting uptake and elimination of waterborne radioiodine in zebrafish

Experimental animals and rearing condition

Laboratory bred zebrafish (*Danio rerio*) (TAB strain) were kept in 3 L aquaria in a temperature controlled room (28 ± 1 ◦C) with 15 fish per dietary treatment. The pH and conductivity was buffered and controlled at the range of 7.5 ±0.3 and 400±50 µs respectively. Fish were kept at a day/night cycle of 16/8 h with day-light fluorescent lamps and were fed a dry diet (Aqua Schwarz Gmbh, Göttingen, Germany) at 1.5% body weight twice a day.

Experimental set-up

All experiments were conducted in 800 ml beaker glasses that were filled with 600ml of incubation medium E3 (the salts content of E3 water is shown in table 3.4.1.). The beakers were placed in a water bath that was kept at a constant temperature of 28°C. A minimum of six fish per tank and two tanks per treatment (or sampling time point) were used in all experiments.

To reduce the number of fish for the experiments as well as the variability among the individuals, the fish were anesthetized for measurements instead of sacrificing them at each time point. In all experiments only male fish were.

Fish were sampled at predetermined time points and anesthetized using metacain. Individual fish were placed in glass vials and the amount of radioiodine was measured using a gamma-counter (1470 Wallac Wizard Gamma Counter, Perkin-Elmer, Turku, Finland). After the measurement, fish were rinsed in clean water and then transferred to the original medium to continue the experimental exposure. At the end of the experiment, the fish were killed on ice-water and measured. The concentration of radioiodine in the water was 150 cpm ml^{-1} medium in all experiments. The isotope ^{125}I in the form of sodium iodide (NaI), pH 10.2 was used as radioiodine source (PerkinElmer, Norway).

Chemicals and E3 water was freshly made for each experiment and fish were not fed during the experiments.

3.1. The effect of the anesthetic metacain

The effect of the anesthetic metacain was tested on the uptake and depuration of radioiodine. The experiment consisted of the following three groups: six times anesthesia, two times anesthesia and no anesthesia (control) for the uptake stage. In the depuration stage the three groups continued with five times, two times or no anesthesia. For each treatment two times six zebrafish were tested and the total amount of radioiodine in the individual fish was

measured at various sampling points. The radioiodine exposure lasted for 72 hours and afterwards fish were transferred to clean E3 water for 96 hours (depuration fase). Metacain (100 mg L^{-1}) was used as anesthetic and sodium hydrogen carbonate ($NaHCO_3$) was added to the solution to buffer the pH.

The measured values were compared between the groups and analysed for significance using t-test. These comparisons were conducted at different time points for both the uptake and depuration stage. At the end of the uptake and depuration stage all values were compared with the control group. One-way ANOVA and Tucky test was used to compare the mean of different groups.

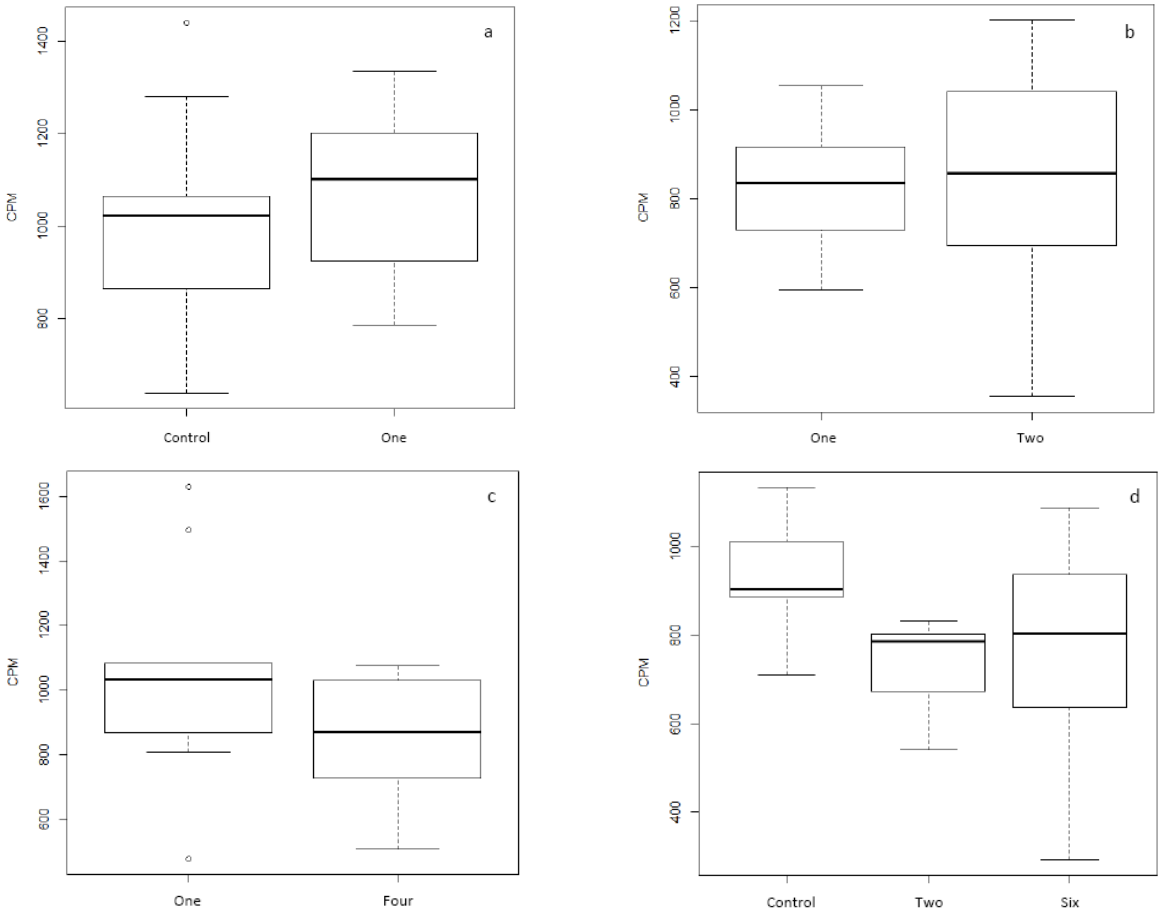

Fig. 3.1.1. Effect of anesthesia on waterborne radioiodine uptake in male zebrafish at different time points. Vertical axis shows the measured radioiodine in cpm and horizontal axis shows the times fish was anaesthetized; a) after 4 hours, b) after 24 hours, c) after 48 hours, d) after 72 hours.

No significant differences (p=0.05) were found between groups in all cases. Therefore it was concluded that anesthesia up to six times in a single batch of fish in this experimental design, has no significant effect on uptake nor on the depuration of radioiodine. This allowed for repeated measurements with the same fish over time and allowed us to reduce the amount of fish used in the experiments.

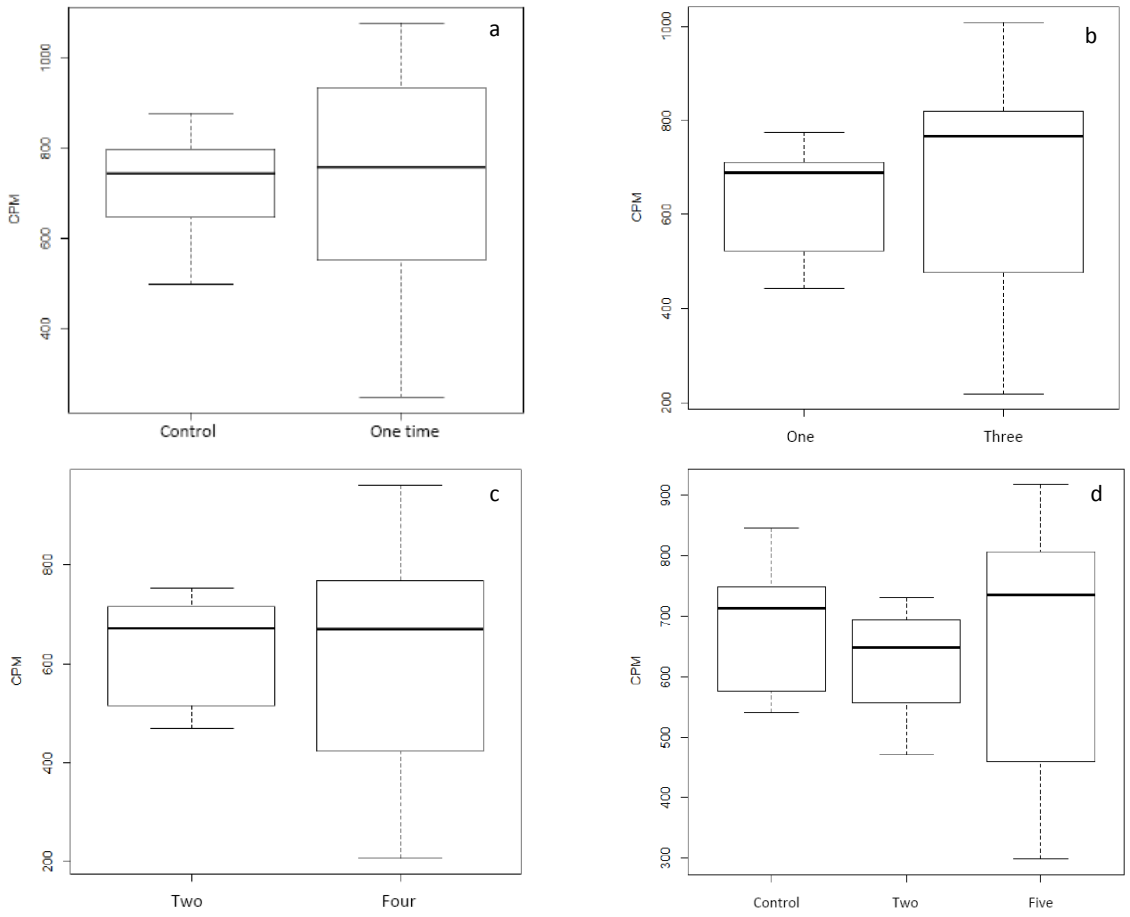

Fig. 3.1.2. Effect of anesthesia on the release of radioiodine from male zebrafish at different time points. Vertical axis shows the measured radioiodine in cpm and horizontal axis shows the times of anesthesia. a) after 4 hours, b) after 52 hours, c) after 72 hours, d) after 96 hours

3.2. Comparing the effect of metacain on the uptake of waterborne radioiodine in male and female zebrafish

Uptake of waterborne radioiodine was examined in male and female fish. In this experiment, males and females were treated separately. Six fish per group in two parallels were measured. Values for male and female were compared at the following time points: after 0, 2, 4 and 6 times of anesthesia. Results are presented in Fig. 3.2.1.

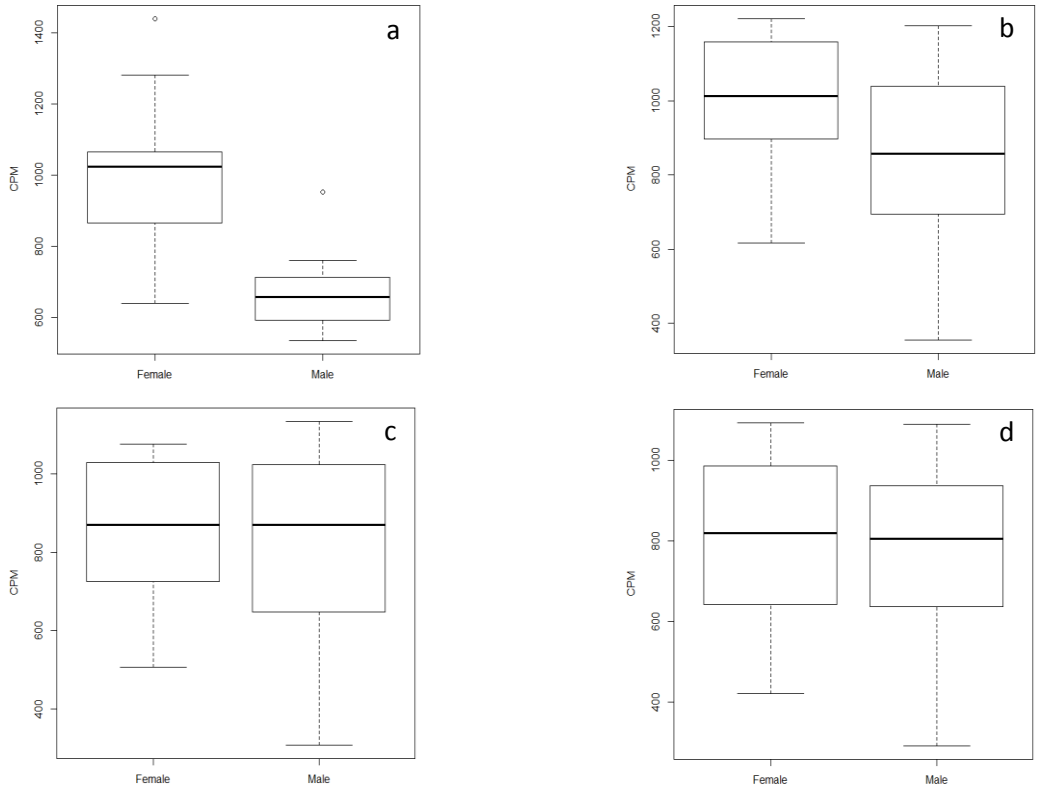

Fig. 3.2.1. Effect of anesthesia on waterborne radioiodine uptake in male and female zebrafish at different time points a) after 4 hours and no anesthesia, b) after 24 hours and two times anesthesia, c) after 48 hours and four times anesthesia, d) after 72 hours and six times anesthesia

The data collected from males and females were tested by t-test. Only after 4 hours, the accumulation of radioiodine in the fish was significantly higher in females than males ($p<0.05$), but not at later time points. Additional data have confirmed that the uptake of radioiodine occurs faster in females than in males; however the total amount of radioiodine is similar after 24-48 hours.

3.3. Effect of pHs on radioiodine uptake

In this experiment the effect of pH was studied on the uptake of waterborne radioiodine in male zebrafish. Two parallel tanks and 4 fish per tank were used for each pH treatment. 400ml of medium was added to each tank with a concentration of 150cpm radioiodine mL^{-1}. Next, the solution was adjusted for pH 4, 6 and 8 using HCl (0.1M) or NaOH (0.1M). The tanks were incubated at room temperature for four days to stabilize I speciation. Tanks were transferred to a water bath (28°C), acclimated and the fish were added. The uptake of radioiodine was measured as described earlier. There was no mortality during the experiment. As it is presented in Fig. 3.3.1 the result clearly shows that by increasing the pH the uptake of radioiodine decreases.

The maximum amount of radioiodine in the fish was measured after 5 hours of uptake in all pHs. This trend during the time point is in agreement with the first eight hours uptake in seen in previous experiments.

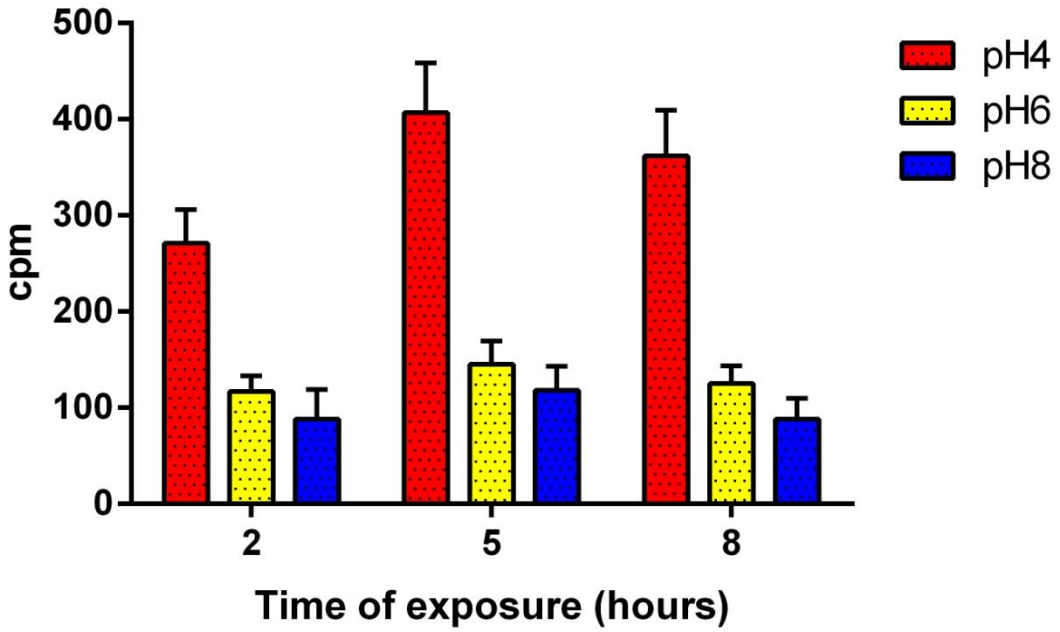

Fig. 3.3.1. The effect of pH on the uptake of waterborne radioiodine at 2, 5 and 8 hours after exposure in male zebrafish (Mean±SD, n=6).

In a second experiment the hypothesis was tested that either the previous result is caused by the effect of pH on the uptake route in the fish or by affecting the speciation of radioiodine. In this experiment, the same set-up as described above was used. To adjust the pH, the radioiodine stock (pH 10.2) was adjusted to pH 4 and pH 8. Then acidic and basic radioiodine

was added to each tank and the pH adjusted to 4, 6 and 8 using HCl (0.1M) and NaOH (0.1M). The fish were transferred to the tanks and the uptake of radioiodine was measured after 24 hours. The result is presented in Fig. 3.3.2. The radioiodine that was acidified before pH was adjusted was taken up in larger amounts than the radioiodine that came from a basic solution, which was taken up ijn equal amounts between treatments. The change in uptake seen in the radioiodine stock that was first acidified is most likely due to the formation of iodine species that are more bioavailable to zebrafish than to an effect of pH on the physiology of the fish (e.g. leaking of gill epithelium).

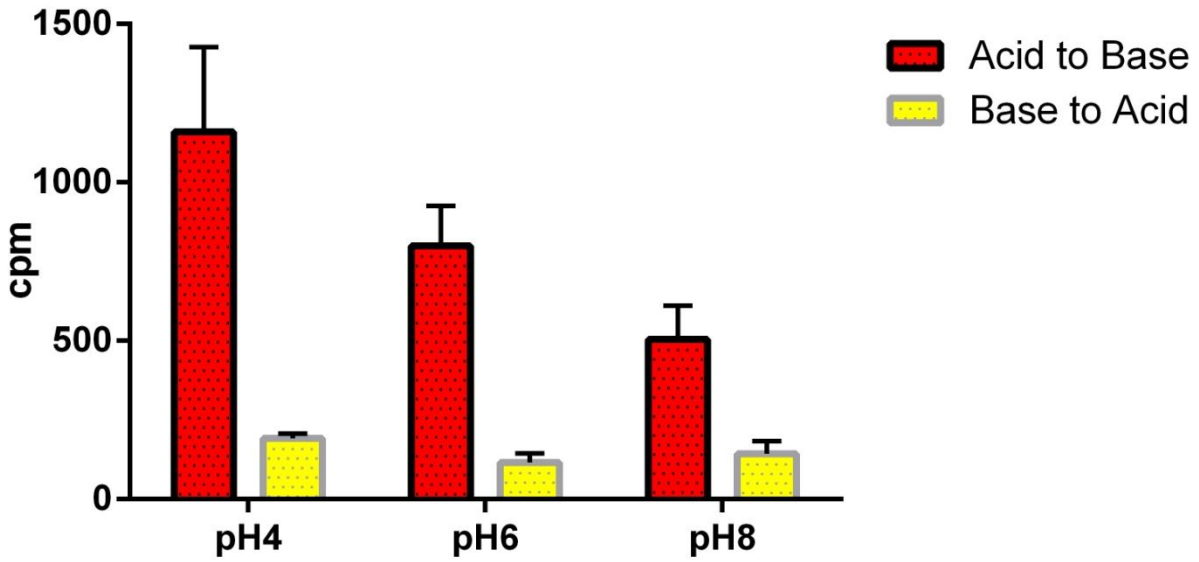

Fig. 3.3.2. The effect of pH on the uptake of radioiodine at different pHs (Mean±SD, n=4). Two different source of radioiodine were used: pH 4 and pH 8. The values are after 24 hours of exposure in male zebrafish.

3.4. Stability of iodine species in E3 water

According to the results from the pH experiment, iodine speciation is a significant factor that plays an important role in the uptake of radioiodine. Because of the effect of factors such as pH, a method based on liquid chromatography coupled with ICP-MS was developed to measure various species of iodine in a single water sample. Details of method set-up are presented in Chapter 2. By using this method it was possible to measure iodide, iodate, bromide and bromate in the same run with acceptable LOD and LOQ. To use this method for our experiments, it was needed to test the effect of E3 water on the stability of species and interference with the method since E3 contains various salts in relatively high concentrations. The concentration of salts in E3 water is presented in table 3.4.1.

Table 3.4.1. Salt content of E3 water.

Compound	NaCl	KCl	$CaCl_2$	$MgSO_4$
Concentration (mg L^{-1})	286.7	12.7	48.3	81.67

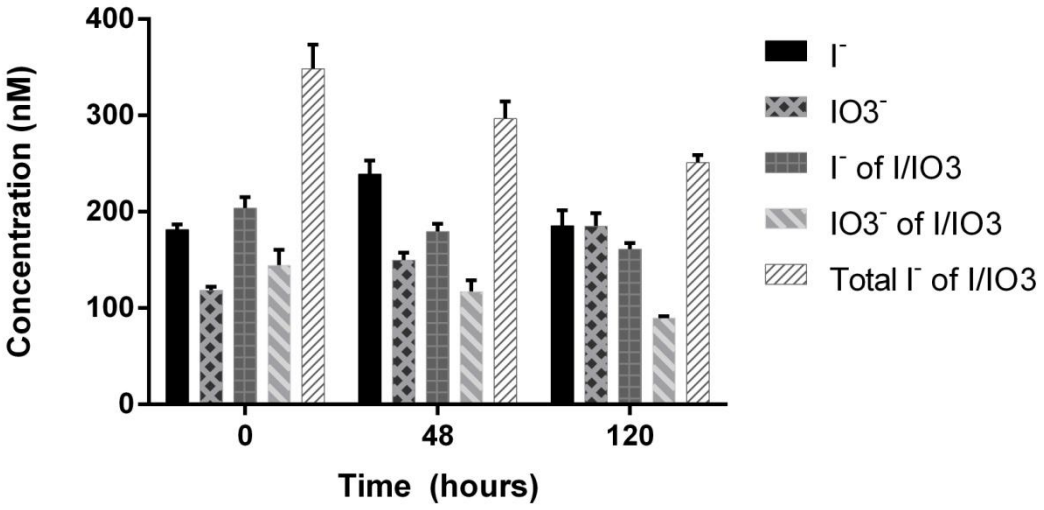

Fig. 3.4.1. Stability of Iodide/Iodate in E3 water (Mean±SD, n=5).

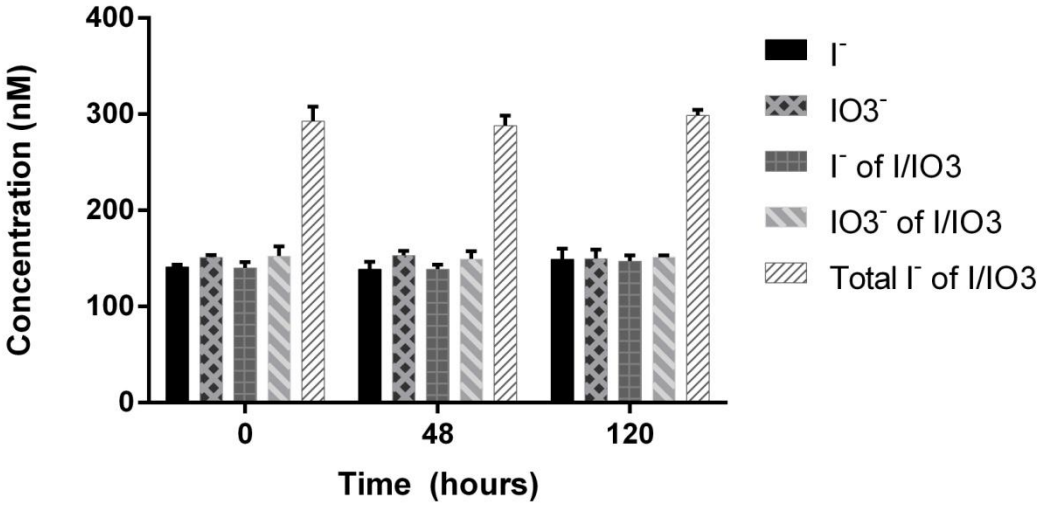

Fig. 3.4.2. Stability of Iodide/Iodate in MilliQ water (Mean±SD, n=5).

In this experiment six containers were filled with E3 and MilliQ water and 150 nM of iodide (I^-), iodate ($IO3^-$) and a mixture of iodide/iodate ($I^-/IO3^-$) was added to the separate

containers. Next, the concentration of iodide and iodate were measured at 0, 48 and 120 hours after mixing. The caps of the containers remained closed during the experiment.

The results presented in Fig. 3.4.1 and Fig. 3.4.2 show a fluctuation in concentration of iodine species in E3 medium. In contrast these species are stable in MilliQ water. The pattern of alteration in E3 is erratic for iodide and the trend is increasing for iodate. The trend for iodide/iodate in mixture is decreasing and could be explained due to changes in speciation into production of species that are not measured in this method or volatile species that can evaporate from the solution. These results show that the slats in E3 can interfere with both iodide and iodate either in mixture or separately. The instability of species can be another reason for having different values after 48 and 120 hours.

The same experimental condition was administrated in order to test the effect of different salts of E3. The aim of this experiment was to find out which salt is interfering with the method and ultimately to replace it with an alternative. The same concentration of each salt including NaCl, KCl, $CaCl_2$ and $MgSO_4$ were mixed separately with iodide and iodate with concentrations of 150nM. Then after 0, 24, 96 and 168 hours iodide and iodate were measured in different salt solutions. The results are presented in Fig. 3.4.3. All the four salt compounds from E3 are interfering with the method at a similar level. It was concluded that it is not possible to use this method for the measurement of iodine species in experiments containing fish in E3.

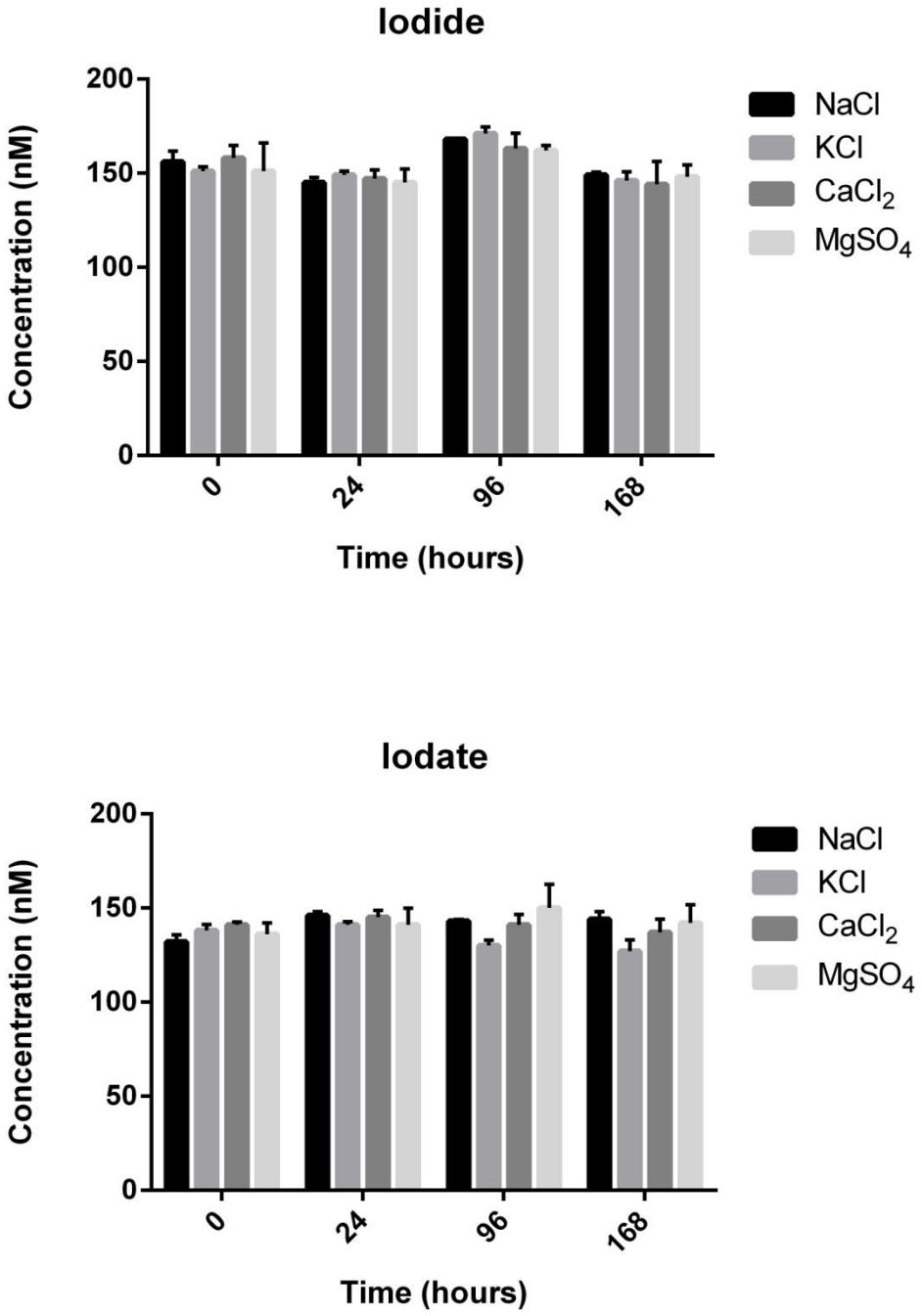

Fig. 3.4.3. Stability of Iodide and Iodate in E3 components showed in different colors for salts after 0, 24, 96 and 168 hours (Mean±SD, n=3).

CHAPTER 4.

Modelling of uptake and depuration of waterborne radioiodine in zebrafish

4.1. Method

The whole body residue of radioiodine through uptake and depuration stages was measured at predefined time points. In this experiment six tanks containing six fish each were used. The sampling from the tanks was designed to have two tanks for each sampling time point and the number of anesthesia for each fish did not exceed six times as was tested in 3.1. The fish were exposed to radioiodine for 24 hours. The uptake was measured at 0.5, 1.5, 2.5, 4, 6, 9 and 24 hours after start of exposure. Next, the fish were transferred to clean E3 to investigate the depuration of radioiodine. The amount of remaining radioiodine was measured at 4, 6, 9, 24 and 48 hours after transfer to clean E3 water.

4.2. Triphasic uptake of waterborne radioiodine

Radioiodine accumulated rapidly from 0 to ~ 196 cpm after 6 hours. This period of increasing radioiodine consists of two phases with different slopes. During the first 1.5 hours the uptake is very quick with the slope of 77.3 and reaches ~ 119 cpm. After this the uptake is followed by a second phase with a slope of 16.4 (between 1.5 and 6 hours) that reaches a maximum of ~ 196 cpm.

After 6 hours, the increase of radioiodine stopped and was followed by a period of decreasing radioiodine. This third phase was between 6 and 24 hours and went from ~ 196 to ~ 181 cpm. The slope of this decreasing phase is very low (-0.78).

In an overall overview three main phases are recognizable. By using a triphasic approach the linear models can better predict the uptake trend of radioiodine during 24 hours. The regression coefficient in the triphasic model significantly improved in comparison with a monophasic model. The uptake trend is presented in Fig. 4.2.1.

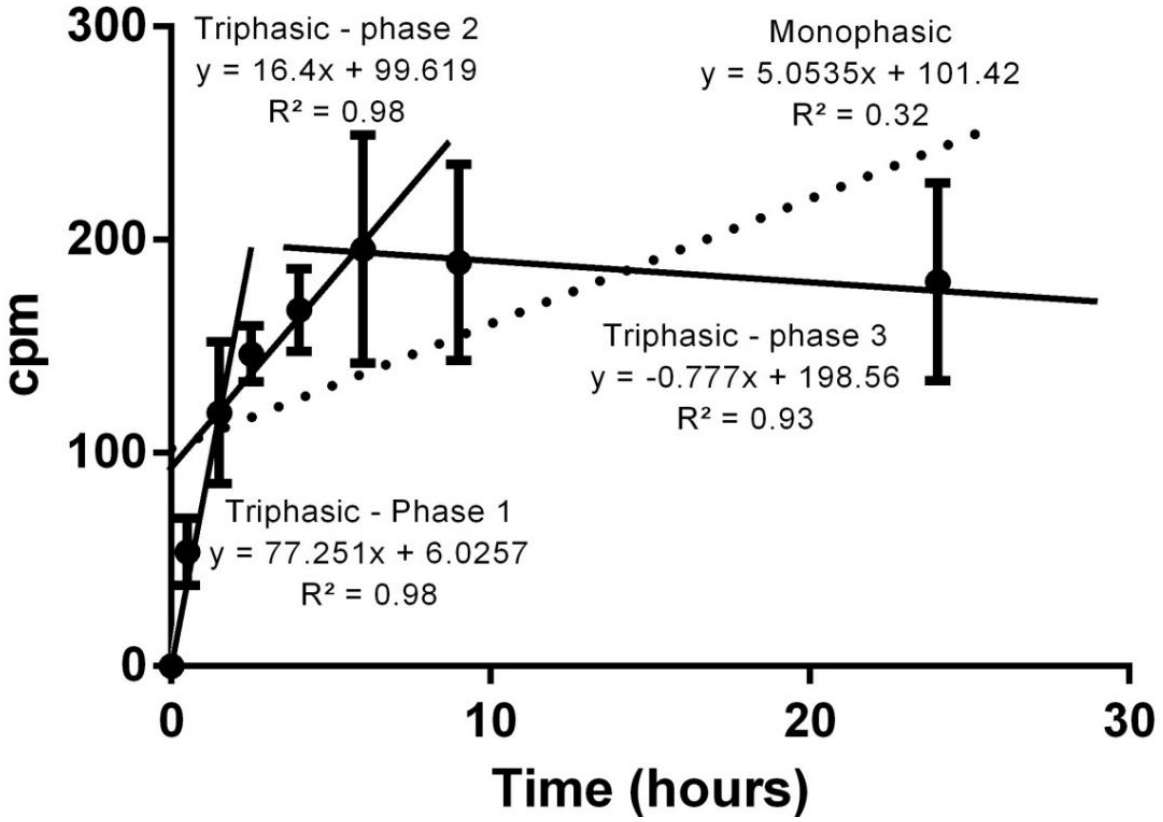

Fig. 4.2.1. The whole body residue of radioiodine (Mean±SD, n=12 for each time point) in zebrafish during the uptake stage. Multiphasic models are presented by solid lines and dotted line represents a monophasic model. A triphasic model clearly improved the monophasic uptake model.

During the first phase of uptake within 1.5 hours, iodine influxed into the fish and the concentration increased in blood circulation. Then in the second phase the uptake continued but it seems that the excretion is also started and reduced the net amount of iodine retention in the body. Finally in third phase the elimination process was reached to it maximum capacity therefore the influx and excretion reach equilibrium.

4.3. Biphasic depuration stage

The depuration stage consists of only two phases. During the first phase the radioiodine residue was excreted and cpm counts in whole fish decreased from ~ 180 cpm to ~140 cpm. This reduction is seen immediately after transfer from a medium containing 150 cpm mL^{-1} radioiodine into clean E3. This transfer allowed the fish to excrete the ingested radioiodine in the intestine as well as the excess iodine in circulation according to new equilibrium in a new

condition where no radioiodine was present in the water. The same biphasic excretion is reported for radio iodide in rats. Pavelka in 2004 showed that the first phase was very quick with a half-life of 12 hours due to elimination from the body and the second phase with half-life of 108 hours characterizing the elimination from the thyroid.

This first decreasing stage in our zebrafish was followed by a more or less steady phase with a slope of -0.14 that signifies the fish released the excess radioiodine in the intestine and circulation in the first phase. As it is shown in Fig. 4.3.1 the first phase is quick and took place in the first 9 hours then the steady phase continued until the end of the depuration stage.

According to the characteristics of uptake and depuration stages the main time points were selected at 2, 5, 8 and 24 hours for uptake and at 5, 24 and 48 hours for depuration to cover the main critical and turning points for halide salts experiments.

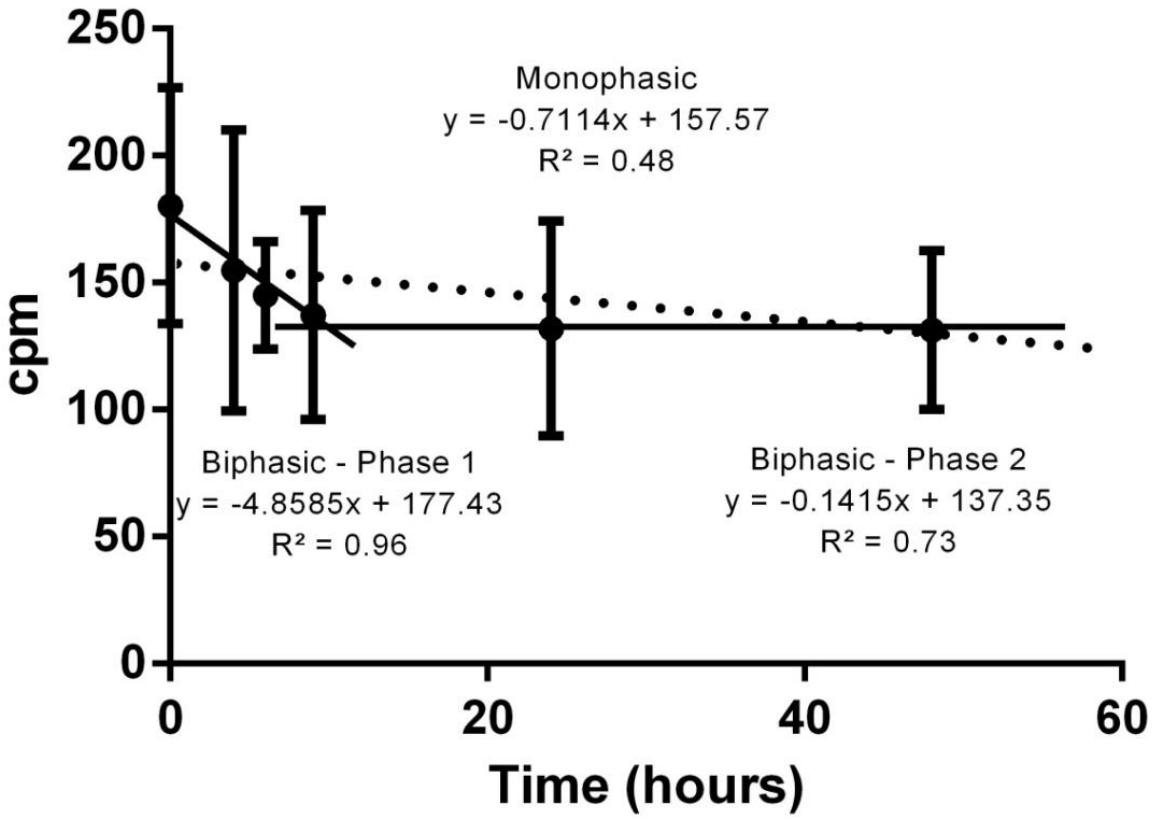

Fig. 4.3.1. The whole body residue of radioiodine (Mean±SD, n=12 for each time point) in male zebrafish during depuration stage in clean E3. Multiphasic models are presented by solid lines and dotted line represents monophasic model.

CHAPTER 5.

Effect of halide salts and sodium perchlorate

5.1. Method

The effect of sodium perchlorate ($NaClO_4$) was examined on the uptake and depuration of radioiodine in zebrafish. In this experiment, fish were pre- exposed to 6 mg L^{-1} of sodium perchlorate for 13 hours in duplicate groups. In a study by Moren and her colleagues (2008), tested sodium perchlorate to block the iodine uptake with concentration between 0.5 to 8.6 mg L^{-1} and showed that using 50 μM (6 mg L^{-1}) make the maximum uptake inhibition in halibut larvae and this later concentration was used for this study.

After the incubation radioiodine was added and the uptake of radioiodine was measured at 2, 5, 8 and 24 hours. After 24 hours fish were transferred to clean E3, without sodium perchlorate and radioiodine, and the depuration was measured at 5, 24 and 48 hours.

In a second experiment, the effect of five halide salts including potassium iodide (KI), potassium iodate (KIO_3), potassium bromide (KBr), potassium bromate ($KBrO_3$) and sodium fluoride (NaF) were tested on the uptake and depuration of radioiodine in zebrafish. Since bromine was the main halide of interest, concentration of bromine in the seawater was considered. The concentration of bromine in sea water is between 41 to 71 mg L^{-1} (Wegman et al., 1983), so 50 mg L^{-1} was considered as an average. In a pilot experiment the tolerance of zebrafish was tested in 50, 500 and 5000 mg L^{-1} as 1x, 10x and 100x of seawater concentration. It was more than 40% mortality in two higher concentrations. On the other hand the concentration of bromine in freshwater is very low, between 0.014 to 0.2 mg L^{-1}. Therefore to be in the safe concentration which does not damage the fish and to have maximum possible effect of bromine, half of the seawater concentration or 25 mg L^{-1} was selected for final experiment. Different halide salts was used in the pre-exposure medium and measurements of radioiodine were carried out as described above.

5.2. The effect of sodium perchlorate

Perchlorate is a classic and well known environmental contaminant that inhibits the iodine uptake and reduces the thyroid hormone levels by blocking the sodium-iodine symporter. These effects of perchlorate are described in many different organisms including fish species (Schmidt et al., 2012; Crane et al., 2005).

In this experiment, 13 hours of pre-exposure to perchlorate did not affect the influx of radioiodine compared to control. If any, after two hours the uptake seemed even higher than control although it was not significant. The effect of perchlorate on the first two increasing

phases of uptake was not significant ($p>0.05$) and the radioiodine was still flooding into the body. Apparently, the uptake was passive or through another channel or symporter that was not blocked by perchlorate. Since zebrafish lives in freshwater with an osmolality lower than the body, zebrafish do not drink much water. In contrast, marine fish drink lots of water to compensate for water loss. Therefore with regards to rapid uptake of radioiodine in the beginning, the place of radioiodine uptake most likely are the gills and in a minor degree the intestine.

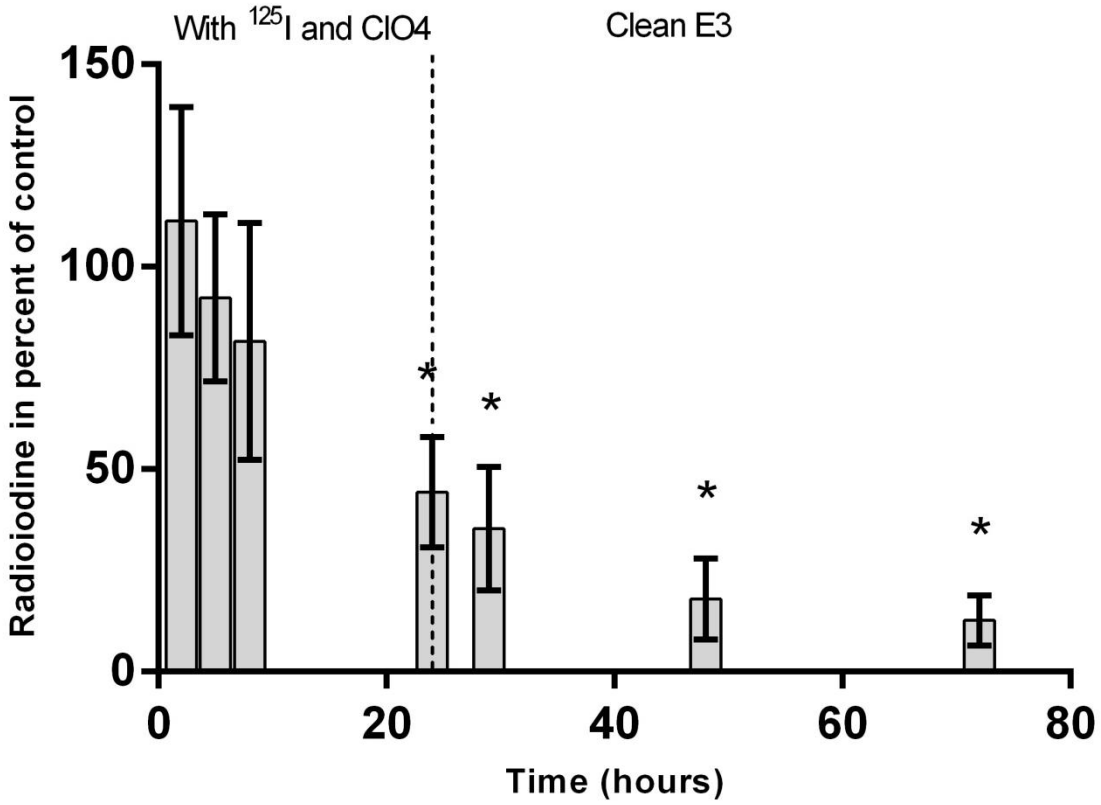

Fig. 5.2.1. Effect of sodium perchlorate on the uptake and depuration stages of radioiodine in male zebrafish. Values are the ration of measured radioiodine in exposed fish compared to control values in percent (Mean±SD; n=12 for each time point). * = significantly different compared to control ($p=0.05$).

After eight hours of exposure, the beginning of the third phase of uptake, the effect of perchlorate became visible. The radioiodine residue was significantly lower ($p<0.05$) after 24 hours in perchloride compared to control. Radioiodine concentration in the perchloride group was only 44% of control.

Moren and colleagues (2008) tested the gradually increase of perchlorate concentration on the uptake of radioiodine in halibut larvae and showed that exposure to the high concentration (8.6 mg L^{-1}) of perchlorate reduced the uptake by 55%. This number is in good agreement with the reduction of uptake by 56% in our experiment.

During the depuration stage, where the fish were transferred to clean E3 water, this efflux process continued exponentially. At the end of depuration after 48 hours the remaining residue of radioiodine was 13% of control in average. This value can be considered the real accumulated

radioiodine in the thyroid and reflects the blocking of perchlorate.

The difference between the amount of radioiodine blocked by perchlorate at the start of the depuration and at the end reflects the unbound amount of radioiodine point found in the intestine and in circulation. The results are shown in Fig. 5.2.1.

5.3. The effect of iodine species

In this experiment male zebrafish were pre-exposed to iodide and iodate for 13 hours and then exposed to radioiodine. Based on previous results (the uptake and depuration of radioiodine), the uptake from water is very rapid. The design of this experiment, with 13 hours pre-exposure, is enough time for the fish to take up sufficient iodine to saturate the thyroid. By adding radioiodine to pre-saturated fish, we wanted to: 1) confirm that the initial uptake of radioiodine is passive, 2) confirm that 48 hours of depuration is enough to measure the actual retained amount of iodine. Moreover, in this experiment the bioavailability of iodide and iodate as sources of iodine could be compared.

The results are presented in Fig. 5.3.1. The findings showed both iodide and iodate were successful in saturating the thyroid. The bioavailabilities of both species appear to be the same at this concentration and pre-exposure duration time.

Interestingly, the influx of radioiodine in the first two fases of uptake and the beginning of the third phase is exactly the same as control. This part is a bit higher for iodate but still follows the same slope as control.

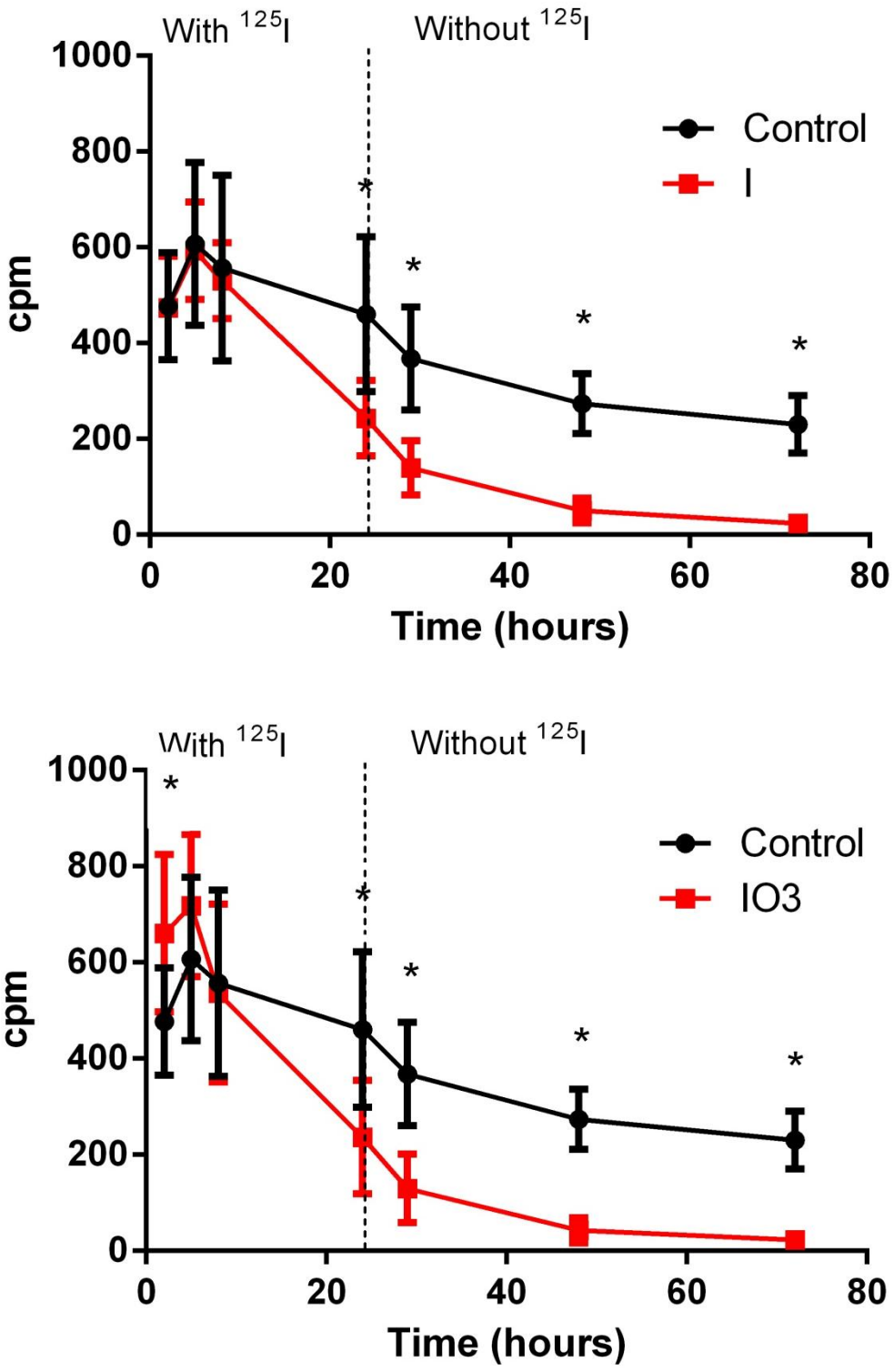

Fig. 5.3.1. The effect of potassium iodide and potassium iodate on the uptake and depuration of radioiodine (Mean ±SD; n=12 for each time point) in male zebrafish. * = significantly different compared to control (p=0.05).

During the third phase of uptake the radioiodine residue reduced to 53% and 51% of control for iodide and iodate respectively at 24 hours. This elimination process continued during the

depuration phase when fish were transferred to clean E3 and had time to excrete the excess radioiodine from the circulation. After 48 hours of elimination the average residue of radioiodine was reduced to 10% of the control group for both iodine species.

The overall effect during both uptake and depuration stages were similar for iodide and iodate as well as perchlorate.

The ratio of radioiodine residue to control at the end of both stages including 24 hours exposure and 48 hours depuration were very similar for iodide and iodate as well as for perchlorate. This clearly confirms that 48 hours in clean E3 is enough time for the kidney to eliminate the excess radioiodine.

5.4. The effect of bromine species

The goitrogenic effect of bromine especially bromide is confirmed in laboratory mammals. It is demonstrated that administrating high dose of bromide in rat decreased the accumulation of iodine in thyroid, reduced the half-life of iodine in thyroid and significantly elevated the elimination of iodine through kidney (Pavelka, 2004).

In this part the possible goitrogenic effect of bromine species including bromide and bromate were tested on both uptake and elimination processes of radioiodine in male zebrafish. The results are shown in Fig. 5.4.1. According to the results potassium bromide did not affect the uptake and depuration of radioiodine. Although the radioiodine residue was little higher during the second and third uptake phases, it was not significant. During the depuration stage the trend and average values were the same as control. These results suggest that bromide has no inhibitory effect on the uptake of iodine in zebrafish. Although the blocking effect of bromide is clearly demonstrated in rats, this function of bromide was not observed in zebrafish as a freshwater fish. This could be explained by the different uptake route of iodine in fish compared to mammals. In mammals, when iodine is administrated orally through food or drinking water, the main uptake route is the intestine. However, the main uptake route in freshwater fish is the gills and to a lesser extend the intestine. This process is in agreement with the very quick uptake in the first hours of exposure.

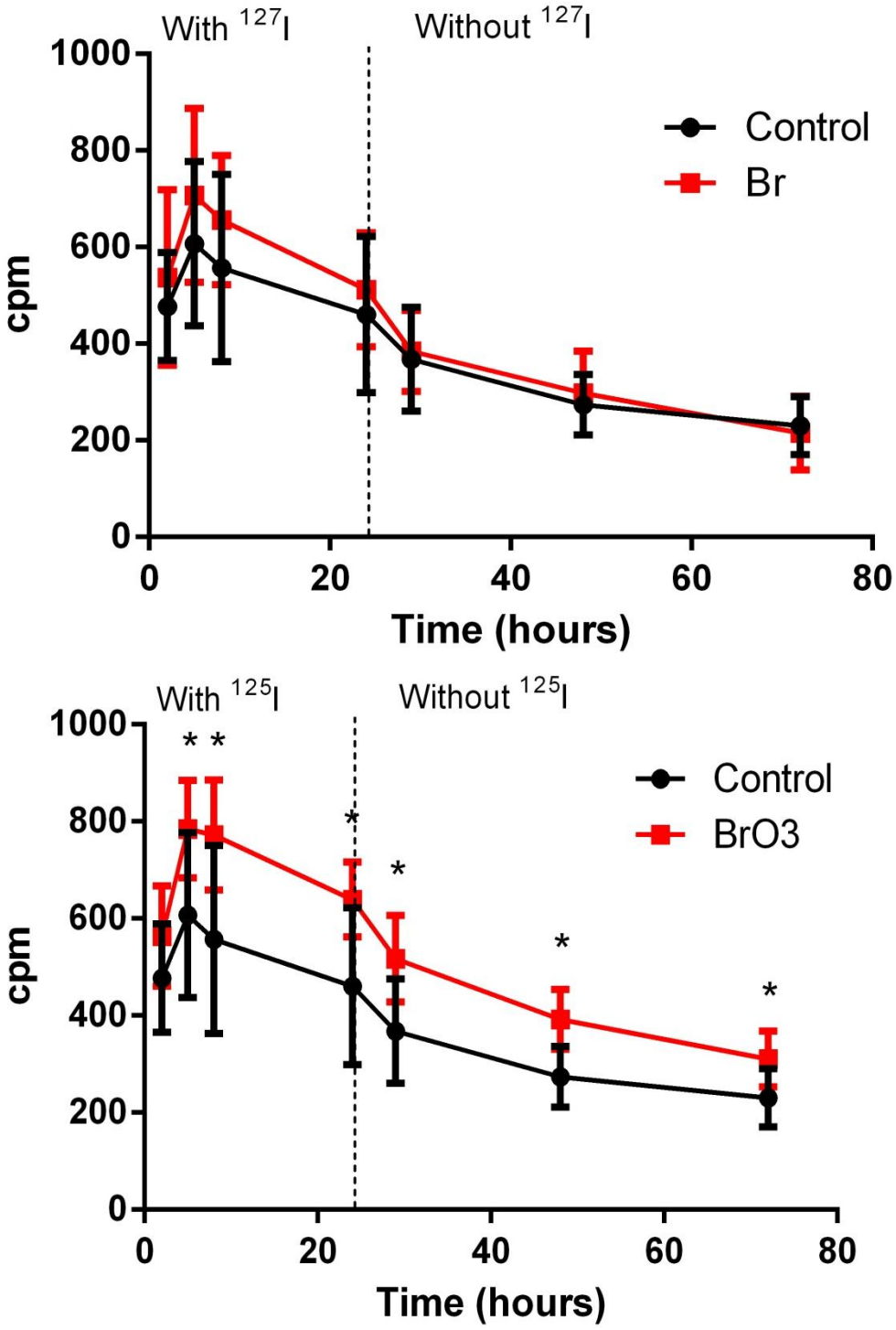

Fig. 5.4.1. The effect of potassium bromide and potassium bromate on the uptake and depuration of radioiodine (Mean ±SD; n=12 for each time point) in male zebrafish. * = significantly different compared to control (p=0.05).

The effect of potassium bromate was even more interesting; the radioiodine residue in both uptake and depuration stages were significantly higher ($p<0.05$) than control. Based on this result it was obvious that potassium bromate did not change the uptake capacity of zebrafish

but the elimination could be changed under the effect of potassium bromate. It is confirmed that exposure to even low concentrations of bromate caused damage to the renal system and in severe and long term exposure it can resulted in tumor growth in laboratory animals (Geter et al., 2006; Pfaff and Brockhoff 1990 and Kurokawa et al., 1990).

In general, based on findings of uptake and depuration modeling (Chapter 4) during the second phase of uptake the excretion has started and continued to play a significant role in the third phase of uptake as well as during the whole depuration stage.

Kidney is considered to be the most important route for excretion of iodine. Therefore, the significant higher residue of radioiodine in zebrafish treated with potassium bromate could be related to an effect of bromate on the functioning if the renal system. We believe that the increased level of radioiodine is caused by a disrupted of kidney function resulting in a delay of the elimination process. It was hypothesized that a prolongation of the depuration stage would result in the elimination of excess radioiodine by the kidney.

5.5. The effect of sodium fluoride

Administration of fluorine in patients with hyperthyroidism can reduce the basal metabolism. Galletti and Joyet in 1958 showed that oral administration of fluorine in human reduces the accumulation of iodine in thyroid without reducing the ability of thyroid hormones production, given that enough iodine is available. Ubom in 1991 stated that fluorine in the soil of some area in Nigeria is one of the reasons for higher levels of hypothyroidism and goiter in these areas.

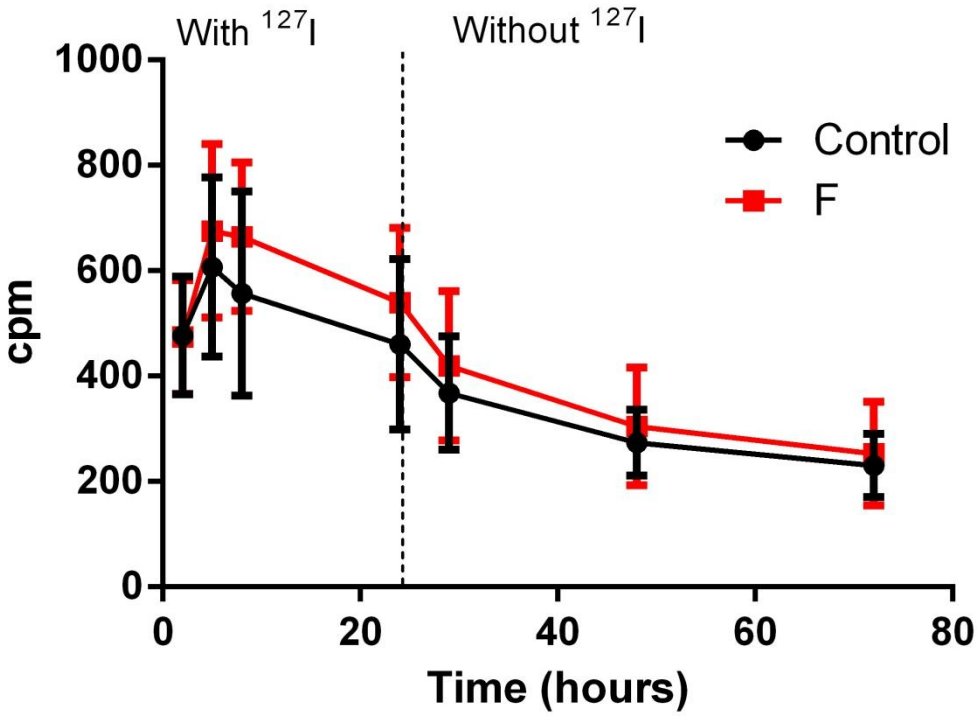

Fig. 5.5.1. The effect of sodium fluoride on the uptake and depuration of radioiodine (Mean ±SD; n=12 for each time point) in male zebrafish. No Significant differences found.

In this experiment (shown in Fig. 5.5.1), pre-exposure to sodium fluoride had no significant effect on the uptake and depuration stages of radioiodine in zebrafish. Although there are some reports on the goitrogeneic effect of fluorine (Burgi et al., 1984), there is no consensus in the scientific community on this effect of fluorine on thyroid function. Burgi and colleagues in 1984 reviewed the effect of fluorine on the thyroid and mentioned that there is no convincing evidence for thyroid disrupting effect of fluorine, however they mentioned that there are studies with proper condition and population numbers for both treatment and control showing no goitrogenic effect of fluorine in human subjects.

Our results clearly confirmed that there is no effect of fluoride on the uptake and elimination processes in zebrafish under these experimental conditions.

CHAPTER 6.

Summary, general discussion and future perspectives

6.1. Determination of inorganic iodine and bromine species

A new method was developed to quantify the iodide, iodate, bromide and bromate in aqueous solution using AE-HPLC-ICP-MS with detection limit of 0.87, 0.40, 0.76 and 0.51 µg L^{-1} respectively. This method was successfully tested on bottled mineral waters, as well as samples from surface and municipal waters.

The limit of detection and repeatability of measurements was better than the required values by European Commission with regards to the accuracy of methods for drinking waters. The results of analyzed samples showed that two out of 22 samples contained iodine species. About two third of the bottled mineral waters containing bromide in the range of 2.74 – 1923.18 µg L^{-1}. Surface and municipal waters only contained bromide in a concentration lower than 11.5 µg L^{-1}. Bromate was only detected in two mineral waters with a concentration lower than 4 µg L^{-1}, while the legal maximum limit is 10 µg L^{-1} (WHO, 2011; Fawell and Walker, 2006). According to these results all of analyzed waters are safe for drinking with regards to bromate.

6.2. The effect of pHs on the uptake of iodine

In general sodium-iodine symporter is the main channel through which iodine is taken up into the thyroid follicle cells. Amachi and his colleagues in 2007 studied the uptake of iodine in the marine bacterium Flavobacteriaceae and showed that hydrogen peroxide, the result of a glucose oxidase, is needed for iodine uptake. They tested the effect of enzymatic oxidation of iodide and found an increase in uptake while adding a reducing agent inhibited the uptake of iodine. Finally they concluded that iodide first was oxidized to hypoiodus acid (HOI) by hydrogen peroxide and then this uncharged molecule passes the cell membrane passively.

In another study in marine algae (Laminariales) Kupper and colleagues in 1998 showed that adding haloperoxidase, which catalyzes the oxidation of iodide by hydrogen peroxide, increased the iodide uptake. Oxidative stress and adding hydrogen peroxide to the medium also increased the uptake. They concluded that this was the result of a facilitated diffusion mechanism that happens through formation of HOI and molecular iodine (I_2) after oxidation of iodide.

In our study the addition of HCl and reducing pH to 4, it is clearly showed that the uptake of radioiodine was drastically increased in comparison to adding NaOH or a pH up to 8. HCl is a strong acid and oxidizing chemical that can change the iodine species to HOI which on its turn can be taken up passively in the zebrafish. On the other hand adding a reducing agent like NaOH can prevent the formation of HOI and molecular iodine and reduce the total influx

of iodine. These findings are in agreement with the main mechanism found for uptake of iodine and in conclusion we propose that the same mechanism found in marine bacterium and algea exists in zebrafish.

Because HOI is a volatile species, the use of gas chromatography coupled with online ICP-MS can be a helpful technique to quantify the concentration of hypoiodus acid in solutions with different pHs. By administrating this method in combination with the results from radioiodine uptake from different pHs, it is possible to complete the findings in this field in terms of bioavailability of iodine species for fish.

6.3. Modeling of the uptake and depuration of waterborne radioiodine

In this part, the normal curve for uptake and depuration was studied based on whole body residue of radioiodine. During the uptake stage, three significant phases was recognized with different slopes. During the first phase within 1.5 hours, very rapid uptake was measured. In the second phase the uptake was continued but with less slope and finally in third phase between 9 to 24 hours the reducing trend with very low slope was observed. According to these results and data from halide salts experiment, we propose that first phase is only representing the passive or facilitated uptake. It is in agreement with rapid influx of radioiodine in the beginning of the uptake stage in pre-exposed fish in iodide/iodate and perchlorate experiments. In the beginning of second phase the elimination through kidney is started. This elimination was reached the maximum capacity during third phase of uptake where these processes reached the equilibrium. This triphasic model successfully improved the monophasic model.

During depuration stage only two phases was identified. During first phase within 9 hours the fish released the excess radioiodine from the intestine and blood circulation when they transferred to clean E3 without radioiodine. The third phase is a steady line with very low negative slope, showing the main accumulation of radioiodine in the thyroid.

6.4. The Effect of halide salts

In this section the effect of iodine species in the form of potassium iodide and potassium iodate were tested on the uptake and elimination of radioiodine as well as bioavailability of these species. Our findings showed that at a concentration of 25 mg L^{-1} and a pre-exposure of 13 hours, there were no differences between iodide and iodate in terms of bioavailability. According to our results they both satisfied the thyroid in zebrafish by up to 90 percent. The bound radioiodine was measured after 48 hours depuration stage in clean E3. To discuss and

compare the bioavailability of these species in more detail, a concentration range lower than 25 mg L^{-1} in combination with different pre-exposure time (less than 13 hours) should be tested.

The pre-satisfied thyroid with iodide and iodate showed the same trend during uptake and depuration. During the third phase of uptake and whole depuration stage the values for pre-exposed fish were significantly lower than control. The trend from both iodide and iodate pre-exposure showed influx in the beginning to the circulation and then elimination through the kidney during third phase of uptake and depuration. Since the thyroid was saturated with iodine during the pre-exposure period, little radioiodine accumulated in the thyroid (about 10 percent).

In the last part the goitrogenic effect of bromine and fluorine salts were tested. According to the existing information bromide has 1) inhibitory effect on the uptake of iodine 2) reduces the accumulation in the thyroid and 3) increases the excretion of iodine in the kidney in rats.

In this study no effect of bromide was detected on iodine uptake in adult zebrafish. There were no significant differences between pre-exposure to bromide and control values in different time points. The overall trend was similar to the control for both species. Thienpont and colleagues in 2011 showed that sodium bromide significantly reduced intrafollicular T4-content in zebrafish eleutheroembryos. This result is from zebrafish larvae and they may have different thyroidal characteristics and iodine uptake capacity than adults or through a cytotoxic effect of bromide on thyrocytes of the larvae.

On the other hand fish evolved in bromine rich environment in the ocean. The average concentration of bromine in the seawater is between 41 to 71 mg L^{-1} (Wegman et al., 1983). Therefore they are adapted to high concentration of bromine and evolved in the condition with even higher concentration of bromide than our experimental condition (25 mg L^{-1}). So, it is not far from the expectation that fish have the ability to take up the waterborne iodine despite a high amount of bromide in the aqueous environment. This situation is completely different for mammals that have evolved in a bromide (and iodide) poor environment.

It is suggested that bromide can replace iodide in the thyroid hormones (Pavelka, 2004; Buchberger et al., 1990). In 1996, Vobecky and colleagues showed that one third of iodine in the thyroid of rat is replaced by bromine after elevated ingestion of bromide. This change resulted in a decrease of iodinated thyroid hormones and an increase of brominated ones. According to our findings on the effect of bromine on the iodine metabolism, no change in iodine metabolism was detected. In case of replacement of iodide by bromide in thyroid hormone the accumulated radioiodine at the end of depuration should be higher than control.

This was not detected because a possible substitution would be smaller than the detectable level of our method.

Bromate as another species of bromine was tested and the results demonstrated that the radioiodine residue in third phase of uptake and depuration stage were significantly higher than control. This phenomenon is explainable through the effect of bromate on the kidney function. The same effect of bromate is reported in rats (Geter et al., 2006; Pfaff and Brockhoff 1990 and Kurokawa et al., 1990). We suggest bromate delayed the elimination via damaging the kidney which resulted in higher values in pre-exposed fish than control.

In case of fluoride there are contradictory reports on its effect on the thyroid. However it is reported that it has inhibitory effect on the thyroid function in mammals but our findings showed very similar effect as bromide and it didn’t change the uptake nor elimination process in zebrafish.

CHAPTER 7.

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