ACKNOWLEDGMENTS:

ABSTRACT:

The development of a marking technique which could be efficiently used to mass-mark different groups of salmonids, would be a great tool for fisheries and aquaculture management. The present study was conducted in order to evaluate a series of rare earth elements (REEs) as potential markers in scales of Atlantic salmon (*Salmo salar* L.) by addition of these elements to the feed. The results demonstrated that the five tested elements were clearly incorporated into the scales of the fish fed the supplemented diets. Fish marked with these elements can be successfully identified by chemical analysis of the scales by inductively coupled plasma mass spectrometry (ICP-MS). The elemental levels (except for lanthanum) in the scales of the treated fish were still significantly higher than those of the untreated fish 2 months after the labeled diets were administered, although these levels were markedly lower than the ones present right after the labeling. A long-term monitoring of the chemical concentrations in the scales is required to study the longevity of the induced marks.

The background levels of the tested markers and some other elements were also analyzed and found to show interesting patterns. It is suggested that, in some instances, the natural chemical fingerprint of the scales may be enough to distinguish among fish groups. However, more research about the elemental background levels in the scales is needed in order to conclude with this.

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1. INTRODUCTION:

Aquaculture has grown into a major industry in Norway in the past three decades, contributing to economic growth and employment especially along the western and northern coastline (The Norwegian Ministry of Fisheries and Coastal Affairs 2010). Atlantic salmon is the dominating species within this industry, making up 93% of the Norwegian fish farming production in 2011, with more than one million tons produced (Statistics Norway 2012). Moreover, Norway has become the world's largest producer and exporter of Atlantic salmon with a total export weight of 978 048 metric tons representing a value of 29 197 million NOK in 2011 (Norwegian Directorate of Fisheries 2012). Farmed salmon is therefore an important export product for Norway and aquaculture industry contributes substantially to the country's economy (Hindar et al. 2006; Liu et al. 2011).

The Norwegian salmon industry has grown very fast. It began as a small local family business in the early 1970s and it rapidly developed into a modern, intensive, integrated and globalised industry controlled by only a few multinational companies (Liu et al. 2011; Pettersen & Alsos 2007). This great growth is mainly due to new technologies and innovations that allow more control over the production process, higher productivity and lower production costs (Asche 2008). A number of environmental concerns have emerged due to this phenomenal growth. Escapees of farmed fish into the wild is one of them, as the rapid expansion of salmon farming has resulted in increased numbers of escaped farmed salmon from the marine net pens and smolt farms (Lund et al. 1991; Thorstad et al. 2008). The escaped fish have the potential to survive and invade natural salmon rivers (Glover et al. 2012).

In Norway farmed salmon has been reported to represent on average 11-35% of the wild spawning populations, reaching up to 80% of some small populations in rivers located close to fish farms (Hindar et al. 2006). The risks that these intrusions pose to native salmon populations (see Fig. 1) are a highly debated topic, especially in the countries where salmon farming and wild salmon coexist (Ford & Myers 2008). Adverse environmental impacts, including ecological and genetic effects, caused by escaped farmed salmon on wild salmon populations are scientifically documented (Thorstad et al. 2008). Fleming et al. (2000) reported on the significant potential for resource (such as space and prey) competition between farmed and hybrid juveniles and their wild

counterparts due to the overlap in their habitat use and diet. In addition, escaped juveniles have a faster growth rate and are generally more aggressive, which can cause stress and lead to the displacement of native fish, even increasing their mortality (Fleming et al. 2000; McGinnity et al. 1997; 2003). The same authors also demonstrated that escaped farmed salmon are able to successfully interbreed with wild salmon, although their breeding performance is lower. Farmed Atlantic salmon has been subject to selective breeding and domestication throughout its production and therefore differs genetically from wild populations (Gjøen & Bentsen 1997; Roberge et al. 2007) and displays reduced genetic variation (Skaala et al. 2004). Owing to this fact and as mentioned in Liu et al. (2011), interbreeding between wild and farmed salmon may cause changes in genotypes and loss of genetic variation in wild salmon populations as well as a reduction in the fitness and productivity of wild salmon. Besides the risks associated with the competition and genetic interactions between farmed and wild salmon, other negative effects include the potential transfer of pathogens and diseases through infected escaped fish (Naylor et al. 2005). For instance, furunculosis disease is believed to have been transmitted to wild stocks from a large number of infected farmed salmon that escaped from Norwegian fish farms in 1988-1989 (Naylor et al. 2005).
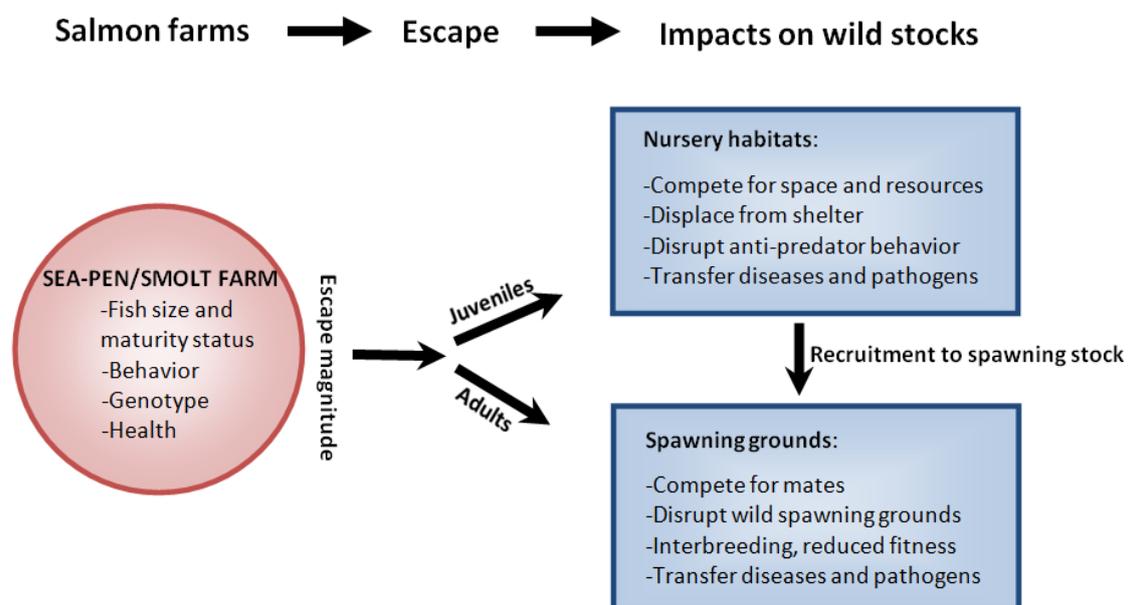

Fig. 1. Summary of the potential risks imposed by farm-escaped salmon on wild populations. Adapted from (Meager & Skjaeraasen 2009).

Apart from the environmental impacts already described, the economic consequences of escapees to fish farmers should also be considered. In Norway, the direct economic cost through loss of stock is relatively small since reported escapes account for less than 0.2% of the salmon that is held in the net pens annually (Jensen et al. 2010), although it can be a significant impact for the specific affected farm. However, the major cost of escapes is indirect since escape events are often reported by the press and thereby generate criticism and a bad reputation to the industry (Jensen et al. 2010).

As a conclusion of the above and if salmon farming and healthy wild salmon populations are to coexist in the future, measures to reduce the number of farm escapees must be implemented (Glover 2010). The Norwegian government is well aware of the problem and has therefore established a national strategy plan against escapes that compiles all the information and actions required to prevent and reduced them. In fact, the number of farmed escaped salmon already seems to have decreased after the Norwegian technical standard NS 9415 was introduced in 2004 for the use of certified equipment in all fish farms (Jensen et al. 2010). Norwegian authorities also mandate immediate reporting and recapture efforts after escape events and there are penalties for the breach of these escape-related regulations (Naylor et al. 2005). Despite these legal obligations there is evidence of unreported escape events (Skilbrei & Jørgensen 2010). These unreported escapes may be unintentional (fish farmers not aware of it) or intentional (fish farmers with-holding information after escape incidents) (Glover et al. 2008). Therefore there is increasing opinion about the need to develop a method for labeling farmed fish in order to identify the origin of escapees, and potentially use it as a tool to detect aquaculture sites in need of better husbandry practices (Adey et al. 2009) and to prosecute fish farmers breaching the regulations (Glover 2010).

In view of the need of a reliable method for identifying escapees, the Norwegian Directorate of Fisheries established a committee to evaluate a series of marking techniques (Glover 2010; Naylor et al. 2005), including physical tags, bar-code and genetic marks, among others. Moreover, a genetic method developed by Glover et al. (2008) has already been successfully implemented in a number of court cases to identify the farm of origin of recaptured escaped salmon. However, this method faces some challenges and Glover (2010) suggested that non-genetic supplementary techniques would be required in the future in order to increase precision and assist genetic assignment tests. For instance, fish scale microchemistry (Adey et al. 2009) and scale

fatty acid profile (Grahl-Nielsen & Glover 2010) could be potential tools since they have been shown to differ amongst reared Atlantic salmon groups.

According to the eventual necessity for alternative tagging techniques, the idea behind this thesis research was to develop a simple and inexpensive method that allows us not only to distinguish between farmed and wild Atlantic salmon, but also to track the escaped salmon back to the specific farm of origin. The results from a pilot study performed by our group (data not published) suggested that the incorporation of rare earth elements (REEs) in fish scales following supplementation to the feed would be worthwhile to investigate as a potential tagging method.

Chemical marking offers the possibility to mark large groups of fish and individual handling is not required, which reduces labor-intensity and improves animal welfare. Furthermore, the REEs seem to have the potential for being successful chemical markers since they are non-radioactive and therefore easy to handle, they are incorporated in the bony tissues and have been shown to have a long retention time, and they are relatively inexpensive. Based on this, the present study was designed in order to evaluate some of the REEs as possible elemental tracers.

2. THEORETICAL BACKGROUND:

2.1. Rare earth elements (REEs):

2.1.1. Introduction:

The term "rare earth elements" refers to a set of 17 chemical elements in the group III of the periodic table (Fig. 2.), specifically to the 15 lanthanide elements plus yttrium and scandium (Humphries 2012). The last two elements are commonly included with the REEs as they occur with them in natural minerals and have similar chemical properties (Tse 2011). The lanthanides include a series of elements with atomic number ranging from 57 to 71; in increasing order of atomic number: lanthanum, cerium, praseodymium, neodymium, promethium, samarium, europium, gadolinium, terbium, dysprosium, holmium, erbium, thulium, ytterbium, and lutetium (Kagan et al. 1988).

Fig. 2. Rare earth elements (highlighted in yellow)and their position in the periodic table (Hou 2006).

The REEs are generally classified into two groups according to their atomic number: the light rare earths (LREEs), which include the elements with atomic numbers 57 through 63 (lanthanum to europium) and the heavy rare earths (HREEs), including those with atomic numbers ranging from 64 to 71 (gadolinium through to lutetium) as well as yttrium (Schüler et al. 2011). The term "rare earths" is misleading since they are neither rare nor earths. In fact, these metallic elements (with the exception of promethium, the only radioactive REE) are moderately abundant in the Earth's crust, with some being

even more abundant than copper, lead, gold, and platinum (Humphries 2012). There is a peculiarity when it comes to the terrestrial contents of the REEs and that is a decreasing content of the elements with increasing atomic weight as well as a higher frequency of those elements with even atomic number (Kabata-Pendias & Pendias 2001). This fact explains that the heavier and odd-numbered REEs are more precious and difficult to obtain and therefore tend to be more expensive. The classification and abundance of the REEs are provided in Table 1. Despite their relative abundance, the REEs are not often found in concentrated form as rare earth minerals, which make them economically challenging to exploit (EPA 2012). Additionally, these metals share many similar properties and therefore tend to occur together in mineral deposits and are difficult to isolate (Castor & Hedrick 2006). A great number of minerals are known to contain REEs but for industrial production they are principally mined from bastnasite and monazite ores, which are enriched in LREEs and account for approximately 95% of the currently used REEs (Redling 2006).

Table 1. Classification and abundances of the rare earth elements.

Element	Symbol	Atomic number[1]	Classification	Crustal abundance[2]
Lanthanum	La	57	Light	30.0
Cerium	Ce	58	Light	60.0
Praseodymium	Pr	59	Light	6.7
Neodymium	Nd	60	Light	27.0
Promethium	Pm	61	Light	10^{-18}
Samarium	Sm	62	Light	5.3
Europium	Eu	63	Light	1.3
Gadolinium	Gd	64	Heavy	4.0
Terbium	Tb	65	Heavy	0.7
Dysprosium	Dy	66	Heavy	3.8
Holmium	Ho	67	Heavy	0.8
Erbium	Er	68	Heavy	2.1
Thulium	Tm	69	Heavy	0.3
Ytterbium	Yb	70	Heavy	2.0
Lutetium	Lu	71	Heavy	0.4
Ytttrium	Y	39	Heavy	24.0
Scandium	Sc	21	Light	16.0

[1] Classification of the REEs according to the atomic number (Schüler et al. 2011).
[2] Crustal abundance in ppm (EPA 2012).

The REEs have a wide variety of applications in several different fields such as catalysts, lighting, metallurgy and many others. Furthermore, their use in modern technology has dramatically increased over the past few years, being incorporated in growing markets such as battery alloys, ceramics and permanent magnets, among others (Goonan 2011). The diverse applications of the REEs are illustrated in Fig.3. and they will continue to expand as research into these elements continues.
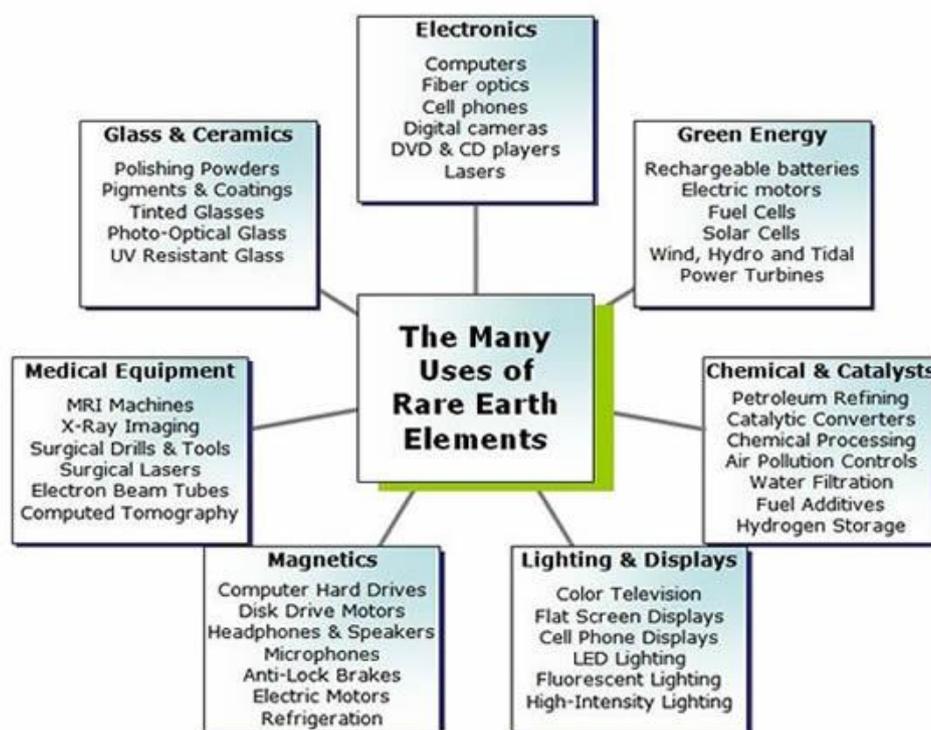

Fig 3. Major end uses and applications of the REEs (Robinson 2011).

2.1.2. Chemical properties of the REEs:

The REEs are inner-transitional elements which share extremely similar chemical, physical and metabolic properties. This similarity makes chemical separation of individual elements very difficult. REEs can only be distinguished by their 4f-electrons. With an increase in atomic number a consecutively filling of the 4f-orbitals (elements 57 to 71, 4f1 to 4f14) instead of the 5d-orbitals takes place while their oxidation states remain the same (Redling 2006). This phenomenon is very important for the understanding of REEs since many of their properties result from the shielding of the inner 4f-orbitals.

The atomic weights of the REEs increase in very small increments (see Table 2). The size of atoms and ions is determined by their nuclear charge, their number and the degree of occupied electronic shells. Therefore it is said that the radius of an ion depends on its valency. Yet, REE ions exhibit a unique physicochemical characteristic in which a decrease in ionic radii is associated with increasing atomic number (see Table 2). This so called "lanthanide contraction" is attributed to the shielded 4f-orbital, which cannot compensate the effect of increased nuclear charge (Bulman 2003). Thereby greater nuclear attraction is exerted over the whole electron cloud which ends up shrinking and leads to the contraction of the ionic structure. Regarding the ionic radius it is important to mention that the ionic radius of the REEs resembles the one of Ca^{2+}, which explains most of their biochemical behavior (Evans 1990).

Table 2. The chemical properties of rare earth elements (Redling 2006).

Element	Symbol	Atomic number	Atomic weight (g/mol)	Ionic radius (Å)
Lanthanum	La	57	138.91	1.061
Cerium	Ce	58	140.12	1.034, 0.92 (Ce^{4+})
Praseodymium	Pr	59	140.907	1.013
Neodymium	Nd	60	144.24	0.995
Promethium	Pm	61	Isotopes 141-151	0.979
Samarium	Sm	62	150.35	0.964
Europium	Eu	63	151.96	0.950, 1.09 (Eu^{2+})
Gadolinium	Gd	64	157.25	0.938
Terbium	Tb	65	158.924	0.923
Dysprosium	Dy	66	162.5	0.908
Holmium	Ho	67	164.930	0.894
Erbium	Er	68	167.26	0.881
Thulium	Tm	69	168.934	0.869
Ytterbium	Yb	70	173.04	0.858
Lutetium	Lu	71	174.97	0.848
Ytttrium	Y	39	88.905	0.88
Scandium	Sc	21	44.956	0.68

REEs generally favor tripositive oxidation state (highly electropositive), therefore their compounds are predominantly ionic in nature. However, divalent (La^{2+}, Sm^{2+}, Eu^{2+} and Yb^{2+}) and tetravalent forms (Ce^{4+}, Pr^{4+} and Tb^{4+}) also exist, yet only Eu^{2+} and Ce^{4+} are stable enough to persist in aqueous solutions.

REEs do not exhibit significant covalent bonding due to the high energy of the outer orbitals. Rare earth compounds are usually based upon ionic binding. In consequence, rare earths attract water molecules in aqueous solutions in order to form a hydration shell around them. The hydration of their tripositive ions is very exothermic and therefore rare earths are strong reducing agents under such conditions. A wide range of rare earth compounds can be formed, each with a varying degree of solubility. Rare earth cations have a great affinity to bond to fluorides, hydroxides and other oxygen-containing ligands (Bulman 2003). The order of preference for donor atoms is: O > N > S and F > Cl. In general, the halides, nitrates, perchlorates, thiocyanates and acetates are relatively soluble in water. The sulphates are moderately soluble, while the oxides, fluorides, hydroxides, oxalates, carbonates and phosphates are insoluble.

Another property worth mentioning is the tendency of the REEs to adhere to particulate matter and surfaces with which they make contact (Luckey & Venugopal 1977). This is sometimes referred to as adsorption and it is exhibited even at very low concentrations in aqueous solutions.

2.1.3. Metabolism and toxicity of REEs:

In order to obtain a good understanding of rare earth toxicity, an overview of their metabolic behavior is needed. REE's metabolism depends on the way of administration and on the chemical form administered.

Oral absorption of REEs is of great relevance for this project since in this case the rare earths were administered via the diet.

In mammals, the absorption of soluble REE salts in the gastro-intestinal (GI) tract has been reported to be minimal (Ellis 1968; Luckey & Venugopal 1977). Once the REE salts enter the GI tract, they either undergo hydrolysis or react with the normal biochemical components to form complexes of insoluble compounds. At physiological pH hydrolysis of the REEs is highly favored. This reaction occurs very quickly and the resulting rare earth hydroxides and phosphates formed in the GI tract are insoluble and therefore precipitate out. Since REEs exhibit a high affinity towards phosphates, insoluble phosphate complexes tend to form. Chelators such as citrates or lactates may be present in the tissues and rare earth cations have a strong tendency to complex with

these compounds, thereby keeping them in solution. These complexes are stable and do not undergo hydrolysis in the biological fluids.

The citrate and ethylenediaminetetraacetic acid (EDTA) forms of the rare earths seem to be more absorbed since these are large complexes that resist hydrolysis reactions and therefore insoluble rare earth compounds do not form (Ennevor 1991).

In addition, as mentioned above the REEs have very strong adsorptive properties that make them to adhere to particulate food matter and move through the tract along with the ingesta (Luckey & Venugopal 1977).

Absorption of rare earths after subcutaneous or intramuscular injection is reported to be negligible, with the REEs predominately remaining at the site of injection (Evans 1990). Intraperitoneal injected REEs have a tendency to stay within the abdominal cavity, although transport of a small fraction of these elements to liver and bony tissues has been reported. For intravenous injection, REEs have been shown to exhibit a high clearance rate from the blood (Redling 2006).

To sum up, the absorption of soluble REE salts increases according to the following sequence: oral administration < subcutaneous < intramuscular < intraperitoneal < intravenously injection. However, the distribution pattern for bioavailable REEs does not change and it is independent of the way of administration, with liver and bony tissues being the sites of greater accumulation (Bulman 2003). This is in accordance with the fact that the REEs are often described as bone-seeking elements (Durbin et al. 1956; Jowsey et al. 1958).

When introducing a foreign chemical into a biological system, possible toxic effects need to be considered. Unfortunately, there are no reported studies on the direct effects of the REEs on fish and therefore the current knowledge about the toxic effects of these elements is limited to toxicological measurements in mammals.

In comparison with other elements, the REEs are generally considered to be of low toxicity (Haley 1965). As expected from the information given on rare earth metabolism, their toxicity varies with their chemical form and the method of administration. REEs appeared to be most toxic when applied through intravenous injection, with median lethal dose (LD_{50}) values ranging between 3-100 mg/kg body weight for rats and mice, whereas oral administration seemed to have the least effect,

with LD_{50} values beyond 1g/kg body weight. This low oral toxicity is ascribed to their poor absorption from the GI tract. Due to slightly greater absorption of subcutaneous or intramuscular injected REEs, their acute toxicity is a little bit higher. According to the LD_{50} values, the medium weight REEs are the least toxic, while the light REEs are slightly more toxic than the heavy ones. Generally, the toxicity of the REEs decreases with increasing atomic weight (Luckey & Venugopal 1977).

According to different studies, the symptoms of rare earth intoxication in rodents include: sedation, labored respiration, twisting, ataxia and immediate defecation (Haley 1965; Luckey & Venugopal 1977). Calcification of soft tissue at the site of injection, fatty liver, liver edema and necrosis, pulmonary edema and hyperaemia are some other clinical symptoms that have been reported (Haley 1965; Luckey & Venugopal 1977).

2.1.4. Analytical methods:

Similarity among REEs makes their determination unusually complicated, especially if the desired REE must be determined in the mixture of the other REEs, because of numerous interferences and coincidences. The most common techniques used for the determination of REEs are inductively coupled plasma mass spectrometry (ICP-MS), inductively coupled plasma optical emission spectrometry (ICP-OES), X-ray fluorescence (XRF) and neutron activation analysis (NAA) (Zawisza et al. 2011). NAA is a very sensitive technique but it suffers from serious interferences from the major elements and from a long irradiation time used in the analysis, especially with solid samples. In the case of ICP-OES, solid samples cannot be analyzed and main constituents may also cause some matrix effects. However, this technique is used due to its capability for rapid multi-element detection over a wide concentration rate. ICP-MS has become one of the most powerful techniques in REE determination. This is ascribed to its high sensitivity, large dynamic linear range, multi-element capacity and possibility to carry out isotopic measurements. Spectral interferences are the main limitation of ICP-MS. Interferences in ICP-MS can usually be solved by different means, including high resolution, reaction/collision cells and separation. Also in most instances a mathematical correction is all that is needed.

Determination of REEs is also difficult from the point of view of separation and preconcentration steps. REEs present at trace level (ppm) do not usually require these

steps while determination of ultra trace levels (ppb) of REEs often requires a preconcentration method. This step in the analysis is generally the most reagent-, cost- and time-consuming. Digestion processes can also be hard and demanding. Therefore, direct analysis of materials for REE determination gains importance. This is possible with the XRF technique, which is widely used for multi-elemental analysis. Unfortunately, spectral interferences and poor detection limits for REEs are a serious problem in XFR.

In the present study, ICP-MS was the technique chosen for the determination of REEs in the fish scales and therefore this methodology will be discussed in more detail.

Fig. 4. shows a schematic diagram of an inductively coupled plasma-mass spectrometer. Samples are introduced into a plasma as aerosol droplets. The plasma is usually produced in argon at atmospheric pressure, sustained by a high frequency (30 MHz) energy field of 1000-2000 W. The high temperature in the plasma, ranging from 6000-10000 K, leads to atom excitation and ionization of the elemental species in the aerosol. The quartz torch consists of three concentric tubes into which different argon flows are introduced. Once the samples are introduced into the plasma, they undergo vaporization, dissociation or atomization, excitation and finally ionization (Pröfrock & Prange 2012). The resulting ions are extracted into the low-pressure mass spectrometer interface through the sampling and skimmer cones. The ions are then focused on to the mass analyzer using a series of ion lenses. Most commercial ICP-MS systems utilize a quadrupole mass spectrometer, which rapidly separates the ions according to their mass to charge ratio (Nageswara Rao & Kumar Talluri 2007). At any given time, only one mass to charge ratio will be allowed to pass through the mass spectrometer from the entrance to the exit.

Upon exiting the mass spectrometer, ions hit the first dynode of an electron multiplier, which serves as a detector. The impact of the ions produces a cascade of electrons, which are then amplified until they become a measurable pulse. Finally, the data collection software compares the intensities of the measured pulses to those from standards, which make up the calibration curve, to determine the concentration of the element.
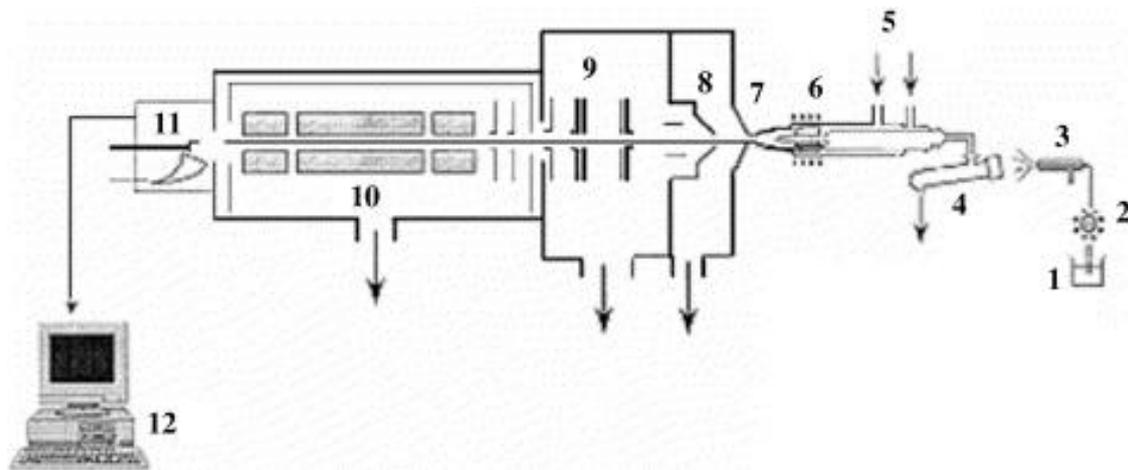
Fig. 4. Schematic diagram of inductively coupled plasma-mass spectrometer: (1) liquid sample, (2) pump, (3) nebulizer, (4) spray chamber, (5) argon gas torch inlets, (6) torch, (7) sampler cone, (8) skimmer cone, (9) ion lenses, (10) quadrupole mass analyzer, (11) electron multiplier detector, and (12) data collection (Nageswara Rao & Kumar Talluri 2007).

2.2. Fish tagging:

2.2.1. Introduction:

Tagging and marking methods have a long history of use as tools in the study of animal populations to provide information related to stock identification, population size, migration patterns, growth and survival rates or the contribution of farmed fish to fisheries programs (Thorsteinsson 2002). Many techniques have been used to mark fish. Some of these include external marks such as morphological characteristics (e.g. sizes of body parts, shape and coloration or scale characteristics), physical attached tags, mutilations (e.g. fin clipping, hot- and cold-branding or tattooing) or externally applied dyes/pigments, which have been conventionally used over the last three centuries (McFarlane et al. 1990). Internal tags and marks, which are not identifiable by external examination, are generally more recent and include implanted tags (e.g. coded-wire tag and PIT-tags), various biological marks (e.g. parasitic or bacterial tags), genetic markers (such as polymorphic isozymes and DNA fingerprints) and chemical marks applied by immersion, feeding or injection (Giles & Attas 1993). Fig. 5. illustrates the most commonly used tags.
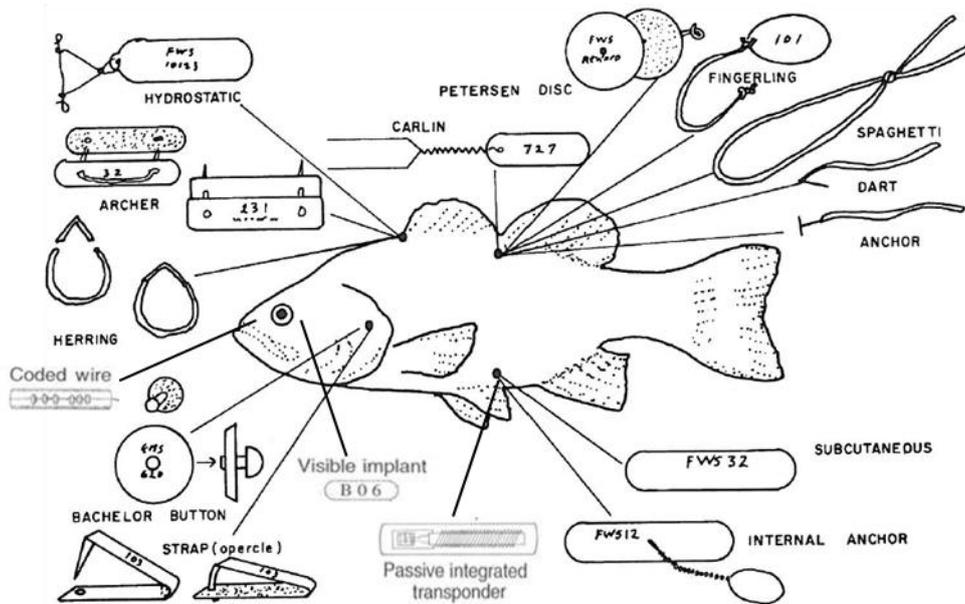
14

Fig. 5. Commonly used tags and their anatomical sites for attachment on fish. Slightly adapted from McFarlane et al. (1990).

Other marks (e.g. thermal-induced marks in the otoliths) and combinations of internal and external marks have also been used. Despite the increasing and rapid development within the field of tagging, no individual method satisfies all the criteria for an ideal mark described by Everhart et al. (1975). Each technique has its advantages and drawbacks relating to fish size requirements, number of fish to be marked, handling, effects on growth, behavior and survival rate, mark permanency, recognition and recovery of the mark, cost, labor-intensity, number of unique mark combinations and other factors (Giles & Attas 1993). New types of tags are continuously being developed to deal with the conflicts arising from information requirements on the one hand, and practical applications (permanency, identifiable, effect on fish behavior, etc) on the other.

2.2.2. Chemical marking:

Internal chemical marks applied through feeding, immersion and injection have received recent attention because they may allow rapid mass-marking of various size fish without individual handling and with less adverse effects on behavior and survival than external tags (Emery & Wydoski 1987). Research on fish nutrition and physiology has increased the knowledge about the incorporation and metabolism of different chemical elements tested as inner markers. In general, when applied through feed or

immersion, metabolically active compounds are absorbed more rapidly; reach greater concentrations in the body and are faster dispersed and excreted than metabolically inactive compounds. Fish growth and metabolism can also dilute or dissipate the induced marks in the instances where the chemicals are not strongly bonded to stable systems such as bony tissues. In contrast, direct injection of less reactive compounds eludes metabolic barriers but exposes fish to increased individual handling (Parker et al. 1990). A great disadvantage of chemical marks is the fact that most of them are not externally visible and may need special equipment or intrusive methods to be detected. In addition, chemical batch-marking impedes the recognition of individual fish (Emery & Wydoski 1987). The use of certain chemicals can be controversial because of later human consumption of marked fish or because of entry of unwanted chemicals into the food chain. Nevertheless, in most cases the final chemical concentrations in the fish are very low and may be negligible (Thorsteinsson 2002).

Researchers have tried to mark batches of fish through feeding, immersion, and injections with chemicals of different nature, including dyes and stains, rare earth compounds, metallic elements, and fluorescent compounds (e.g. calcein and tetracycline). Muncy et al. (1990) reviewed the available literature on chemical marking of fish and the various described methods and application/detection techniques are summarized in Table 3.

Table 3. Chemical methods and detection techniques used for marking fish internally. Application techniques: In, injection; F, feeding; Im, immersion. Detection techniques: V, visual; Uv, ultraviolet; NAA, neutron activation analysis; ASS, atomic absorption spectroscopy; XEOL, X-ray excited optical luminescence; XFS, X-ray fluorescence spectroscopy; DL, dye laser; Fl, fluorometric; EM, electron microscopy; RIS, resonance ionization spectroscopy. The time required to apply a mark and the period of detectability for a specific concentration are given when applicable. Slightly adapted from Muncy et al. (1990).

Chemical	Application		Detection		Amount detected
	Method	Time	Method	Time	
Dyes and stains	Im	3 h	V	6 d	
	F	?	V	77 d	
	In		V	1 year	
Rare earth elements	Im	0.5 h	XEOL	21 d	5
	Im	0.5 h	DL	10 d	0.0002
	Im, F	30 d	AAS	30 d	2000
	F	84 d	NAA	1.5 years	0.6
	F	84 d	RIS	1.5 years	0.1
	F	40 d	NAA	2 years	0.1
	In		NAA	2 years	1
Tetracycline	Im	2 h	V, Uv	8 d	
	F	14 d	Uv	2 years	
	F	40 d	Fl	1 year	0.6
	In		Uv	2 years	
Calcein	Im	2 h	Uv	27 d	
Pollutants	F		EM		?
	F		AAS		0.05
Lead	In		V	2 years	
Cadmium	In		V	4 years	
Mercury	In		V	4 years	
Cobalt	Im	1 d	NAA	36 d	?
Strontium	F	42 d	AAS	42 d	200
	F	80 d	XFS	75 d	1
	Im	49 d	XFS	169 d	1
Manganese	F	60 d	XFS	75 d	1
Natural mixtures	N		EM		1
	N		XFS		1
	N		XFS		1

From the comparative tests, Muncy et al. (1990) inferred that chemical dyes and stains were mainly used for short-term marking since they fade away over time. Externally applied dyes and stains do not require instrumentation to be detected, but they tend to increase predation on marked fish. Injection of metallic compounds also produced visible marks and they were recognizable for up to 4 years. Fluorescent marks can be detected under ultraviolet light or by fluorometric techniques. Feeding and injection of fluorescent compounds also provided long-term internal marks. On the other hand, fluorescent marks induced by immersion did not last long since exposure of marked fish

to sunlight may reduce detectable levels of such compounds. The detection of low levels of non-visible chemical elements requires sophisticated instrumentation and trained operators. Rare earth elements have been detected in fish samples by X-ray-excited optical luminescence, dye laser techniques, resonance ionization spectroscopy, atomic absorption spectroscopy and neutron activation analysis. REE-marks lasted longer and at higher levels when induced by injection than when administered by feeding or immersion techniques. Despite these results, in their review Muncy et al. (1990) concluded that chemical marking of fish is still limited by inadequate field-detection techniques and that development of improved instrumentation is required for more accurate results. They also emphasized that researchers must understand the basis of analytical techniques and be aware of improvements that can lower detection limits, avoid masking effects by background levels, reduce or remove interferences and they must also determine the metabolic pathways of incorporation of the chemical compounds used to mark fish.

2.2.3. Previous works with REEs:

There have been only a few researchers that have investigated the feasibility of using REEs to label fish. Michibata and Hori (1981) and Michibata (1981) attempted to use samarium (Sm) to label medaka (*Oryzias latipes*) and goldfish (*Carassius auratus*). In the first set of experiments, Michibata and Hori (1981) injected Sm into the abdominal cavity. The element was accumulated in the liver, intestine, kidneys, vertebrae, gills, scales and muscle and it was detectable for up to 2 years after the last injection. Although this was an efficient method to induce a chemical label, it required individual handling and it was only useful when the fish were large enough to be injected. Since a mass-marking method seemed more promising, in the next study Michibata (1981) administered the Sm through the diet. Samarium chloride was added directly to the diet at a rate of 66 mg/g of feed and the fish were fed the supplemented diet for 30 days. The author found that the level of Sm decreased dramatically 30 days after the treatment but then it remained constant for the following year. The elemental concentration was determined by neutron activation analysis and it was detected in the fifth branchial arch, scales, gills, intestine and liver. Sm decreased rapidly in the intestine and liver and it was undetectable after 90 days while in the rest of the tissues the amount of element also tended to decrease but still remained one year after labeling. This indicated that

longer term storage of Sm takes place in the bony tissues. Although Sm was clearly incorporated, Michibata (1981) stated that the mechanism of uptake and retention is still unknown and he suggested that the incorporation could take place from the water and not from the diet, since REEs have been reported not to be absorbed in the gastro-intestinal (GI) tract (Ellis 1968; Luckey & Venugopal 1977). Zak (1984) also attempted to use Sm to mark american shad (*Alosa sapidissima*) and atlantic salmon (*Salmo salar*) through feeding and immersion. The element was incorporated but it was undetectable 30 days after the treatment.

Europium (Eu), another REE, has also been successfully used as a fish marker. Kato (1985) induced a Eu mark in chum salmon (*Oncorhynchus keta*) by addition of this element to the diet. Europium chloride was mixed into pellet-type feed at a mean Eu content of 817 ± 85 ppm and the fish were fed the enriched diet for 40 days. Scales and liver were used as the detection organs for Eu but the element was detected more effectively from the scales, where Eu was present for up to 2 years after the labeling. These results also indicate long-term storage of Eu in the bony tissues. Similarly, Shibuya (1979) reported accumulation of Eu in fish scales after administration of the element through the diet, although the marks only lasted for 3 months after the treatment.

Muncy & D'Silva (1981) successfully labeled walleye eggs (*Stizostedion vitreum*) by immersion in terbium (Tb) dicitrate solution (100-1250 μg/l). Tb was determined by X-ray-excited optical luminescence (XEOL) spectroscopy. The element was detected in sac fry hatched from Tb-labeled walleye eggs and remained detectable through the early juvenile stage. Juvenile fish that had developed scales and spines (3 weeks or older) contained inorganic elements such as Ca and Mg that prevented the detection of Tb. Other solutions (terbium chloride, sodium terbium citrate, europium chloride and neodymium dicitrate) were tested but they did not seem to be suitable to mark walleye eggs.

Dysprosium (Dy) was also tested as a chemical marker for chinook salmon (*Oncorhynchus tshawytscha*) fingerlings by Miller (1963) and Babb et al. (1967). These researches used various methods to apply the Dy, including immersion, feeding and intramuscular injection. Neutron actvation analysis was the analytical method used to detect the Dy. The results were similar in both experiments, with the Dy being detected only in the injected fish. The Dy was present in the bones for up to 5 months after

injection, indicating a long-term storage of the element in the bony tissues. The fish fed the Dy-supplemented diets and those immersed in Dy-solutions (0.1-1 μg/l) did not accumulate detectable amounts of the element in the bony tissue. The authors suggested that a more sensitive analytical method might have been able to detect lower elemental levels present in these treatment groups and that a longer exposure time (> 24 h once a week for 5 weeks) at higher concentrations may result in higher amounts of accumulated Dy in the tissues. From these results, the intramuscular injection appeared to be the most effective way of producing a detectable Dy-mark. However, as mentioned before, injection requires individual handling and therefore is time consuming and can result in increased mortality.

Ennevor (1991), Ennevor and Beames (1993) and Ennevor (1994) carried out a series of experiments to evaluate the mass marking of coho salmon (*Oncorhynchus kisutch*) by addition of REEs to the water supply. The researchers used Inductively Coupled Plasma-Mass Spectometry (ICP-MS) for the elemental determination. In the preliminary work described by Ennevor (1991), coho salmon alevins were exposed to dysprosium, lanthanum, cerium, samarium and ytterbium acetates at 20, 100 and 300 μg/l and steelhead (*Salmo gairdneri*) alevins were treated with lanthanum and samarium acetates at 0, 10 and 100 μg/l. Both species were shown to be too sensitive to the REEs for this stage to be suitable for marking. The light REEs appeared to be more toxic than the heavier ones. Coho salmon fry were then tested and demonstrated to be less sensitive to these elements. In addition, when the fry were exposed to lanthanum acetate at 100 μg/l for 3 weeks, detectable levels of this element were accumulated in vertebral columns, otoliths and scales. The author found that a suitable method for administering the REEs was by addition of a constant proportion of a concentrated REE acetate solution to a constant flow of ambient inlet water, which allows for the replenishment of the elements absorbed by the fish. In a similar experiment, Ennevor and Beames (1993) exposed juvenile coho salmon to lanthanum and samarium acetates at 10 and 100 μg/l for 3 and 6 weeks. La and Sm were found to be present in the vertebral columns, otoliths and scales of all treatment groups (except for the 10 μg/l and 3 weeks) 10.5 months after the labeling. In the same work, Ennevor and Beames (1993) demonstrated the feasibility of inducing multi-elemental marks in Coho fry and smolt after exposing the fish to different treatment combinations of lanthanum and cerium. Ennevor (1994) investigated the difference in toxicity and uptake between the chloride and acetate forms of lanthanum and cerium. Uptake and deleterious effects of the REEs did not differ

between the treatment groups. Ennevor (1994) also recommended to mark the fish with a low concentration of REE over an extended period of time.

Dysprosium, europium and samarium were also investigated by Giles and Attas (1993) as internal batch markers for rainbow trout (*Oncorhynchus mykiss*) fingerlings. A chloride suspension with 12.7 μg of Dy or Eu, or with 127 μg of Sm, or with a mixture containing 12.7 μg Dy, 12.7 μg Eu and 254 μg of Sm, was administered to the fish by interperitoneal injection. REE concentrations in the fish were determined by neutron activation analysis. Elemental retention and tissue distribution were similar in fish receiving the REEs singly or in combination. The levels of Dy and Eu decreased by 40-50% during the first 6 months but remained stable for the following 1.5 years. On the other hand, Sm levels kept stable over the 2-year study period. All the elements were primarily retained in the gut while small amounts were detected in the kidneys and gonads as the fish was reaching sexual maturation. Effects of marking on fish growth and sexual development were negligible. Physiological effects were restricted to a transitory increase in gut weight relative to body weight and a slight edema in the kidney.

The results from the labeling trials described above show that the REEs are taken up by the fish and accumulated in the bony tissues. As stated by Michibata and Hori (1981) and Michibata (1981) for samarium, REEs would be suitable chemical elements to mark fish since they offer several advantages: (1) the apparent absence of harmful effects on the fish, (2) the small probability of interference from naturally occurring REEs, (3) a long biological half-life of the deposited REEs in the fish, (4) no radioactive contamination of the fish and other organisms, and (5) safety in handling.

3. OBJECTIVES:

The main objective of this project was to develop a method that, both simply and inexpensively, allows for the discrimination between farmed and wild salmon, and at the same time track the salmon back to its specific site of origin.

In particular, we aimed to:

- Evaluate a series of rare earth elements (praseodymium, neodymium, dysprosium, cerium and lanthanum) as potential chemical markers in scales of Atlantic salmon (*Salmo salar* L.) by addition of these elements to the feed.
- Monitor the concentration of the elements in the scales during the experimental feeding period in order to study the uptake of the supplemented elements.
- Monitor the concentration of the elements in the scales 2 months after terminating the supplemented diets in order to study the dilution of the induced marks.
- Evaluate general effects of the supplemented chemicals on the fish performance.

4. MATERIALS AND METHODS:

4.1. Chemicals:

The REE chlorides (markers) supplemented to the different experimental diets were as follows: lanthanum (III) chloride (LaCl₃), cerium (III) chloride (CeCl₃), dysprosium (III) chloride (DyCl₃), neodymium (III) chloride (NdCl₃) and praseodymium (III) chloride (PrCl₃), and they were all purchased from Treibacher Industrie AG (Althofen, Austria). The chloride form was chosen because it is water-soluble (therefore easy to include in the feed) and it was demonstrated to be incorporated into the fish scales in a previous pilot study (data not published). The solution used for the digestion of all samples (scales, feed and faeces) was a sub-boiled nitric acid (HNO_3) ($\geq$ 65%, puriss. p.a.) supplied by Sigma-Aldrich (Steinheim, Germany). The hydrogen peroxide used to decrease the fat content in the feed and faeces samples was H_2O_2 Trace SELECT Ultra, $\geq$ 30%, also supplied by Sigma-Aldrich (Steinheim, Germany). The water used throughout the sample preparation was deionized (DI) water prepared by a Milli Q System (18.2 MΩ cm, Millipore Corp., Billerica, USA). The internal standard solution added in order to correct for the differences in the dilutions and to reduce the matrix interferences contained 1 mg/l Ge, In and Tl and it was prepared from 1000 mg/l single-element standard solutions obtained from Spectrapure Standards (Oslo, Norway). The solution used for the calibration of the ICP-MS instrument was prepared from a 50 mg/l multi-element standard solution containing REEs and from 1000 mg/l single-element standard solutions containing U, As and Sr, all of them obtained from Spectrapure Standards (Oslo, Norway).

4.2. Dietary treatments:

The basis for all diets was commercially extruded pellets (3 mm) manufactured by Skretting AS, Averøy, Norway. The different experimental diets were obtained by coating 20 kg batches of the common feed pellet with each of the markers in a blender. The coating procedure was equal for all diets. First, the REE chloride (10 g/l) was dissolved and the yttrium oxide (Y_2O_3) (2 g/l) suspended in distilled water at room temperature. The obtained solution was then added to the pellets (25 ml/kg) in the blender. After the mixing with the REE + Y_2O_3 solution the pellets were dried on a tray

for 24 h. The feeds were finally top-dressed with rapeseed oil (10 ml/kg) in order to prevent leaching of the supplemented elements.

4.3. Fish, rearing conditions and experimental design:

The trial was performed at Nofima Marin fresh water Research Station, in Sunndalsøra, Norway (62°40′31″N 08°33′05″E) from early May to mid September in 2012. Atlantic salmon (*Salmo salar* L.) yearling smolt (1[+]) with a mean initial body weight of 87.3 ± 1.57 g were placed into 10 square tanks (1 x 1 m), each of them containing 50 fish. The tanks were supplied with seawater at ambient temperature (average of 9.4 °C) ranging from 7.5 °C (min. temperature reached in June) to 13.9 °C (max. temperature reached in September). The fish were acclimatized to tank environment and fed a commercial diet before the start of the experiment. The trial consisted of a 10-week labeling period (from 9 May till 18 July) during which a REE supplemented diet was administrated to all fish, followed by a two-month "dilution" period (from 18 July till 19 September) with the fish being fed untreated commercial feed. The five experimental diets were randomly assigned to duplicate tanks as showed in Fig. 6.
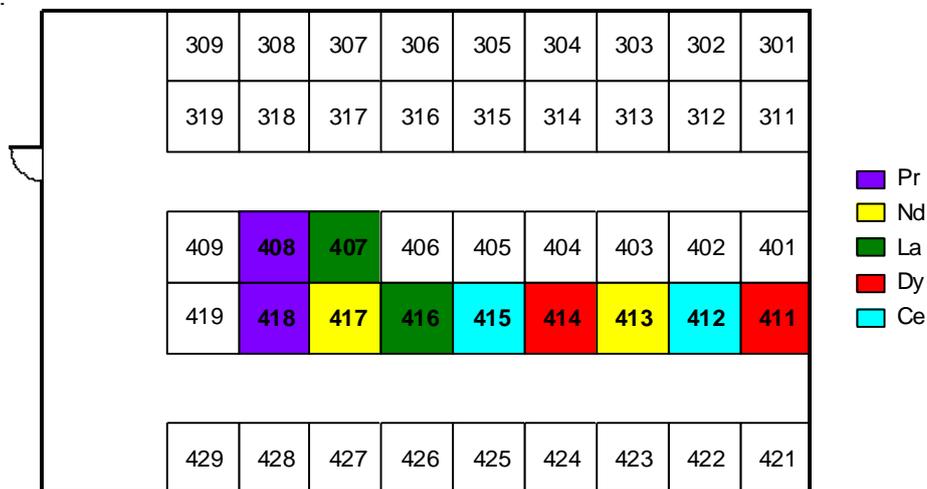

Fig. 6. Allocation of the five experimental diets in the trial room (Pr= praseodymium, Nd= neodymium, La= lanthanum, Dy= dysprosium, Ce= cerium).

4.4. Sampling:

Before the onset of the experiment an initial sample of 10 fish (May, S0) was taken for the determination of the elemental background levels in the scales. Similarly, all experimental diets were sampled for chemical analyses. Thereafter 10 fish from each tank were randomly sampled 5 (June, S1) and 10 (July, S2) weeks after the start of the labeling period. At the termination of the "dilution" phase (September, S3) the same sampling procedure was conducted in order to examine the elemental dilution in the scales. At sampling, all fish were anaesthetized using MS-222 (Metacaine 0.1 g/l; Alpharma, Animal Health Ltd, Hampshire, UK) and individual body weight and length were recorded before storing at -20 °C. During S2, faeces samples were also collected for studying the apparent digestibility of the tested elemental tracers. Twenty fish were picked at random from each tank and faeces were obtained by stripping from the posterior part of the intestine as described by Austreng (1978). An overview of the sampling scheme is showed in Table 4.

Table 4. Details on sampling regimes throughout the experiment.

	Labeling period		Dilution	
Sampling dates	9-May	13-Jun	18-Jul	19-Sep
	S0	S1	S2	S3
Diet sampling	X			
Fish sampling	X[1]	X[2]	X[2]	X[2]
Weight and lenght	X	X	X	X
Feaces sampling			X[3]	

[1] Initial sample containing 10 fish was taken.
[2] Samples were made of 10 individual fish per tank.
[3] Faeces samples were stripped from 20 fish per tank.

4.5. Sample preparation and chemical analysis:

In preparation for chemical analyses scales were scraped with a sharp scalpel from the area between the lateral line and the dorsal fin, the 5 experimental feeds were crushed with a mortar and the faeces samples were pooled (faeces from 20 individual fish within each tank). Before removing the scales, dirt and mucilage were gently cleared away from the fish surface. A mean wet weight of 0.37 g of fish scales, 0.16 g of all feeds and 0.35 g of the pooled faeces samples were weighed and stored in 15 ml polypropylene

test tubes (Sarstedt, Nümbrecht-Rommelsdorf, Germany). All the samples were then oven-dried to constant weight, dry weight recorded, and finally submitted for analysis.

Prior to elemental analyses 100 µl of internal standard was added to all the samples to account for instrumental changes. Then all samples were digested in 2 ml of sub-boiled HNO_3 in a laboratory oven set at 90 °C for 3 hours. In the case of the feed and faeces samples 1 ml of H_2O_2 was added in order to reduce the fat content, the heating process was repeated and the samples were finally centrifuged for 2 min at RCF = 1300 in a Sigma 4K15 Laboratory Centrifuge (Osterode am Harz, Germany) . After the digestion all samples were made up to 14 ml using DI water. Blank solutions (N = 40) were similarly prepared for blank corrections and calculation of limits of detection (LOD) (see equation 3 and Table 5). A further 5-time dilution of all the samples was required in order to avoid salt deposition on the instrument's cones and to reduce matrix effects.

Table 5. Limits of detection (LOD) of the 5 elemental isotopes analyzed in the trial.

	Tested elemental isotopes				
	^{139}La	^{140}Ce	^{141}Pr	^{143}Nd	^{163}Dy
LOD (ng/l)*	0.35	0.65	0.11	0.49	0.14

* The LOD were calculated according to equation 3 in the materials and methods section.

The elemental composition of scales, experimental diets and faeces was determined by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) using a Thermo Scientific Element2 High Resolution Sector Field ICP-MS (Bremen, Germany). The ICP-MS instrument was calibrated with acid matrix matched calibrating standard solutions and it was operated in low (LRM, $m \Delta m$ approx. 300), medium (MRM, $m \Delta m$ approx. 4000) or high (HRM, $m \Delta m$ approx. 10000) resolution mode. Details of instrumental operating conditions and measuring parameters are given in Table 6. A total of 25 isotopes were quantified from which just 5 were considered for the labeling study (^{139}La, ^{140}Ce, ^{141}Pr, ^{143}Nd and ^{163}Dy). In the instances where the REE had more than one isotope the one with greater abundance and no isobaric overlap (when possible) was chosen. A blank and a quality control sample containing the analytes and prepared independently from the calibrating solutions were run after every 10 samples.

Table 6. ICP-MS operating conditions and measurement parameters.

RF power (W)	1500		
Sample uptake rate (ml/min)	0.2		
Gas flow rates (l/min)			
Coolant (Ar)	15		
Auxiliary (Ar)	1.25		
Nebulizer (Ar)	0.8 - 1.0		
Additional (CH$_4$)	0.1		
Ion sampling depth (mm)	3.8		
Ion lens settings	Adjusted to obtain max. signal intensity		
Torch	Fassel torch, 1.5 mm i.d.		
Nebulizer	MicroMist		
Spray chamber	Cyclonic		
Sample cone	Platinum, 1.1 mm orifice diameter		
Skimmer	Platinum, 0.8 mm orifice diameter		
	Low resolution mode	*Medium resolution mode*	*High resolution mode*
Isotopes	^{88}Sr, ^{139}La, ^{140}Ce, ^{141}Pr, ^{142,143,144}Nd, ^{147,149}Sm, ^{153}Eu, ^{157,158}Gd, ^{159}Tb, ^{161,163}Dy, ^{165}Ho, ^{166}Er, ^{169}Tm, ^{172,173}Yb, ^{175}Lu, ^{238}U ^{115}In* and ^{205}Tl*	^{45}Sc, ^{89}Y and ^{115}In*	^{75}As and ^{73}Ge*
Acquisition mode	E-scan	E-scan	E-scan
No. of scans	9 (3 runs x 3 passes)	9 (3 runs x 3 passes)	9 (3 runs x 3 passes)
Mass window (%)	100	125	125
Search window (%)	150	50	50
Integration window (%)	80	60	60
Dwell time per sample (ms)	10	10	30
No. of samples per nuclide	10	20	20

* Used for internal standard correction.

4.6. Calculations and corrections:

Calculations and figures were made using Microsoft® Excel 2007 (Microsoft, Redmond, WA, USA).

The growth rate of the fish is presented as the thermal growth coefficient (TGC). The TGC incorporates both fish size and temperature and it was calculated according to Cho (1992) as:

$$TGC = (W_1^{1/3} - W_0^{1/3}) \times (\Sigma T)^{-1} \times 1000, \qquad \textbf{(1)}$$

where W_0 and W_1 are the initial and final weights (tank means, g), respectively and ΣT is the sum of day degrees during the period (feeding days x average temperature, °C). The factor of 1000 is included in order to simplify the numbers.

*The overall weighted TGC was corrected for weight difference during the different periods.

Apparent digestibility coefficients (ADC) for the tested elemental tracers were estimated by the indirect method, as described by Maynard and Loosli (1969), using Y_2O_3 as the inert marker (Austreng et al. 2000):

$$ADC\,(\%) = 100 - \left[100 \; x \; \left(\frac{\text{elemental tracer in faeces}}{\text{elemental tracer in diet}} \; x \; \frac{Y^2O^3 \text{in diet}}{Y^2O^3 \text{in faeces}}\right)\right] \qquad \textbf{(2)}$$

The limits of detection (LOD) for the different elemental isotopes were determined as follows:

LOD = 3 x SD of blank samples, $\qquad$ **(3)**

where SD is the standard deviation of the blank average for each of the isotopes.

Isotopic counts were blank-subtracted to account for background metal contamination levels, corrected for the dilution factor and finally converted to elemental concentration per gram of dry weight (of scales, feed or faeces):

$$[Isotope]\left(\frac{\text{ng}}{\text{g}}\right) = \frac{\left([\text{Isotope}] \text{ in sample} \left(\frac{\text{ng}}{\text{l}}\right) - \text{ Isotope blank average} \left(\frac{\text{ng}}{\text{l}}\right)\right) \text{x } 0{,}014}{\text{Dry weight of the sample (g)}} \qquad \textbf{(4)}$$

4.7. Statistical analysis:

The data were analyzed by one-way analysis of variance (ANOVA), with the elemental content in scales, body weight or TGC as a dependent variable and sampling date, period or diet as a class variable using the General Linear Model (GLM) procedure in the Statistic Analysis Software (SAS) release 9.3 for Windows (SAS Institute Inc. Cary, NC, USA). During the statistical model run, tank was used as the experimental unit. Significant differences were indicated by Duncan's multiple range test. The level of significance was $p \leq 0.05$, and $p \leq 0.1$ was considered as a trend. Linear, potential or exponential regression analysis was performed using Microsoft Excel to estimate the relationship between two variables. The proportion of the total variation that is explained by the regression model is expressed by R^2. The results are presented as mean $\pm$ standard error of the mean (SE) in the cases where tank means are used or as mean $\pm$ standard deviation (SD) in the instances where individual variations are to be studied.

5. RESULTS:

5.1. Growth, body weight and mortality:

Throughout the 10-week labeling period there were only two mortalities, both of them during the first week of the trial. The dead fish were noticed in tanks 412 (being fed Ce-supplemented diet) and 417 (being fed Nd-supplemented diet) and they weighted 81 and 64 g respectively. During the 2-month dilution period no mortalities were recorded.

The development of the body weight for the different dietary groups is shown in Fig. 7. Significant differences in body weight were not observed in any of the sampling dates between the fish groups fed the different experimental diets. The overall initial body weight averaged 87.3 ± 1.57 g and the fish reached a mean body weight of 503.2 ± 37.19 g at the final sampling.
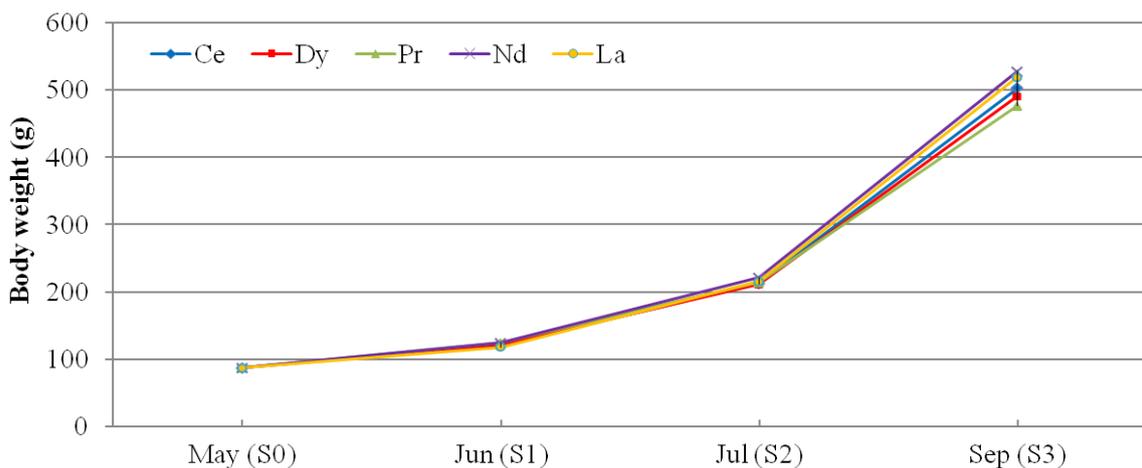
Fig. 7. Development of body weight for Atlantic salmon smolt fed 5 different REE-supplemented diets (Ce, Dy, Pr, Nd and La) during the experimental period.

The calculated TGC for each of the dietary groups within the different experimental periods is presented in Table 7. No significant differences in TGC were found between the different dietary groups within the different experimental periods, nor in the total experimental period.

Table 7. Thermal growth coefficient (TGC) for each of the dietary groups (means ± SEM; n = 2) within the two main experimental periods and overall TGC for the total experimental period.

	TGC					
Period	**Ce**	**Dy**	**Pr**	**Nd**	**La**	*P-value*
Labeling period	3.23 ± 0.07	3.13 ± 0.19	3.15 ± 0.05	3.36 ± 0.29	3.40 ± 0.22	0.79
Dilution period	2.88 ± 0.16	2.72 ± 0.16	2.69 ± 0.16	2.94 ± 0.10	2.88 ± 0.19	0.74
Total period	2.99 ± 0.08	2.85 ± 0.17	2.84 ± 0.12	3.08 ± 0.02	3.03 ± 0.19	0.65

5.2. Digestibility and feed composition:

The chemical analysis of the feed and the faeces showed that the five tested REE chlorides were very poorly, if at all, absorbed in the gastrointestinal (GI) tract (see Table 8).

Table 8. Concentration of the five tested REE-chlorides and Yttrium in each of the experimental feeds and in the faeces from the different dietary groups and estimated apparent digestibility coefficient (ADC) for each of the tested tracers. Values are presented as means of tanks (n= 2).

	Dy-diet		Nd-diet		Ce-diet		Pr-diet		La-diet	
	^{163}Dy	^{89}Y	^{143}Nd	^{89}Y	^{140}Ce	^{89}Y	^{141}Pr	^{89}Y	^{139}La	^{89}Y
Conc. faeces (µg/g)	200.04	226.17	289.71	213.30	261.27	202.32	317.25	237.06	293.55	221.41
Conc. feed (µg/g)	63.41	74.16	91.51	68.51	84.05	67.62	103.92	79.04	87.13	68.45
Ratio faeces/feed	3.15	3.05	3.17	3.11	3.11	2.99	3.05	3.00	3.37	3.23
ADC (%) *	-3.45		-1.68		-3.89		-1.79		-4.16	

*ADC was estimated according to equation 2 in the materials and methods section.

5.3. Labeling:

The chemical analysis of fish scales revealed some interesting findings considering the low absorption of the elemental tracers observed from the analysis of the feed and faeces. First, a large individual variation in the tested elemental concentrations in the scales was noticed within the different REE-treated tanks, especially during the labeling period (S1 and S2) (see Fig. 8 to Fig. 12).
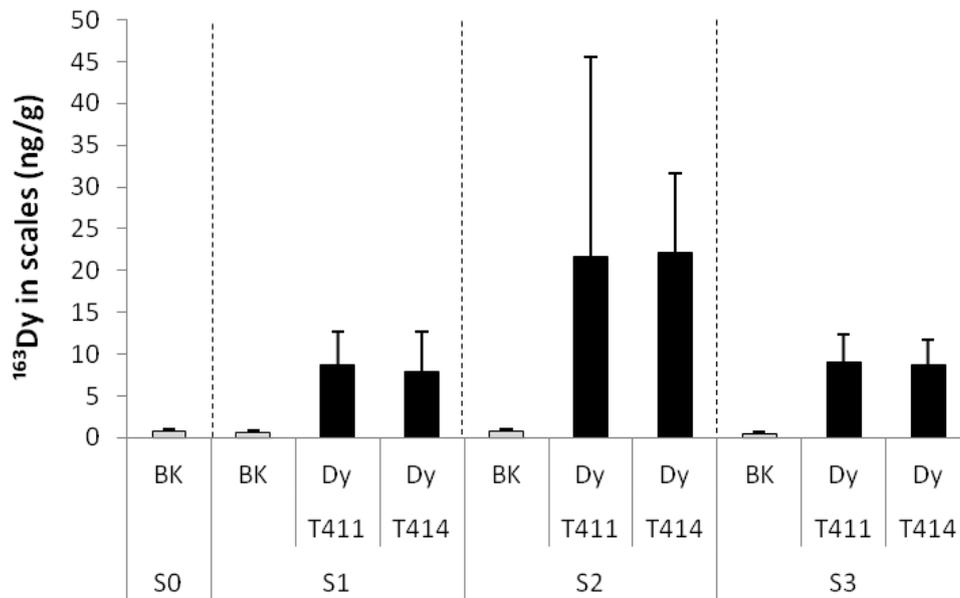
Fig. 8. Concentration of [163]Dy in the scales of Atlantic salmon 1[+] smolt fed dysprosium supplemented diet (Dy) and not dysprosium supplemented diets (BK) at the different sampling dates (S0, S1, S2 and S3, see Table 4). Values are presented as means of individual fish and the variation within the tanks (T411 and T414) and within the BK groups is given as the standard deviation (SD T411 and T414: n = 10, SD BKS0 = 10, SD BKS1,S2,S3: n = number of sampled fish not fed Dy at each sample date).
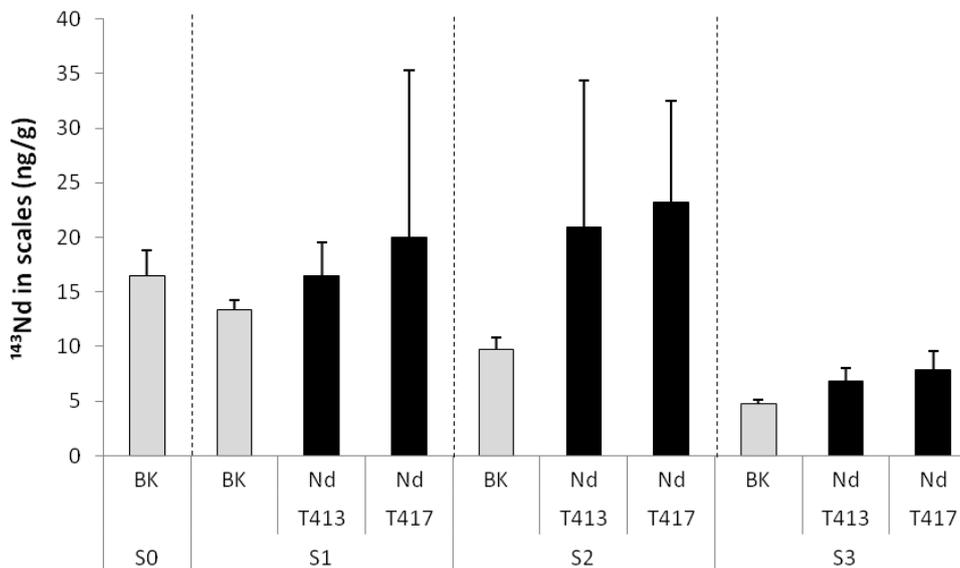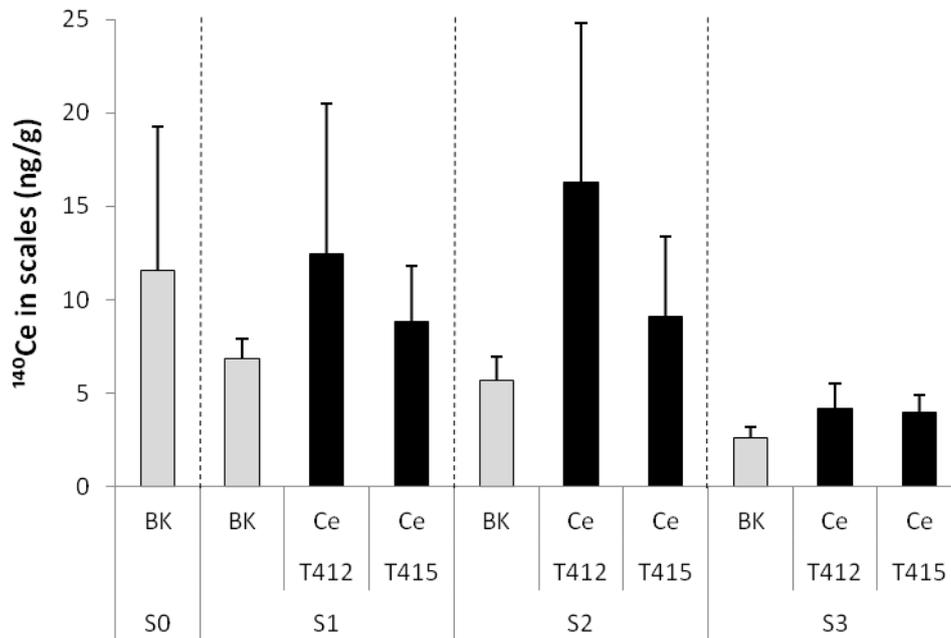

Fig. 9. Concentration of [143]Nd in the scales of Atlantic salmon 1+ smolt fed neodymium supplemented diet (Nd) and not neodymium supplemented diets (BK) at the different sampling dates (S0, S1, S2 and S3, see Table 4). Values are presented as means of individual fish and the variation within the tanks (T413 and T417) and within the BK groups is given as the standard deviation (SD T413 and T417: n = 10, SD BKS0 = 10, SD BKS1,S2,S3: n = number of sampled fish not fed Nd at each sample date).
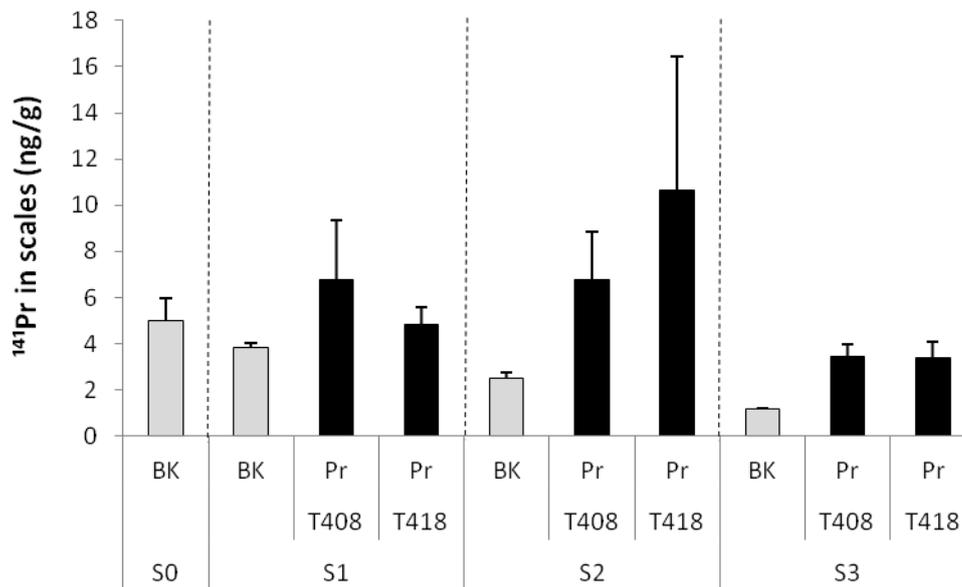
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Fig. 10. Concentration of ^{140}Ce in the scales of Atlantic salmon 1^+ smolt fed cerium supplemented diet (Ce) and not cerium supplemented diets (BK) at the different sampling dates (S0, S1, S2 and S3, see Table 4). Values are presented as means of individual fish and the variation within the tanks (T412 and T415) and within the BK groups is given as the standard deviation (SD T412 and T415: n = 10, SD BKS0 = 10, SD BKS1,S2,S3: n = number of sampled fish not fed Ce at each sample date).
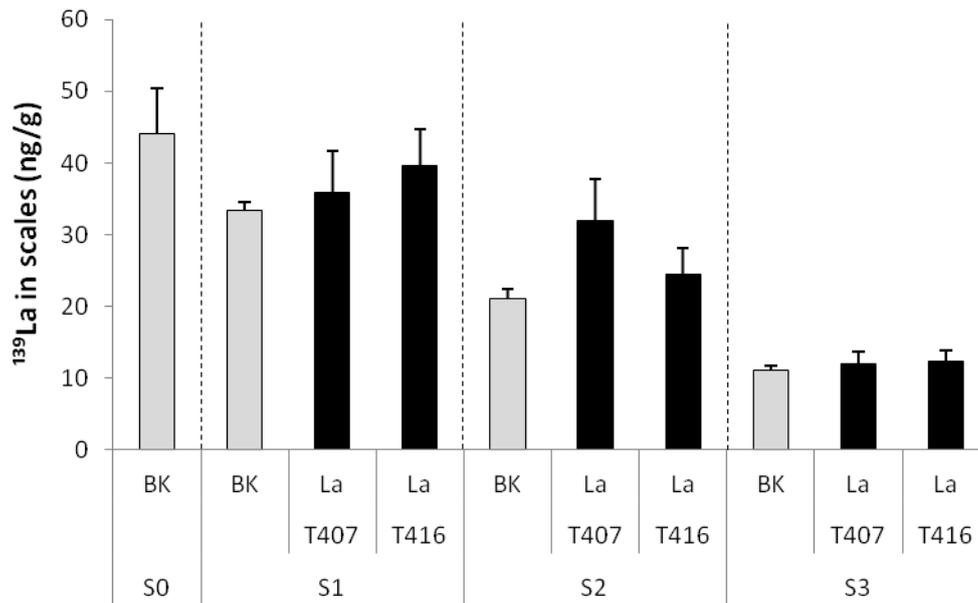

Fig. 11. Concentration of ^{141}Pr in the scales of Atlantic salmon 1^+ smolt fed praseodymium supplemented diet (Pr) and not praseodymium supplemented diets (BK) at the different sampling dates (S0, S1, S2 and S3, see Table 4). Values are presented as means of individual fish and the variation within the tanks (T408 and T418) and within the BK groups is given as the standard deviation (SD T408 and T418: n = 10, SD BKS0 = 10, SD BKS1,S2,S3: n = number of sampled fish not fed Pr at each sample date).

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Fig. 12. Concentration of ^{139}La in the scales of Atlantic salmon 1^+ smolt fed lanthanum supplemented diet (La) and not lanthanum supplemented diets (BK) at the different sampling dates (S0, S1, S2 and S3, see Table 4). Values are presented as means of individual fish and the variation within the tanks (T407 and T416) and within the BK groups is given as the standard deviation of the mean (SD T407 and T416: n = 10, SD BKS0 = 10, SD BKS1,S2,S3: n = number of sampled fish not fed La at each sample date).

One of our hypotheses to explain these individual variations was a possible difference in individual feed intake and growth rate within the tanks. Therefore the elemental concentrations in the fish scales after the labeling period (S2) were plotted against the relative fish weight increase during this period (S0 to S2) (see Fig. 13 to Fig. 17). No strong correlation was found between the concentration of any of the five tested elements in the scales and the fish relative weight increase during the labeling period.
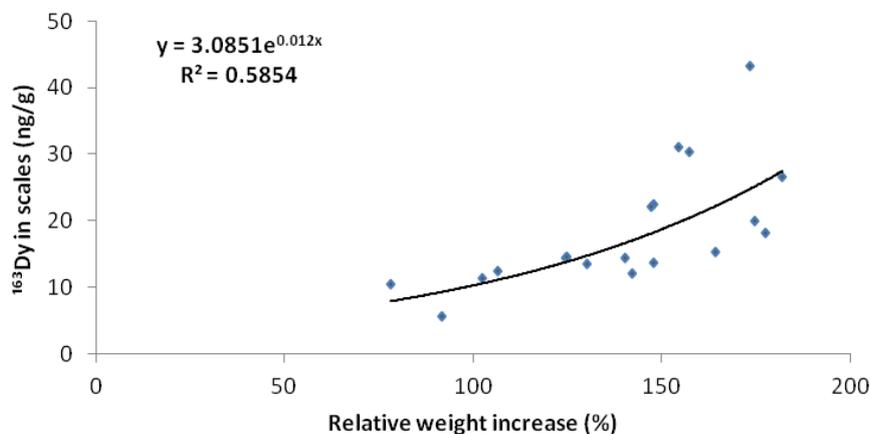

Fig. 13. Relationship between the ^{163}Dy concentration in the scales of the fish fed Dy-supplemented diet at the end of the labeling period (S2) and the relative fish weight increase during this period (S0 to S2).
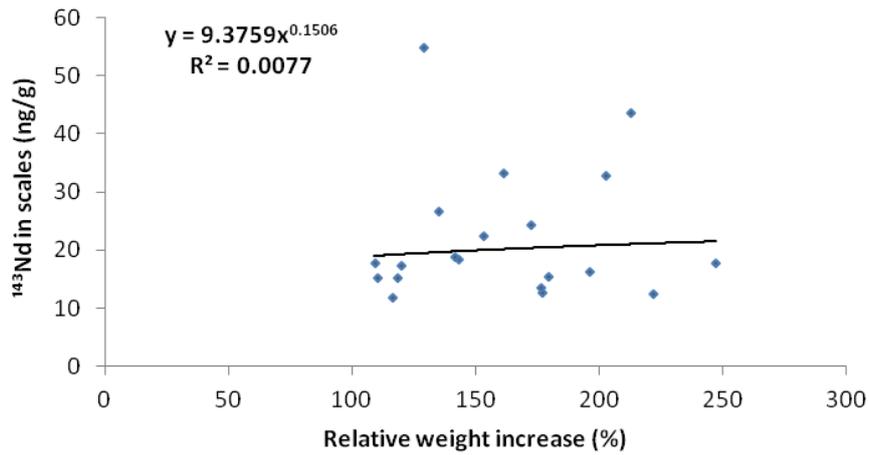
Fig. 14. Relationship between the [143]Nd concentration in the scales of the fish fed Nd-supplemented diet at the end of the labeling period (S2) and the relative fish weight increase during this period (S0 to S2).
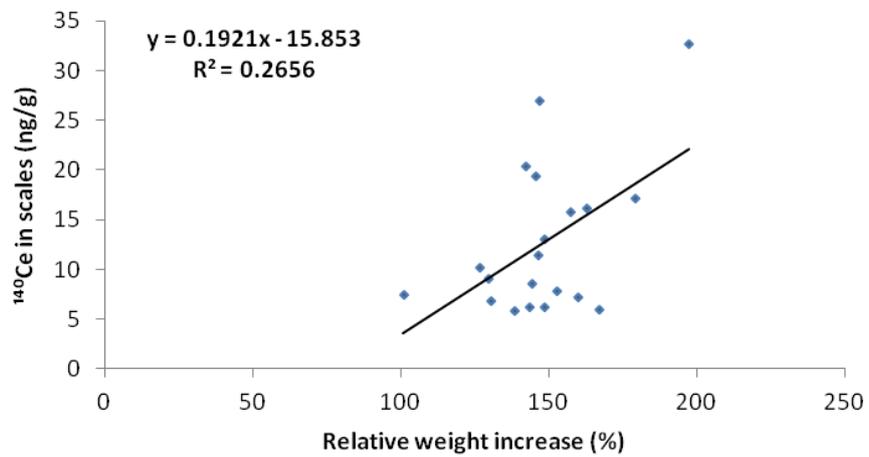

Fig. 15. Relationship between the [140]Ce concentration in the scales of the fish fed Ce-supplemented diet at the end of the labeling period (S2) and the fish relative weight increase during this period (S0 to S2).
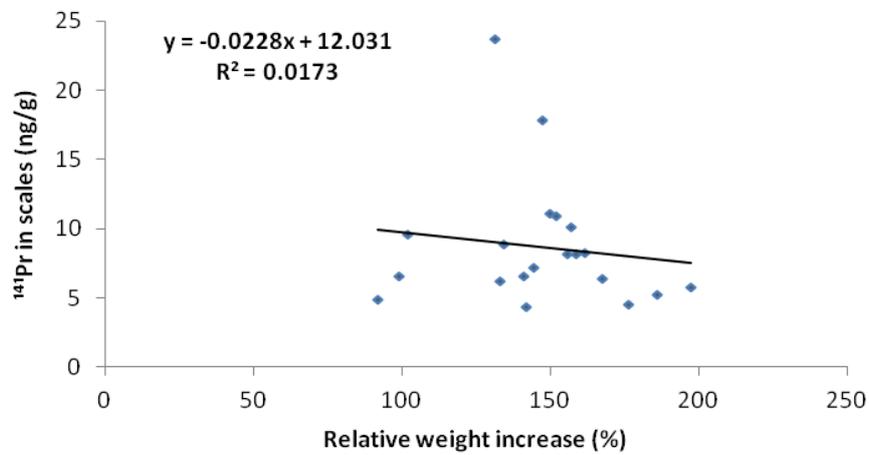

Fig. 16. Relationship between the [141]Pr concentration in the scales of the fish fed Pr-supplemented diet at the end of the labeling period (S2) and the fish relative weight increase during this period (S0 to S2).
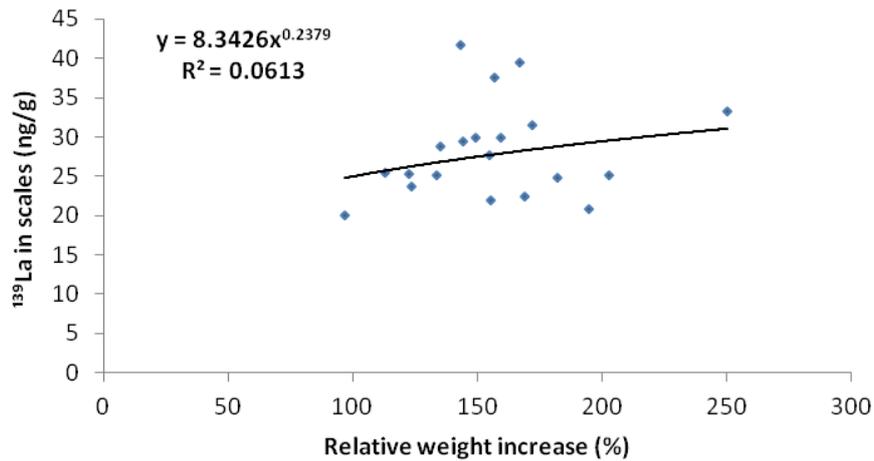
34

Fig. 17. Relationship between the [139]La concentration in the scales of the fish fed La-supplemented diet at the end of the labeling period (S2) and the relative fish weight increase during this period (S0 to S2).

Despite the individual differences the concentration of the five tested tracers in the scales of the REE-treated fish was significantly higher than that of the untreated fish (background levels) in both of the sampling dates within the labeling period (S1 and S2), demonstrating that all five elements were clearly incorporated into the fish scales. The incorporation of all elements (except for lanthanum) increased continuously during the labeling period (S1 and S2). Furthermore, significant differences in the concentration of all tracers (except for lanthanum) were found between the treated and untreated fish groups after the 2-month dilution period, although these concentrations were markedly lower than the ones present right after the labeling period. This suggests that the induced chemical marks (except for the lanthanum marks) may be detected in a long-term period after the labeling. The concentrations of the tested REE isotopes ([163]Dy, [143]Nd, [140]Ce, [141]Pr and [139]La) in the fish scales of the different dietary groups within the different sampling dates are illustrated in Fig. 18 to Fig. 22, respectively.

In particular, the Dy-supplemented diet resulted in the strongest scale marking, with a 28-time increased concentration of this element in the scales of the treated fish at the end of the labeling period (see S2 in Fig. 18). Moreover, the Dy-diet had a highly significant effect ($p < 0.0001$) on the elemental concentration in the scales after the dilution period (S3). Diet effect at S3 was also highly significant for Nd ($p < 0.0001$), Pr ($p < 0.0001$) and Ce ($p = 0.0114$). The La-diet had no significant effect on elemental concentration in the scales at S3 but it showed a strong trend ($p = 0.0724$).
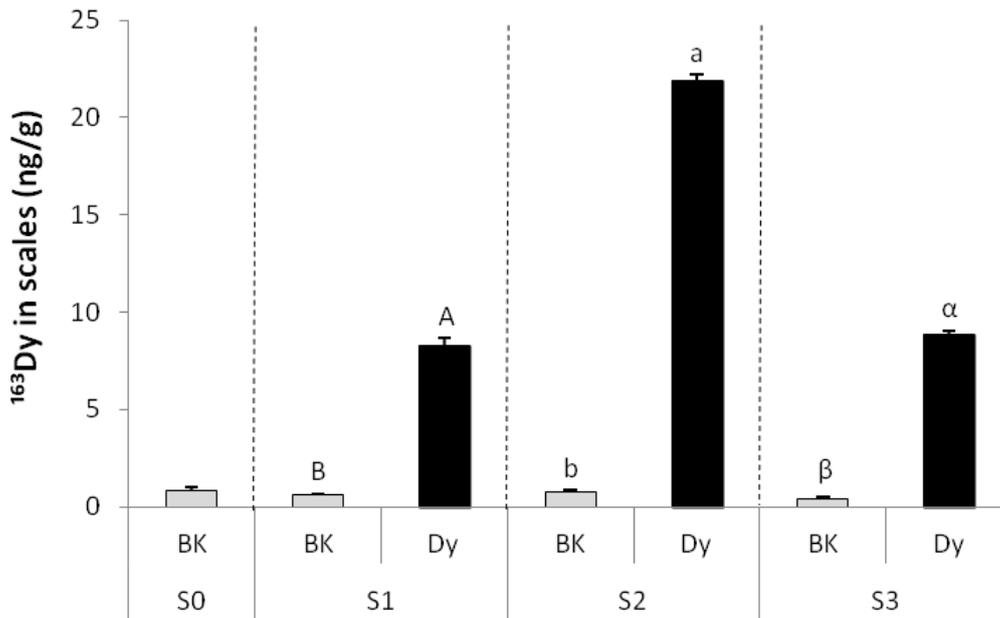
Fig. 18. Concentration of ^{163}Dy in the scales of Atlantic salmon 1$^+$ smolt fed dysprosium supplemented diet (Dy) and not dysprosium supplemented diets (BK) at the different sampling dates (S0, S1, S2 and S3, see Table 4). The value for S0 is presented as the mean of individual fish within one tank and the rest of the values are presented as means of tanks. The variation between the individuals/tanks is given as the standard deviation and the standard error of the mean, respectively (SD BKS0: n = 10, SEM Dy: n = 2, SEM BKS1, S2, S3: n = 8). Significant differences between dietary treated and untreated groups within each sampling period are indicated by different letters on the bars.
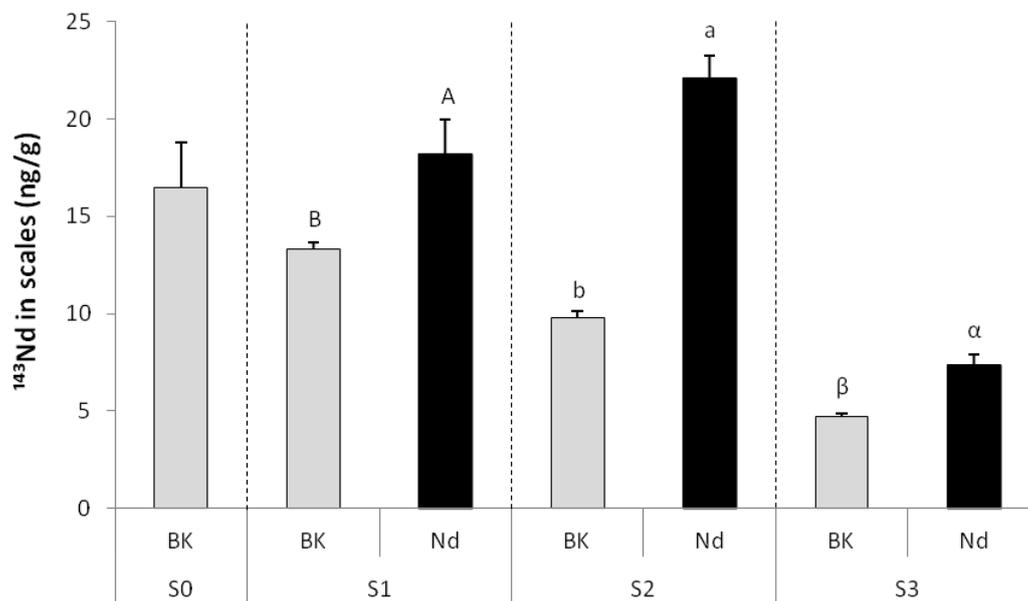

Fig. 19. Concentration of ^{143}Nd in the scales of Atlantic salmon 1$^+$ smolt fed neodymium supplemented diet (Nd) and not neodymium supplemented diets (BK) at the different sampling dates (S0, S1, S2 and S3, see Table 4). The value for S0 is presented as the mean of individual fish within one tank and the rest of the values are presented as means of tanks. The variation between the individuals/tanks is given as the standard deviation and the standard error of the mean, respectively (SD BKS0: n = 10, SEM Nd: n = 2, SEM BKS1, S2, S3: n = 8). Significant differences between dietary treated and untreated groups within each sampling period are indicated by different letters on the bars.

Fig. 20. Concentration of ^{140}Ce in the scales of Atlantic salmon 1^+ smolt fed cerium supplemented diet (Ce) and not cerium supplemented diets (BK) at the different sampling dates (S0, S1, S2 and S3, see Table 4). The value for S0 is presented as the mean of individual fish within one tank and the rest of the values are presented as means of tanks. The variation between the individuals/tanks is given as the standard deviation and the standard error of the mean, respectively (SD BKS0: n = 10, SEM Ce: n = 2, SEM BKS1, S2, S3: n = 8). Significant differences between dietary treated and untreated groups within each sampling period are indicated by different letters on the bars.
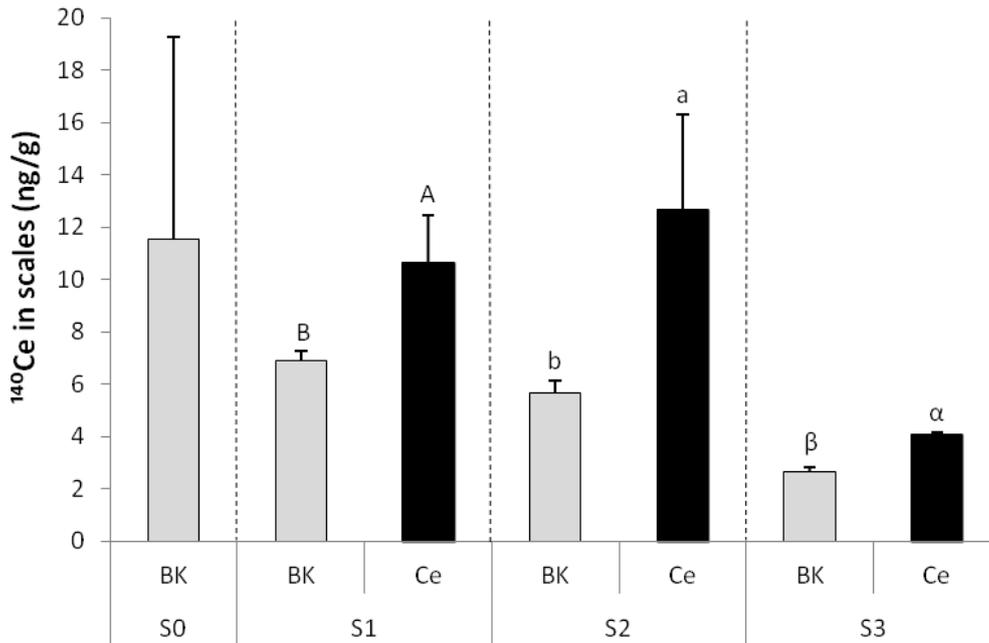

Fig. 21. Concentration of ^{141}Pr in the scales of Atlantic salmon 1^+ smolt fed praseodymium supplemented diet (Pr) and not praseodymium supplemented diets (BK) at the different sampling dates (S0, S1, S2 and S3, see Table 4). The value for S0 is presented as the mean of individual fish within one tank and the rest of the values are presented as means of tanks. The variation between the individuals/tanks is given as the standard deviation and the standard error of the mean, respectively (SD BKS0: n = 10, SEM Pr: n = 2, SEM BKS1, S2, S3: n = 8). Significant differences between dietary treated and untreated groups within each sampling period are indicated by different letters on the bars.
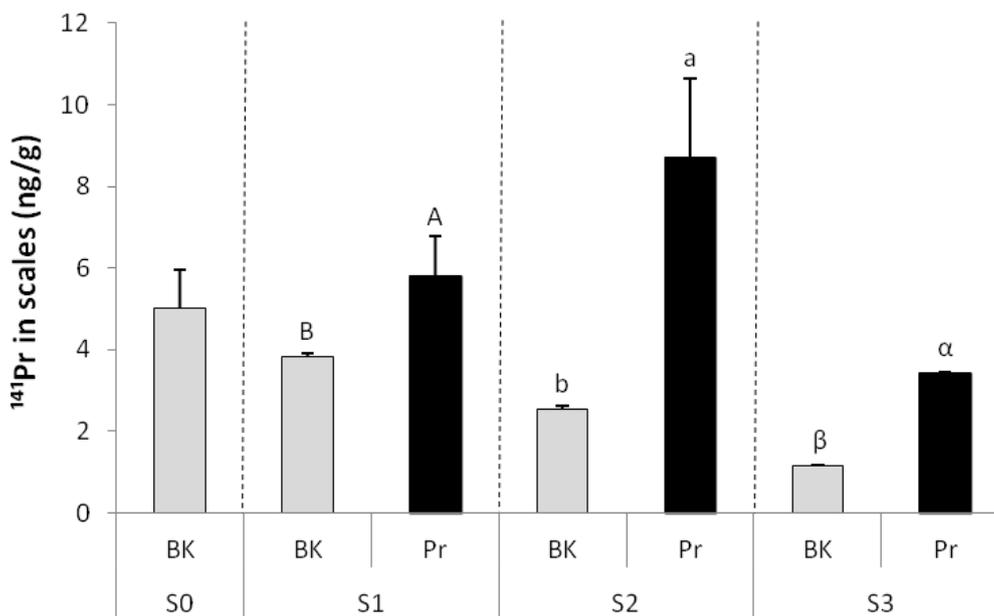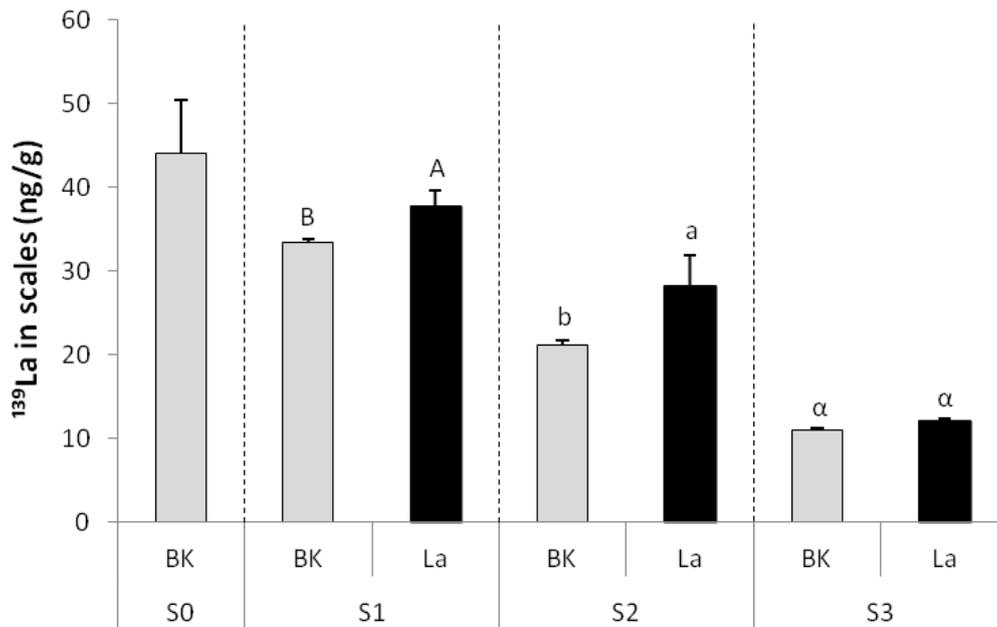
Fig. 22. Concentration of [139]La in the scales of Atlantic salmon 1[+] smolt fed lanthanum supplemented diet (La) and not lanthanum supplemented diets (BK) at the different sampling dates (S0, S1, S2 and S3, see Table 4). The value for S0 is presented as the mean of individual fish within one tank and the rest of the values are presented as means of tanks. The variation between the individuals/tanks is given as the standard deviation and the standard error of the mean, respectively (SD BKS0: n = 10, SEM La: n = 2, SEM BKS1, S2, S3: n = 8). Significant differences between dietary treated and untreated groups within each sampling period are indicated by different letters on the bars.

Surprisingly, the five elemental background levels were also found to steadily decrease with time (see BK-groups in the different sampling dates in Fig. 18 to Fig. 22). Therefore the concentration of the five tested elemental isotopes in the scales of the untreated fish was plotted against the fish body weight (see Fig. 23 to Fig. 27). A strong negative correlation was observed between the background levels of [143]Nd ($R^2 = 0.85$), [141]Pr ($R^2 = 0.89$) and [139]La ($R^2 = 0.92$) in the scales and the fish body weight. The correlation between the [163]Dy and [140]Ce levels and the fish body weight was lower, although still negative.

Fig. 23. Relationship between the [163]Dy concentration in the scales of the untreated fish (background levels) and the fish body weight.

Fig. 24. Relationship between the [140]Ce concentration in the scales of the untreated fish (background levels) and the fish body weight.

Fig. 25. Relationship between the [143]Nd concentration in the scales of the untreated fish (background levels) and the fish body weight.

Fig. 26. Relationship between the ^{141}Pr concentration in the scales of the untreated fish (background levels) and the fish body weight.
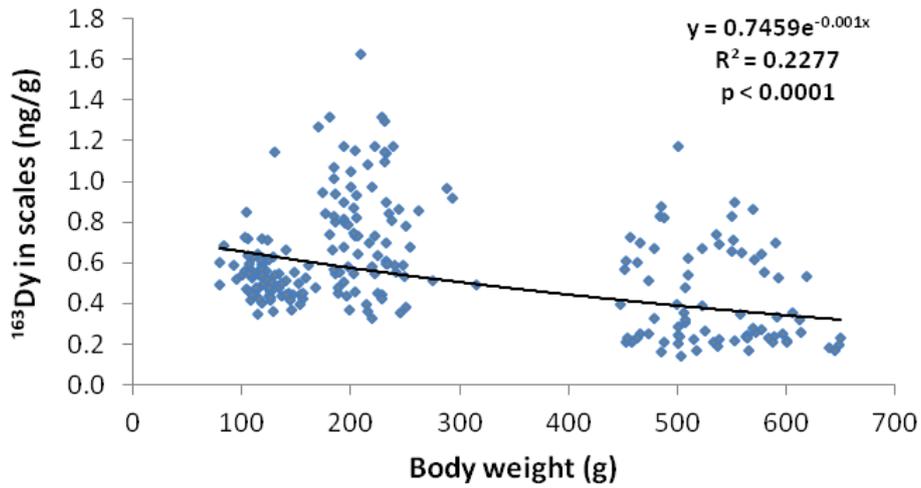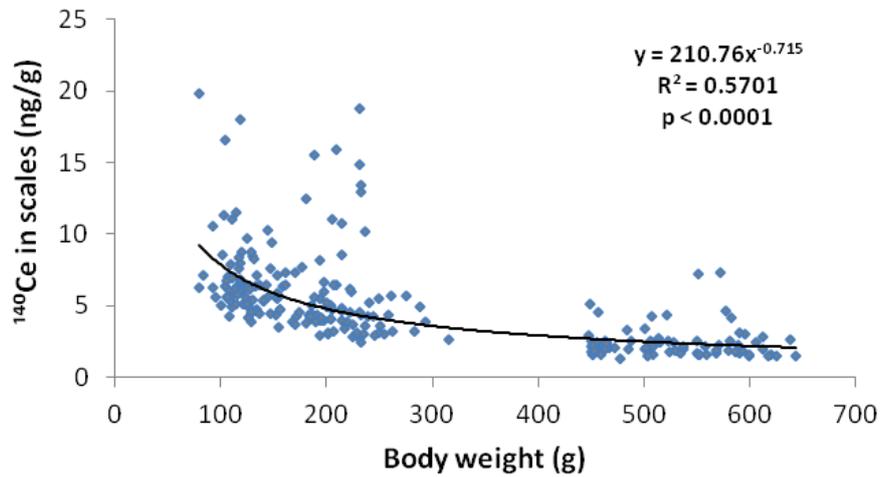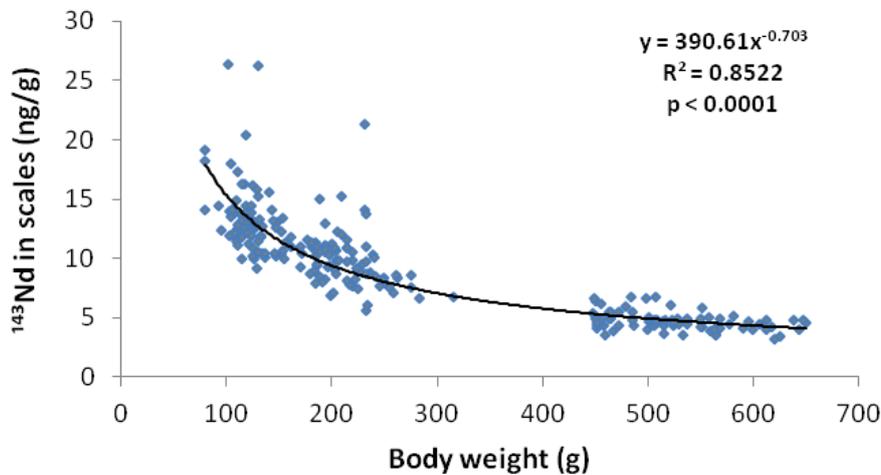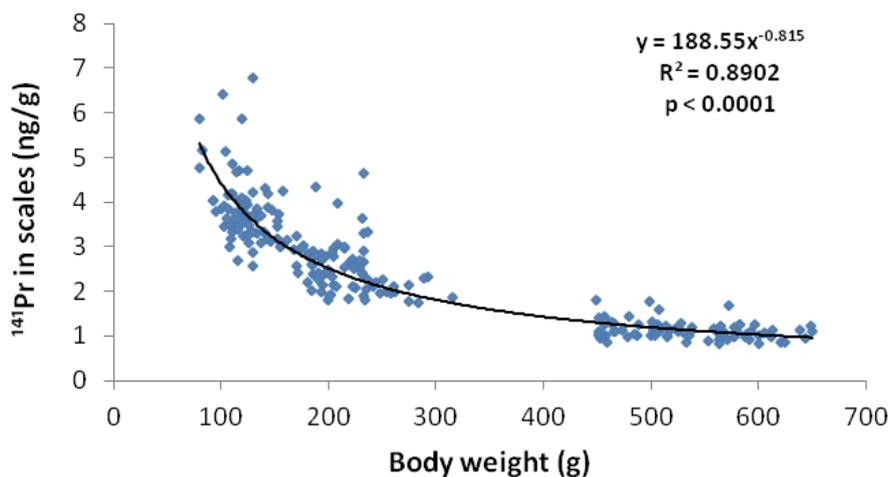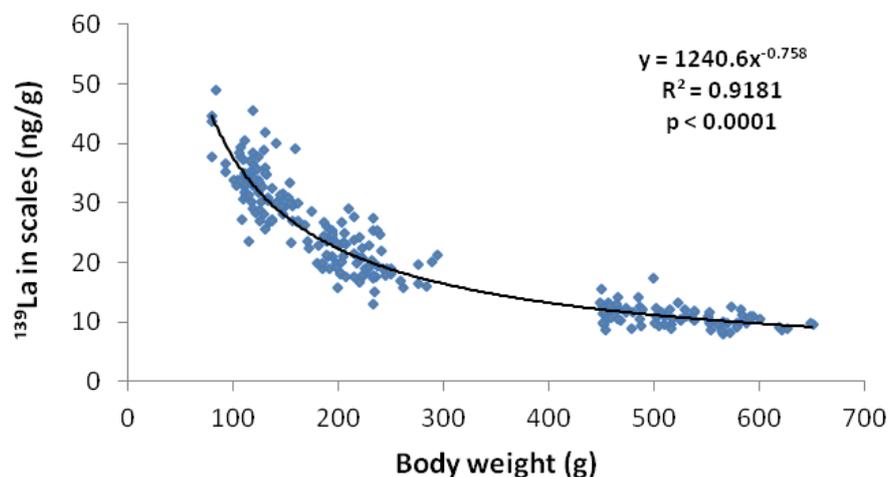

Fig. 27. Relationship between the ^{139}La concentration in the scales of the untreated fish (background levels) and the fish body weight.

5.4. Other elemental background levels:

The interesting finding about the steady decrease of the tested elemental background levels led us to study the chemical fingerprint of the scales a bit further. Hence, the concentration of other elements (not the ones supplemented to the different experimental diets) in the fish scales was plotted in time (see A in Fig. 28 to Fig. 34) in order to monitor the chemical incorporation into the scales. The background levels of some of the elements (^{165}Ho, ^{158}Gd, ^{147}Sm and ^{75}As) showed the same clear decreasing pattern as the supplemented ones (see A in Fig. 28 to Fig. 31, respectively). Interestingly, some of the elemental background levels (^{88}Sr, ^{238}U and ^{45}Sc) presented an

opposite increasing tendency (see A in Fig. 32 to Fig. 34, respectively), suggesting that these elements were more present and therefore more incorporated into the scales in seawater than in freshwater, since the experimental salmon smolt were transferred to seawater shortly before the start up of the experiment.

Fig. 28. Concentrations of ^{165}Ho in the fish scales within the different sampling dates (A) and the relationship between these concentrations and the fish body weight (B). The value for S0 in A is presented as the mean of individual fish within one tank while the rest of the values are presented as means of tanks. The variation between the individuals/tanks is given as the standard deviation and the standard error of the mean, respectively (SD S0 = 10, SEM S1,S2,S3: n = 10). Significant differences between sampling dates are indicated by different letters on the bars.

Fig. 29. Concentrations of ^{158}Gd in the fish scales within the different sampling dates (A) and the relationship between these concentrations and the fish body weight (B). The value for S0 in A is presented as the mean of individual fish within one tank while the rest of the values are presented as means of tanks. The variation between the individuals/tanks is given as the standard deviation and the standard error of the mean, respectively (SD S0 = 10, SEM S1,S2,S3: n = 10). Significant differences between sampling dates are indicated by different letters on the bars.
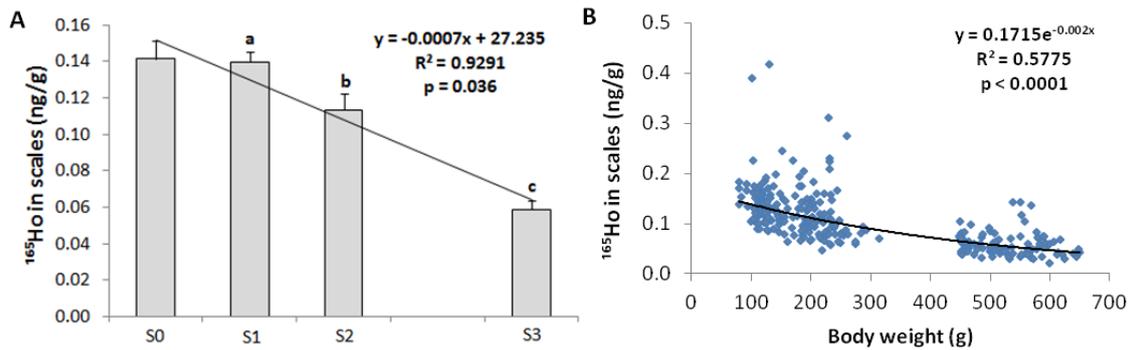
Fig. 30. Concentrations of ^{147}Sm in the fish scales within the different sampling dates (A) and the relationship between these concentrations and the fish body weight (B). The value for S0 in A is presented as the mean of individual fish within one tank while the rest of the values are presented as means of tanks. The variation between the individuals/tanks is given as the standard deviation and the standard error of the mean, respectively (SD S0 = 10, SEM S1,S2,S3: n = 10). Significant differences between sampling dates are indicated by different letters on the bars.

Fig. 31. Concentrations of ^{75}As in the fish scales within the different sampling dates (A) and the relationship between these concentrations and the fish body weight (B). The value for S0 in A is presented as the mean of individual fish within one tank while the rest of the values are presented as means of tanks. The variation between the individuals/tanks is given as the standard deviation and the standard error of the mean, respectively (SD S0 = 10, SEM S1,S2,S3: n = 10). Significant differences between sampling dates are indicated by different letters on the bars.
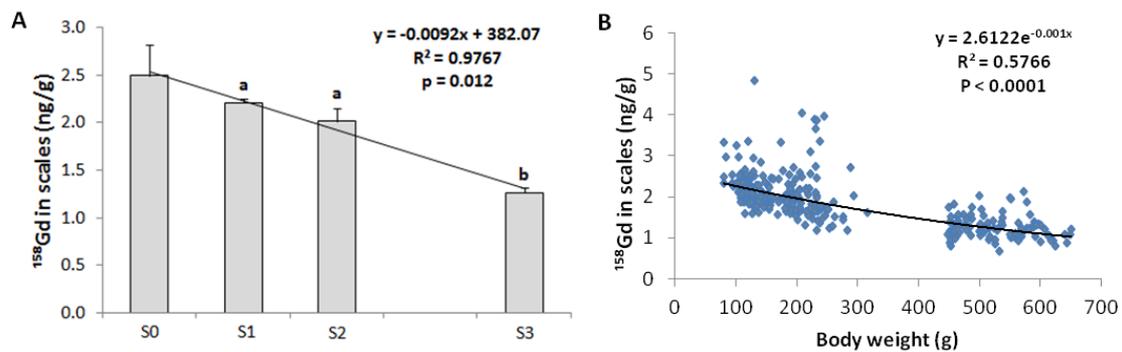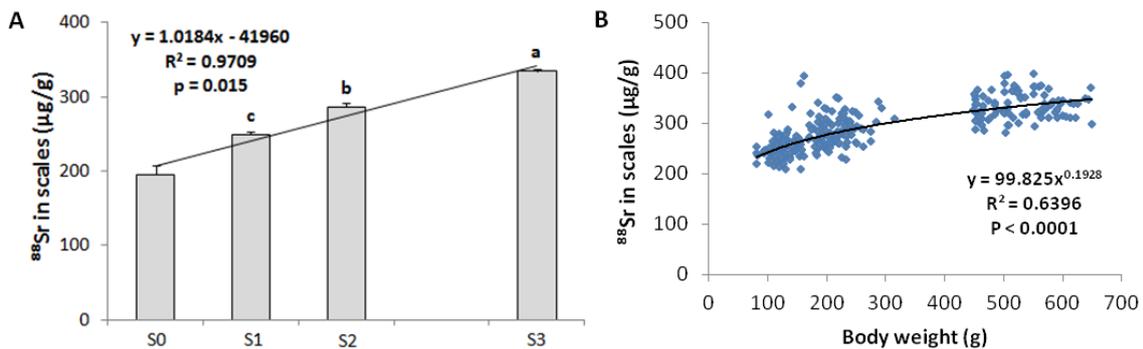

Fig. 32. Concentrations of ^{88}Sr in the fish scales within the different sampling dates (A) and the relationship between these concentrations and the fish body weight (B). The value for S0 in A is presented as the mean of individual fish within one tank while the rest of the values are presented as means of tanks. The variation between the individuals/tanks is given as the standard deviation and the standard error of the mean, respectively (SD S0 = 10, SEM S1,S2,S3: n = 10). Significant differences between sampling dates are indicated by different letters on the bars.
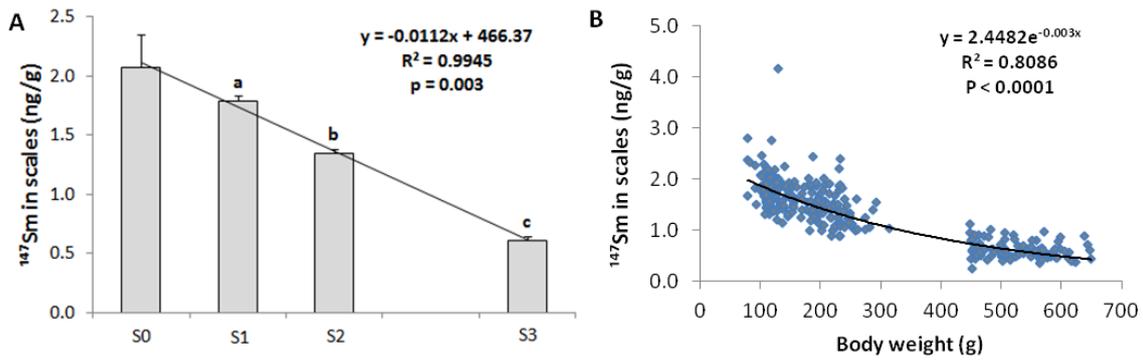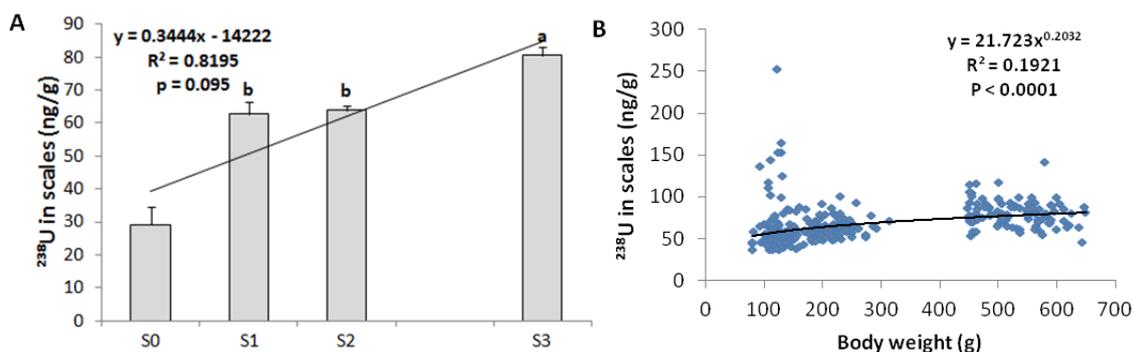
Fig. 33. Concentrations of ^{238}U in the fish scales within the different sampling dates (A) and the relationship between these concentrations and the fish body weight (B). The value for S0 in A is presented as the mean of individual fish within one tank while the rest of the values are presented as means of tanks. The variation between the individuals/tanks is given as the standard deviation and the standard error of the mean, respectively (SD S0 = 10, SEM S1,S2,S3: n = 10). Significant differences between sampling dates are indicated by different letters on the bars.
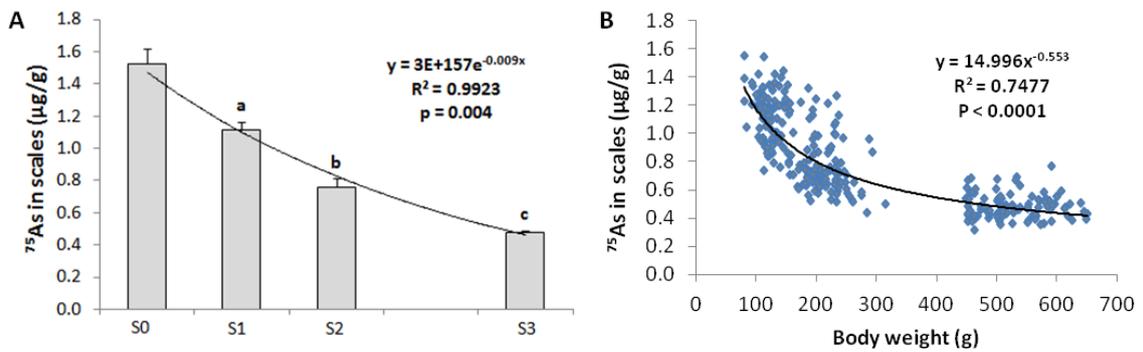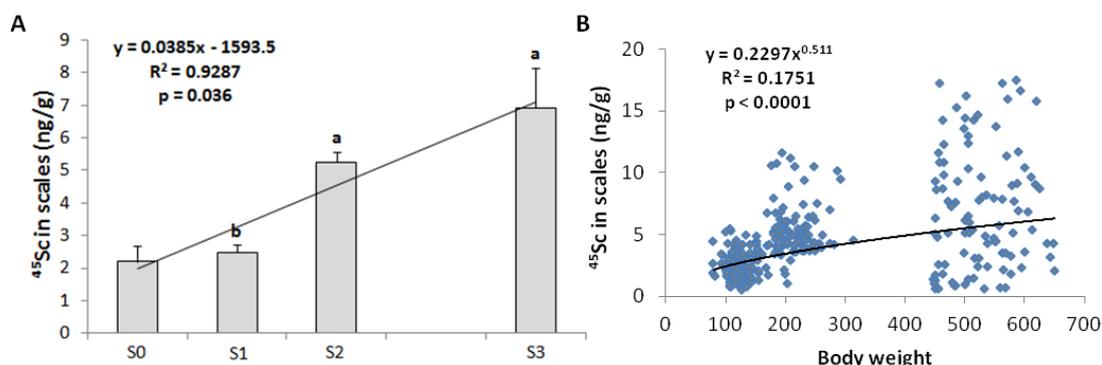

Fig. 34. Concentrations of ^{45}Sc in the fish scales within the different sampling dates (A) and the relationship between these concentrations and the fish body weight (B). The value for S0 in A is presented as the mean of individual fish within one tank while the rest of the values are presented as means of tanks. The variation between the individuals/tanks is given as the standard deviation and the standard error of the mean, respectively (SD S0 = 10, SEM S1,S2,S3: n = 10). Significant differences between sampling dates are indicated by different letters on the bars.

The concentration of these elemental isotopes in the fish scales was again plotted against the fish body weight in order to study the relationship between the two parameters (see B in Fig. 28 to Fig. 34). As expected, a negative correlation was observed between the concentrations of ^{165}Ho, ^{158}Gd, ^{147}Sm and ^{75}As in the scales and the fish body weight. This correlation was strongest for ^{147}Sm ($R^2 = 0.81$), followed by ^{75}As ($R^2 = 0.75$) and ^{165}Ho and ^{158}Gd ($R^2 = 0.58$ for both). In contrast, a positive correlation was found between the concentrations of ^{88}Sr, ^{238}U and ^{45}Sc in the scales and the fish body weight. This correlation was higher for ^{88}Sr ($R^2 = 0.64$) than for ^{238}U ($R^2 = 0.19$) and ^{45}Sc ($R^2 = 0.18$).

6. DISCUSSION:

Atlantic salmon 1^+ smolt were successfully labeled with dysprosium, neodymium, praseodymium, cerium and lanthanum by addition of these elements to the diet. The REEs were detected in the scales, which is consistent with previous findings (Ennevor 1991; Ennevor & Beames 1993; Ennevor 1994; Michibata 1981) and with the bone-seeking characteristic of these elements (Durbin et al. 1956; Jowsey et al. 1958). Ennevor (1991), Ennevor and Beames (1993) and Ennevor (1994) have shown that lanthanides were deposited in the scales, otoliths and vertebral column of coho salmon (*Oncorhynchus kisutch*) after adding the elements to the water supply and remained in these tissues for at least 10.5 months after labeling. Michibata (1981) also succeeded in marking medaka (*Oryzias latipes*) and goldfish (*Carassius auratus*) with dietary samarium (Sm), another REE, which was retained in the fish scales one year after marking.

The incorporation of all tested REEs (except for lanthanum) into the scales increased over feeding time (see S1 and S2 in Fig. 18 to Fig. 22). Muncy et al. (1988) experienced the same accumulation pattern when analyzing for Sm in striped bass (*Morone saxatilis*) scales 28 and 84 days after the start of a Sm enriched diet. On the other hand, the concentration of the REEs in the scales decreased dramatically within two months after marking (see S3 in Fig. 18 to Fig. 22). This is most probably due to the continual deposition of calcium in the scales diluting the original REE deposited. Because fish continuously accumulate calcium in their bony tissues after the labeling, the relative amount of the REEs will gradually decline as the fish grow (Yamada et al. 1979; Yamada & Mulligan 1982). This dilution effect was demonstrated in all the treatment groups. Retention of the REEs within the bony tissues as fish grow and the ability to detect the elemental mark in the scales of labeled adults are therefore a matter of concern if REEs are to be used as long-term markers. If the initial amount accumulated is not sufficiently large, the dilution effect could impede the identification of marked adult fish. A possible solution to this dilution problem would be to analyze only the zone of the scales formed during the labeling time, where the element concentration would be about the same as when the fish were marked (Yamada & Mulligan 1982). Such analysis could be achieved using Laser Ablation Inductively Coupled Plasma Mass Spectrometry (LA-ICP-MS), since this technique has the ability to sample specific

parts of individual scales (Farrell et al. 2000; Wells et al. 2000). In fact, LA-ICP-MS has been successfully used by Flem et al. (2005) to analyze the pre-smolt phase of scales from four Norwegian salmon stocks for differences in trace elemental concentrations, which allowed to distinguish between different fish stocks.

Despite the dilution effect, in the present experiment there were still significant differences in the concentration of all tracers (except for lanthanum) between the treated and untreated fish after the 2-month dilution period (see S3 in Fig. 18 to Fig. 22). In previous studies (Kato 1985; Michibata & Hori 1981) REE-marks laid down in fish scales were detectable up to 2 years after labeling. Besides, results from Michibata (1981) showed that although the amount of Sm in the scales decreased rapidly 30 days after marking, which is in line with our results, the level of Sm detected remained almost constant for the following year. These facts suggest that the difference between the REE concentration in the scales of marked and unmarked fish may be maintained over a long period of time and therefore marked adults could be possibly identified. However, a long-term monitoring study would need to be performed in order to be able to conclude about this.

As mentioned above, the La level in the scales of fish fed the La-diet did not increase over feeding time and there was not a significant difference in the concentration of La between this fish group and the untreated fish after the dilution period (see Fig. 22). This could be explained by the higher background levels of this element in the scales (44.0 ± 6.41 ng/g at S0) compared to the background levels of the other tested elements (0.8 ± 0.19 ng/g for Dy to 16.5 ± 2.33 ng/g for Nd at S0). The incorporation of the supplemented La may be masked by the high elemental background level and therefore it can be assumed that a longer-lived mark could possibly be induced by increasing the La concentration in the feed or by feeding the La-diet for a longer period. Considering the above, an important step in the experimental design would be to choose a marker with low background levels. In this case dysprosium, which had the lowest background levels, was shown to induce a very clear label and therefore appears to be a very suitable marker.

Surprisingly, the levels of the five REEs in the scales of the untreated fish did not remain constant throughout the experimental period, but also decreased steadily with time (see BK-groups in Fig. 18 to Fig. 22). In previous works where the ICP-MS technique was used (Ennevor 1991; Ennevor 1994), the untreated fish had undetectable

amounts of element present in the scales and therefore the elemental background levels could not be monitored. These were relatively old studies and recent developments in ICP-MS have led to improved detection limits (Rodushkin & Axelsson 2000), allowing for the monitoring of the background levels. The decrease in the background levels can also be explained by the fish growth. A negative correlation was observed between the five elemental background levels and the fish body weight (see Fig. 23 to Fig. 27). This correlation was especially strong for Nd, Pr and La. The negative correlation suggests that some uptake of the REEs might have taken place during the freshwater rearing phase and that the elemental background levels also experienced the already mentioned dilution effect once the fish were transferred to seawater. The incorporation of Ce, La and Sm into the scales following addition of these elements to the water supply has been previously demonstrated (Ennevor 1991; Ennevor & Beames 1993; Ennevor 1994). Since the elemental concentrations in the scales of both the treated and untreated fish decrease as fish grow it is likely that the background levels remain lower than those of the treated fish also in the long term, allowing the identification of marked adult fish.

Although the five tested tracers were clearly incorporated into the fish scales the mechanism of their uptake remains to be resolved. The chemical analysis of the feed and the faeces indicated that the markers were very poorly, if at all, absorbed from the gastrointestinal (GI) tract (see Table 8). Shibuya (1979) reported that salmon fed europium (another REE) retained the mark for 3 months. He stated that the Eu was deposited in the scales after being absorbed through the GI tract. However, in mammals, Ellis (1968), Luckey and Venugopal (1977) and Kennelly et al. (1980) demonstrated that REEs salts were negligible absorbed from the intestine, which is consistent with our results. In agreement with Michibata (1981), it is suggested that REEs deposited in the scales in the present experiment do not result from absorption in the GI tract but from direct absorption from the water, where the REEs are dissolved from the labeled feed or/and the excreted faeces. If true, the labeling of the scales may only be feasible in closed systems, where the dissolved elements could remain in high enough concentrations to be incorporated. In order to be able to conclude with this statement the labeling study should be reproduced in an open-cage system. A possible alternative is to test the citrate and ethylenediaminetetraacetic acid (EDTA) forms of the REEs, which appear to be more absorbed in the GI tract (Ennevor 1991). In spite of this, our findings still show that the procedure used in the present experiment can be used to label fish under these conditions.

Large individual variations in the elemental concentrations in the scales were noticed within the treated tanks, especially during the labeling period (see Fig. 8 to Fig. 12). This could represent a drawback for the labeling method since it may impede the identification of single marked fish. Nevertheless, large escape events (>10 000 individuals) have been reported to account for the majority of escaped fish (Jensen et al. 2010) and therefore identifying such events should be the greatest focus. Our results have demonstrated that this may be possible by marking the fish with dietary REEs.

A potential explanation for the large individual variations is a possible difference in individual feed intake and growth rate within the tanks. However, no strong correlation was observed between the concentrations of the tested elements in the scales and the fish relative weight increase (see Fig. 13 to Fig. 17). This is in line with the digestibility results for the tested tracers. Since there is poor or no absorption of these elements in the GI tract, a difference in individual feed intake would not result in large elemental variations in the scales. Therefore it can be assumed that this is not the sole reason explaining the individual variations. As mentioned before, Ennevor (1991) demonstrated that coho salmon exposed to La in the water supply accumulated this element in the bony tissues. The researcher reported high individual variations between scale samples compared with the otolith and vertebral column samples. The fact that the REEs tend to adhere to surfaces even at very low concentrations in aqueous solutions (Luckey & Venugopal 1977) could explain the high variation between scale samples since they are exposed surfaces, while the otoliths and vertebral column are internal structures. This is supported by the fact that the individual variation in all the elemental concentrations in the scales was reduced after the 2-month dilution period (see S3 in Fig. 8 to Fig. 12). It is likely that adhered REEs were rinsed from the scales during this period, thus reducing the possible variation caused by surface REE. In Ennevor (1991), as well as in the present experiment, fish were only wiped clean of mucus prior to scale scrapings. In similar studies where ICP-MS was used to investigate the trace element composition of fish scales (Adey et al. 2009; Muhlfeld et al. 2005; Wells et al. 2000), a further cleaning of scales was performed during the sample preparation. Adey et al. (2009) reported that the optimal scale cleaning method is a 3 to 5 min sonication in 3% ultrapure hydrogen peroxide followed by two rounds of cleaning with an electric rotary toothbrush and Milli-Q water. This method has been shown to reduce the variability in trace element concentrations in the scales (Wells et al. 2000). Scales are also often lost and regenerated, especially during times of physiological stress (e.g. during

smoltification) (Wells et al. 2000), and this can mislead regarding the elemental content of pooled scale samples. In previous works (Ennevor & Beames 1993; Yamada & Mulligan 1982) scales were first examined under a microscope and only the non-regenerated ones were chosen for analysis. However, these facts were not taken into consideration in the present experiment since it was designed in order to develop a simple and quick technique. A more reliable method for collecting and preparing scale samples would need to be used for more accurate results and for the potential identification of single marked fish.

A major concern with the chemical marking of farmed fish is the ability to induce a mark without affecting growth or survival. Fish were weighed throughout the experimental period. Significant differences in body weight and TGC were not observed in any of the sampling dates or periods between the fish groups fed the different experimental diets, indicating that the supplementation of the five tested REE chlorides to the feed had no different effect on the fish growth. Due to the lack of a negative control throughout the trial it is not possible to conclude that the supplemented elements did not influence the fish growth. However, the body weights reached at the final sampling and the TGCs during the experimental period are within the normal range according to recent growth studies of Atlantic salmon in closed-containment systems (Thorarensen & Farrell 2011). This suggests that it is unlikely that the tested tracers have any adverse effects on fish growth. Furthermore, feeding experiments conducted on rats (He et al. 2003a), poultry (Halle et al. 2002a) and pigs (He et al. 2001; Rambeck et al. 1999a) proved that REE-supplementation can enhance growth performance, although the same could not be reproduced for fish (Renard 2005; Tautenhahn 2004). In addition, no significant mortalities were recorded in any of the treated fish groups, indicating that the concentration of REE chlorides used for the labeling (250 mg/kg feed) was not toxic to the fish. This is in accordance with the low acute toxicity of REEs reported in animal experiments by Richter (2003), with a median lethal dose ($LD_{50)}$ of up to 10 g/kg body weight. The low oral toxicity of the REEs can also be deducted from the fact that its LD_{50} is the same as the one of table salt (NaCl) (Wald 1990).

Another important consideration is the safety of the chemical tracers in labeled fish destined for human consumption. However, the REEs contained in such products would not be harmful since they are not absorbed by the human gastrointestinal tract (Haley 1965; Luckey & Venugopal 1977). Additionally, long term medical studies performed

in man showed that the administration of up to 3 g of lanthanum per day and person for up to 4 years was well tolerated and did not cause any adverse or toxic effects (Harrison & Scott 2004; Hutchison et al. 2004).

The interesting and already discussed fact that the tested elemental background levels decreased over time made us want to further investigate the chemical fingerprint of the scales. Since the ICP-MS analysis allowed for the quantification of 14 more elements other than the tested ones, the monitoring of some of them was performed and illustrated in A in Fig. 28 to Fig. 34. The background levels of some of the elements (^{165}Ho, ^{158}Gd, ^{147}Sm and ^{75}As) showed the same decreasing pattern described for the background levels of the tested tracers (see A in 28 to Fig. 31, respectively). However, the levels of some other elements (^{88}Sr, ^{238}U and ^{45}Sc) had a completely opposite increasing tendency (see A in Fig. 32 to Fig. 34, respectively). This suggests that the latter elements were more present and therefore more incorporated into the scales in seawater than in fresh water since the experimental salmon smolt were transferred to seawater shortly before the start up of the experiment. In fact, strontium for instance has been reported to be 200 - 400 times more concentrated in salt water than in fresh water (Guillou & de la Noüe 1987). The opposite would apply for ^{165}Ho, ^{158}Gd, ^{147}Sm and ^{75}As, which may be, as well as the tested tracers, more abundant in fresh water. These findings are very interesting as they indicate that farmed salmon may get a "double" chemical fingerprint in the scales during its lifecycle and that this signature might be used as a natural tag for the identification of reared fish from specific farming sites. The elemental composition of fish biomineral structures such as bones, scales and otoliths is increasingly used to determine nursery-area residency (Thorrold et al. 1998), study migration routes and habitat utilization (Coutant 1990; Secor et al. 1995) and to discriminate among populations or stocks (Campana et al. 1995; Campana et al. 2000). However, only few studies have attempted to use scale chemistry (Adey et al. 2009; Flem et al. 2005) to differentiate between wild and farmed salmon or to discriminate amongst farmed populations. These studies utilize the relationship between the trace metal content of a bony tissue and that of the ambient water during biomineralization; thereby local differences in water chemistry are reflected in bony structures and hence potentially reflects locality of origin. Since tissues such as otoliths and scales grow concentrically, spatial resolved elemental analysis may provide a record of the location of the fish throughout its life history (Adey et al. 2009).

Veinott and Porter (2005) suggested that a database of otolith elemental fingerprints from different salmon farms may allow for the identification of the origin of escaped salmon from aquaculture sites. Similarly, it is suggested that a database of scale microchemistry signatures from both smolt and on-growing farms could be built for the same purpose. The use of scales offers some advantages over using otoliths (Adey et al. 2009; Flem et al. 2005; Wells et al. 2000): (1) scales are easier to collect than otoliths and therefore the sampling can be done by unskilled personnel, (2) they are removed non-lethally and hence allow for multiple sampling during a fish's life, (3) the scales require minimal preparation before analysis, which reduces the possibility of contamination compared to otoliths.

7. CONCLUDING REMARKS AND FUTURE PERSPECTIVES:

Atlantic salmon 1⁺ smolt scales were successfully labeled by supplementation of REEs to the diet for a short period of time, even though the mechanism of their uptake remains unclear. It is of importance to choose a marker with low natural background levels in order to be able to induce a clear label.

A long term monitoring of the REE levels in the scales after marking is required in order to further study the dilution of the induced labels and the natural background levels. Such study would allow us to investigate the possibility of identifying marked adult fish.

The reproduction of the current experiment in an open-cage system would be necessary in order to find out if the supplemented elemental markers are sufficiently taken up through the gastrointestinal tract and incorporated into the scales under these conditions and therefore get to know whether or not is possible to induce specific labels for different sea-sites.

Another interesting finding from the present study was that the natural elemental background levels themselves give very interesting information and in some instances may be enough to track the site of origin of escaped farmed salmon. More knowledge about the natural REE background levels in salmon scales is needed:

- What determines the elemental background levels?
- Would it be possible to create a database with the elemental fingerprints from the different farming sites?
- Could background levels be enough to track escaped salmon back to the site of origin?

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