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Evaluation of the maximum limits for selenium in Atlantic salmon feeds

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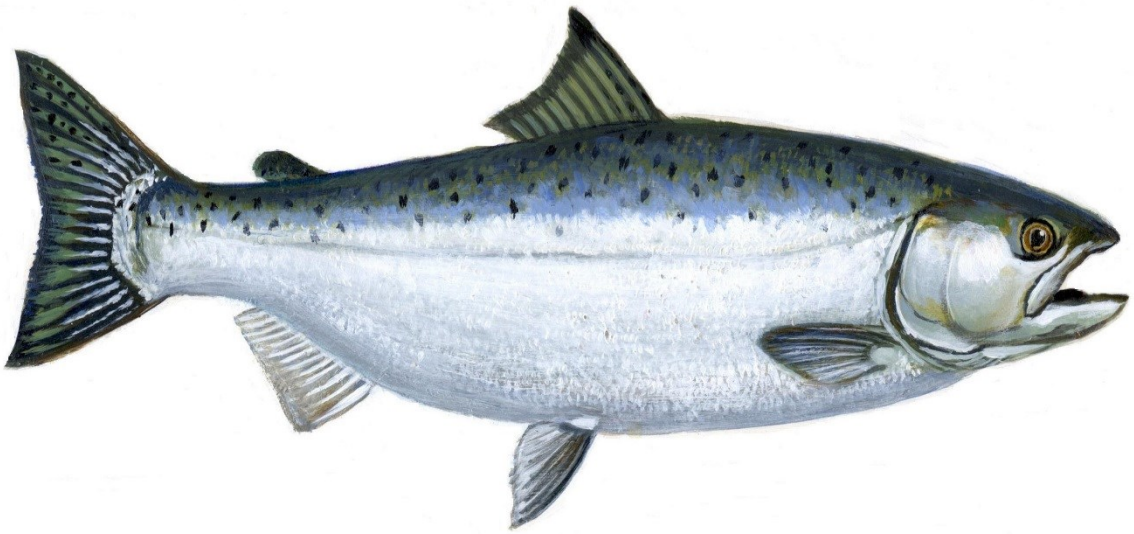

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Content

1 Introduction

The present report evaluated the safe limit of selenite and organic selenium (Se) (as Se-yeast or selenomethionine (SeMet) supplementation in feed to Atlantic salmon (Salmo salar). The study was financed by the Norwegian Seafood Research Fund (project no. 900871); Skretting AS, Biomar AS, Marine Harvest ASA and Cargill Aqua Nutrition AS.

The change from marine to plant feed ingredients will alter the nutritional composition of salmon feeds, reducing essential micro-nutrients such as Se that are naturally high in fish meal. Earlier studies have shown that Se concentrations in Atlantic salmon muscle were lower when fed on plant protein replacement feeds compared to marine protein feeds. Several earlier studies on fish nutrition have suggested Se supplementation to plant based feeds to restore or maintain Se levels in farmed fish as a Se source for consumers. In addition, Se supplementation to plant based feed for marine carnivorous fish has been suggested to be needed to cover animal nutrient requirement and ensure health robustness of the farmed fish. In the European Union (EU), feeds can be supplemented with approved organic Se (e.g. selenized yeasts) to a maximum authorised level of 0.2 mg/kg (Regulations (EU) No 427/2013; 445/2013; 121/2014; 847/2014 and 2015/489). The maximum limit for total Se in animal feeds including fish feed has been set at 0.5 mg/kg feed (EC 1831/2003 and amendments).

In addition to being an essential element, excess Se is known to be toxic at low concentrations and a narrow window exists between requirement and Se toxicity for most vertebrates, including farmed fish and humans consuming Se supplemented food producing animals.

The present report aims to assess safe levels of organic and inorganic Se supplementation to Atlantic salmon feeds with regards to fish welfare and food safety. Safe thresholds levels for fish welfare were assessed as defined by the European Food Safety Authority (EFSA) by the use of lower bound benchmark dose models (BMDL). Safe threshold levels for human consumption of Se supplemented foods were assessed by expanding earlier EFSA assessments for feed Se supplementation to food producing land animals (for meat, milk and dairy products, and eggs), with farmed salmon.

The report consists of four chapters. All chapters are presented as scientific papers, of which the first two are already published in peer-reviewed scientific papers. The last two chapters are in draft form and will be submitted.

-In the first chapter, analyses of Se speciation were reported. As the EU legislation differentiates between organic and inorganic Se and both the relative toxicity and feed-to-fillet transfer depends on the chemical form of Se, analytical methods identifying of Se species is needed.

-In the second chapter, the sensitivity of Atlantic salmon as well as central modes of toxic action for dietary selenite and SeMet (as Se-yeast) were assessed. EFSA has recently published a new guidance document in which the difference between adverse effect, biomarkers of exposure or effect, and mode of action (MOA) were defined (EFSA, 2017a). The chapter assessed the MOA, central biomarkers and adverse effects to be used in the next (third) chapter on safe threshold Se levels in feed with regards to fish welfare.

-In the third chapter, fish welfare safe threshold levels were assessed for selenite and SeMet supplementation to Atlantic salmon feeds. EFSA recently evaluated the methods to assess safe feed levels, and advised to use bench mark dose (BMD) models instead of none observed

adverse effect levels (NOAEL) to assess the safe threshold levels to excess supplementation (EFSA 2017b). This chapter assessed the safe limits of selenite and SeMet supplementation to plant based feed with regards to the welfare of Atlantic salmon by assessment of targeted biomarkers of Se toxicity pathways and general adverse effect parameters in a BMDL evaluation

- In the fourth chapter, a model was established to predict levels in fillet following dietary exposure. The model was based on long term (whole production cycle) feeding with given dietary Se levels and aquaculture production parameter such as feed intake and growth rate. This model was used to assess the impact of long-term dietary selenite and SeMet levels on final fillet Se levels. Safe threshold levels for human consumption of Se supplemented foods were assessed by expanding earlier EFSA assessments for feed Se supplementation to food producing land animals (for meat, milk and dairy products, and eggs), with farmed salmon.

2 Summary

Speciation analysis for Se in salmon feed and muscle were developed using anion-exchange HPLC-ICP-MS for analysis of inorganic Se species and cation-exchange HPLC-ICP-MS for analysis of organic Se species. In addition, a reversed phase HPLC-ICP-MS method was applied for the analysis of SeCys in selected samples. Low background high plant feed were supplemented with selenite or Se- yeast and fed to Atlantic salmon for 3 months. Selenomethionine was the major Se species in the non-supplemented basal diets and in diets supplemented with Se-yeast, whereas selenite was the major Se species in feeds supplemented with inorganic Se. In muscle of Atlantic salmon fed non-supplemented feeds and feeds supplemented with organic Se, SeMet was the major Se species. In salmon fed inorganic diets, SeMet accounted for a lower proportion of the total Se (maximum 30%), and several unidentified Se-containing peaks were detected. Overall, the results show that the methods can be used for assessing the Se supplementation sources in the feeds, and furthermore for determining the Se species retained in tissues of salmon.

To investigate sensitivity and overall metabolic toxic MOA and establish biomarkers of toxic Se exposure and effect, a wide scope metabolomics pathway profiling was performed on Atlantic salmon (572 g) that were fed selenite or Se-yeast fortified diets for 3 months. Apparent toxic MOA high Se levels (15 mg kg^{-1}) included oxidative stress and altered lipid metabolism for both inorganic and organic Se, with higher toxicity for inorganic Se. Dietary Se levels of respectively 1.1 and 2.1 mg kg^{-1} selenite and Se-yeast did not affect any of the above mentioned parameters.

To assess safe levels of selenite and SeMet supplementation with regards to fish welfare, Atlantic salmon (147 g) were fed a low natural background Se diet fortified with five graded levels of selenite or SeMet at five levels for 3 months. Fish fed high selenite levels showed mortality, whereas fish fed SeMet showed no mortality. Excess sublethal Se supplementation was assessed by targeted biomarkers of Se toxicity (e.g. markers of oxidative stress and lipid metabolism), as well as general adverse effect parameters (plasma biochemistry, hematology, liver histopathology, and growth). Safe limit were set by model-fitting the dose-response effect data in a BMDL evaluation. With regards to fish welfare, Atlantic salmon seemed to tolerate the supplementation of selenite or SeMet to a level of total Se of respectively 1-2 or 3 mg kg^{-1} feed (daily dose ~0.01-0.02, or ~0.03 mg kg body $weight^{-1}$ day^{-1}), in a high plant-based salmon feed with background levels of 0.45 mg Se kg^{-1}.

To assess the transfer of dietary selenite and SeMet to the fillet of farmed Atlantic salmon, the uptake and elimination rate constants of the two Se forms were determined from the second feeding trial in which feeding salmon with either selenite or SeMet enriched diets for 3 months was followed by a 3 month depuration period. The elimination and uptake rates were used in simple one-compartmental kinetic model to predict levels in fillet based on long term (whole production cycle) feeding with given dietary Se levels and aquaculture production parameter such as feed intake and growth rate. Model predictions for salmon fed low Se plant based feeds supplemented with either 0.2 mg kg^{-1} selenite of SeMet gave predicted fillet concentrations of 0.042 and 0.058 mg kg^{-1} ww, respectively. Based on these predictions and earlier EFSA risk assessment of Se supplementation to food producing land animals, the supplementation with 0.2 mg kg^{-1} selenite would likely protect food safety for the most sensitive consumer group (toddlers). However, supplementing feed to farmed animals, including salmon, with 0.2 mg kg^{-1} SeMet would give a Se intake that is higher (13%) than the upper limit (UL) set for toddlers.

3 Se speciation

Selenium and selenium species in feeds and muscle tissue of Atlantic salmon

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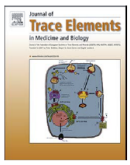

Selenium and selenium species in feeds and muscle tissue of Atlantic salmon

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Abstract
Selenium (Se) is an essential element for animals, including fish. Due to changes in feed composition for Atlantic salmon (*Salmo salar*), it may be necessary to supplement feeds with Se. In the present work, the transfer of Se and Se species from feed to muscle of Atlantic salmon fed Se supplemented diets was studied. Salmon were fed basal fish feed (0.35 mg Se/kg and 0.89 mg Se/kg feed), or feed supplemented either with selenised yeast or sodium selenite, at low (1 - 2 mg Se/kg feed) and high (15 mg Se/kg feed) levels, for 12 weeks. For the extraction of Se species from fish muscle, enzymatic cleavage with protease type XIV was applied. The extraction methods for Se species from fish feed were optimised, and two separate extraction procedures were applied, 1) enzymatic cleavage for organic Se supplemented feeds and 2) weak alkaline solvent for inorganic Se supplemented feeds, respectively. For selenium speciation analysis in feed and muscle tissue anion-exchange HPLC-ICP-MS for analysis of inorganic Se species and cation-exchange HPLC-ICP-MS for analysis of organic Se species, were applied. In addition, reversed phase HPLC-ICP-MS was applied for analysis of selenocysteine (SeCys) in selected muscle samples. The results demonstrated that supplemented Se (organic and inorganic) accumulated in muscle of Atlantic salmon, and a higher retention of Se was seen in the muscle of salmon fed organic Se diets. Selenomethionine (SeMet) was the major Se species in salmon fed basal diets and diets supplemented with organic Se, accounting for 91-118% of the total Se. In contrast, for muscle of salmon fed high inorganic Se diet, SeMet accounted for 30% of the total Se peaks detected. Several unidentified Se peaks were detected, in the fish fed high inorganic diet, and analysis showed indicated SeCys is a minor Se species present in this fish muscle tissue.

Keywords:

Selenium, supplementation, speciation analysis, feed legislation, fish,

Introduction

Selenium (Se) occurs naturally in the terrestrial and marine environment. Marine fish, e.g. Atlantic cod (*Gadus morhua*), Greenland halibut (*Reinhardtius hippoglossoides*) and Atlantic herring (*Clupea harengus*) generally contain high levels (0.25 to 1.4 mg/kg ww) of total Se [1]. Selenium levels found in fish feed can be ascribed to high levels of Se in marine feed ingredients, mainly fish meal. Today's feed (in Norway) for Atlantic salmon (*Salmo salar*) contains an average of 0.6 mg Se/kg feed (ranging from 0.3 to 1.1 mg Se/kg, n = 50) [2], and during the past 15 years a trend towards lower levels of Se in Atlantic salmon feed has been reported [3] and ascribed to the increased replacement of fish meal with plant ingredients. Atlantic salmon and rainbow trout (*Oncorhynchus mykiss*) fed plant based diets have been shown to contain lower muscle or whole body Se concentrations compared to fish fed marine based diets [4-6]. Reduced levels of Se in fish due to lower levels of Se in feed emphasise that it may be necessary to supplement Se to the feeds to meet the physiological requirement of the farmed fish.

In Europe, the use of feed additives is regulated through the European feed legislation. The current maximum limit for total Se in animal feeds including fish feed has been set at 0.5 mg/kg feed (EC 1831/2003 and amendments). Recently, the European Food Safety Authority (EFSA) has issued several scientific opinions on the use of organic selenium-yeast forms as feed additives and based on the apparent higher bioavailability of organic Se compared to inorganic Se concluded that the supplementation level should be limited to a maximum of 0.2 mg/kg feed to ensure consumer safety [7-10]. Subsequently, the European Union recently regulated the use of several Se feed additives, mainly selenised yeasts, at a supplementation level of maximum 0.2 mg Se/kg feed [11-15]. The legislative differentiation between total Se and supplemented organic Se forms warrants the establishment of suitable Se speciation techniques. Furthermore, such methods will help generating data on the occurrence of Se species in feed and fish, which is important for future risk assessments of Se feed additives.

Selenium species have previously been determined in fish and mussels using ion-pair reversed-phase high pressure liquid chromatography (HPLC) or anion-exchange HPLC coupled to inductively coupled plasma mass spectrometry (ICP-MS) after enzymatic digestion [1, 16, 17]. Fish feed is a complex matrix consisting of different sources of lipids and proteins and feed additives, which may cause challenges in the analysis of Se species, e.g. during extraction, chromatographic separation and species identification. In a recent study, Godin and colleagues [18] applied an extensive approach for the determination of Se species in feed and in whole body of rainbow trout fry. Inorganic Se species were determined in feed using anion-exchange HPLC-ICP-MS following extraction with sodium hydroxide, while selenomethionine (SeMet) was determined in feed and fish using anion-exchange HPLC-ICP-MS following enzymatic extraction [18]. For the determination of selenocysteine (SeCys), a derivatisation step was applied prior to enzymatic extraction to stabilise this Se species for analysis using reversed-phase HPLC-ICP-MS [18].

In fish, supplemented organic Se forms appear to have a higher bioavailability and tissue accumulation compared to supplemented inorganic Se forms [6, 19]. The upper limit (0.2 mg/kg feed) for supplementation with selenised yeast (organic Se forms) is based on risk assessments of consumer safety [7-10]. In such assessments, documentation of the feed-to-food transfer in long term (> 3 months) feeding trials is essential. However, the assessments were mainly based on experimental data from trials with terrestrial farmed animals and to a lesser degree for farmed fish. Only a few studies on the speciation on farmed marine fish when subjected to different dietary sources of Se in the feeds exist. Godin and colleagues found through speciation analysis that trout fry fed diets supplemented with selenised yeast

contained higher SeMet concentrations compared to fry given diets supplemented with selenite [18]. In contrast, the SeCys concentration in the fry was not affected by the supplementation source of the diets. Furthermore, speciation analysis of whole fry showed that 24 - 38% of the total Se was not identified [18]. In order to get a better scientific basis for the risk assessment of the use of different Se feed supplements in fish feed, studies need to address the species-related transfer and accumulation in the chain from fish feed to the final aquaculture product.

In the present work, the aim was to study inorganic and organic Se species in fish feed and fish muscle of farmed Atlantic salmon fed diets supplemented with inorganic Se or organic Se at two different inclusion levels and hence, contribute to a better understanding of the species-related transfer of Se in the aquaculture feed chain. Methods for the determination of Se species in feeds and in muscle of Atlantic salmon were established in order to reach this goal, with a focus on optimizing the extraction of Se species.

Materials and methods

Chemicals and reagents

All chemicals used were analytical grade quality or better. Milli-Q water (18.2 MΩcm, EMD Millipore Corporation, MA, USA) was used for sample preparation and analysis. Seleno-DL-methionine (SeMet, ≥ 99% purity), sodium selenate (Se(VI), ≥ 98% purity), sodium selenite (Se(IV), 99% purity), seleno-DL-cystine (SeCys2, ≥ 98%), protease type XIV from *Streptomyces griseus,* lipase from *Candida rugosa*, ammonium phosphate dibasic, ammonium acetate, tris(hydroxymethyl)aminomethane, urea, dithiothreitol, iodoacetamide, heptafluorobutyric acid (HFBA), phosphoric acid and sodium hydroxide were all obtained from Sigma Aldrich (Oslo, Norway). Methanol (HPLC grade), pyridine, hydrochloric acid, ammonia solution), hydrogen peroxide (H_2O_2, Emsure ACS, ISO, 32% w/w) were obtained from Merck (Darmstadt, Germany). Se-methyl-seleno-L-cysteine (SeMetSeCys ≥ 98%) and nitric acid (HNO_3, Trace select, ≥69.0% w/w) were obtained from Fluka (Oslo, Norway). A multi element standard, mercury (Hg), gold (Au), germanium (Ge), rhodium (Rh) and thulium (Tm) standards were obtained from Spectrascan (Teknolab, Ski, Norway). A tuning solution of lithium (Li), yttrium (Y), cesium (Ce) and thallium (Tl) (ICP-MS stock solution, tuning solution B) was obtained from Thermo Fisher Scientific Inc (Waltham, MA, USA). Selenium enriched yeast certified reference material (Selm-1) and TORT-3 (Lobster hepatopancreas) were both obtained from the National Research Council Canada, NRC, Ontario, Canada. Oyster Tissue (SRM 1566 b) was obtained from National Institute of Standards and Technology, Gaithersburg, MD, USA) and fish muscle tissue (ERM-BB422) from Institute for Reference Materials and Measurements, IRMM, Geel, Belgium.

Samples

All samples were obtained from a feeding trial where Atlantic salmon were fed diets supplemented with Se. The overall aim of the feeding trial was to establish biomarkers of early toxicity [20], while this work describes the speciation and the species-related transfer of Se in feed and muscle of the fish. In short, inorganic Se (selenite; DMS, Heerlen, The Netherlands) or organic Se (selenised yeast containing SeMet; AlkoSel®, Lallemand, Malvern Link, England) were added to a basal diet at two nominal levels; 1.4 and 13 mg/kg feed, respectively, resulting in feeds with either low or high Se concentration (Table 1). The basal low diet had a low inclusion of fish meal (8%) and krill meal (2%), and a low Se level. In addition, the experimental design included a basal diet with a higher inclusion of fish meal (15%) and krill meal (7%). Plant-based protein sources were included in both diets, and the protein level of

the diets were balance to similar levels. The measured Se concentrations in the diets are shown in Table 1. The diets were produced by BioMar AS (Trondheim, Norway). Subsamples were shipped to NIFES, homogenised and stored in darkness at 4 °C until further analysis. The feeding trial was conducted at the facilities of EWOS Innovation, Dirdal, Norway. Atlantic salmon were fed the six diets in triplicate tanks (30 fish per tank) for 12 weeks. Muscle samples were taken using a standardised cut; two cm wide cut behind the dorsal fin on the left lateral side of the fish. Skin and bones were removed from the muscle. The samples were stored on ice during sampling and transportation to the laboratory. The samples were then stored at -20°C. The samples were later thawed, homogenised, freeze-dried, and ground and homogenised before being stored at room temperature prior to analysis.

Table 1 Selenium concentrations in diets (mg/kg dw, mean ± SD, n = 3), in muscle tissue (mg/kg ww, mean ± SD, n = 9), and the calculated retention (%, n = 3) of Se in the muscle tissue of Atlantic salmon fed the respective diets. Data points with no letter in common are significantly different ($p < 0.05$).

Description of diets	Nominal Se concentration (mg/kg dw)	Se concentration in diets (mg/kg dw)	Se concentration in muscle tissue (mg/kg ww)	Retention (%)
Basal low	0	0.35 ± 0.02	0.12 ± 0.01[a]	15 ± 1[a]
Inorganic Se low	1.4	1.10 ± 0.03	0.14 ± 0.01[a]	14 ± 3[a]
Inorganic Se high	13	15.0 ± 0.5	0.46 ± 0.07[b]	3.7 ± 0.2
Organic Se low	1.4	2.10 ± 0.05	0.43 ± 0.04[b]	21 ± 1[b]
Organic Se high	13	15.0 ± 0.2	3.4 ± 0.2	24 ± 1[b]
Basal high	0	0.89 ± 0.03	0.22 ± 0.03[a]	70 ± 4

Determination of total selenium

Diets and freeze-dried muscle tissue were digested using the microwave-acid decomposition method described by Julshamn *et al.* [21]. Samples were accurately weighed (0.2 g) in (PTFE) vessels and concentrated HNO_3 (2 mL) and H_2O_2 (0.5 mL) were added. The samples were digested in a microwave system (Ethos Pro Milestone, Sorisole, Italy) using the following temperature programme: 1 min at 250 W, 1 min at 0 W, 5 min at 250 W, 5 min at 400 W and 5 min at 650 W. The digests were diluted to a final volume of 25 mL with Milli-Q water.

For determination of total Se concentration in extracts of fish muscle, diets and reference materials the extracts were diluted 25 or 50 times, depending on Se concentration of the sample, with Milli-Q water and 5% HNO_3, centrifuged at 2.5 g for 5 min, and filtered with 0.45µm, 25 mm membrane filters with syringe (Merck Millipore).

The sample digests and sample extracts were analysed with an iCAP-Q ICP-MS using collision cell and FAST SC-4Q DX autosampler (both Thermo Fisher Scientific Inc., Waltham, MA USA). An external calibration curve was prepared from freshly prepared multi element solutions,

which included Se, diluted to appropriate concentrations by 5% (v/v) HNO_3 and used for the quantification of Se. The instrument was set in He KED (kinetic energy discrimination) mode for interference removal. A solution of internal standard comprising of Ge, Rh and Tm was added on-line for correction of instrumental drift during the analysis. The instrument was tuned daily using a tuning solution (1 ppb tuning solution, Thermo Fisher, in 2% HNO_3 and 0.5% HCl) prior to analysis. Plasma power was set to 1550 W, nebulizer gas flow to 1.05 L/min, the auxilliary gas flow to 0.8 L/min, and the He gas (CCT1) flow was 4.6 mL/min. Nickel interface cones were used, and a microflow PFA-ST nebulizer (Thermo Fisher Scientific Inc.) was used. Isotope ^{78}Se was monitored, and the integration time was 0.1 sec.

The results obtained for total Se (average ± SD) 10.6 ± 0.6 mg/kg; *n* = 8 (TORT-3) and 2.1 ± 0.1 mg/kg; *n* = 8 (Oyster Tissue) agreed well with the certified reference values of 10.9 ± 1.0 mg/kg (TORT-3) and 2.06 ± 0.15 mg/kg (Oyster Tissue), respectively. Selected sample extracts were spiked with known amounts of Se using the standard mixture (Agilent Technology), and the mean recovery (± RSD) of the spiked Se was 103 ± 8 %; *n* = 12.

Determination of selenium species

Sample preparation

Selenium species were extracted by enzymatic hydrolysis using a non-specific protease (Protease type XIV), by modifying the extraction procedure described elsewhere [1]. Diets and freeze-dried muscle tissues were accurately weighed (0.2 g) in polypropylene tubes and suspended in 2.5 mL of 1 mM ammonium phosphate buffer, pH 7, containing 20 mg protease, and mixed vigorously for 1-2 min. The solutions were left in a water bath (OLS200 Grant Instruments, Cambridge, UK), in the dark and at 37 °C, shaking at 100 rpm/min for 24 h. The samples were then cooled to room temperature, before centrifugation (2.5 g for 10 min) followed by filtration using 0.45µm, 25 mm syringe filters (Merck Millipore). Centrifugal filters (Amicon Ultra-0.5 mL 10 kDa, Merck Life Science AS, Oslo, Norway) were used to separate by centrifugation (14,000 g for 20 min) the high molecular weight compounds from the low molecular weight Se species (e.g. SeMet with molecular weight of 196.106 g/mol), as an extra clean-up procedure prior to the chromatographic separation. Based on total Se analysis of extracts analysed pre and post filtration (both 45µm and Amicon filters), the recovery of Se in filtrated extract was 102 ± 16% (n = 6, data not shown). Also, non-filtrated and filtrated samples were compared by chromatographic analysis, and no differences were observed in the signals or in the chromatographic profiles (data not shown). The phosphate buffer was treated in the same way as the samples and used as a procedural blank sample.

The extraction yield for Se in the diets was further studied using different extraction solvents, both waters-soluble solvents and a combination of enzymatic digests, on the basal high diet. The diet was accurately weighed (0.2 g) in polypropylene tubes and suspended in 2.5 mL of 1 mM ammonium phosphate buffer, pH 7, and in 2.5 mL 10% methanol in Milli-Q water to extract the water-soluble and polar Se species. For enzymatic digestion, the weighted sample (0.2 g) was extracted by 10 mg, 20 mg and 30 mg protease type XIV in 2.5 mL of the phosphate buffer. For studying the effect of lipase, the diet was first extracted by 20 mg protease (24h in water bath), following addition of 10 mg lipase (in 2.5 mL phosphate buffer) to the same sample. The sample was left on water bath for additional 24h. Total Se was determined in the final extract by ICP-MS.

Diets were additionally subjected to a sequential proteolytic extraction procedure, where samples were first extracted by protease following the description above, then after

centrifugation (2.5 g, 10 min) the soluble extract of the samples were transferred to new tubes using Pasteur pipettes. A fresh batch of protease (20 mg) in phosphate buffer was then added to the insoluble sample residue, shaken vigorously, and left for additional 24h in water bath. This procedure was repeated twice, and the soluble extracts were determined for total Se by ICP-MS.

The inorganic Se supplemented diets were also extracted using 0.1 M NaOH in MilliQ water for the specific extraction of the inorganic Se species. The diets were accurately weighed (0.2 g) in polypropylene tubes and suspended in 5 mL 0.1 M NaOH and mixed on a rotator (model LD79, Labinco, Breda, The Netherlands) for 12 h. The samples were then centrifuged (2.5 g for 10 min), diluted with MilliQ-water, and filtrated using 0.45µm, 25mm membrane filters with syringe (Merck Millipore).

Selected muscle samples were extracted, derivatised and analysed for SeCys using the protocol described by Bierla et al [16], including the procedure for sample defatting, protein denaturation and derivatised by carbamidomethylation. Freeze-dried muscle (0.1 g) was extracted with 2 mL of 7 M urea solution in 0.1 M Tris-HCl buffer, pH 7.5, and sonicated for 20 min. The solution was then added 30 µL 0.2 M dithiothreitol (DDT) and 40 µL 0.5 M iodoacetamide (both in 0.1 M Tris-HCl buffer, pH 7.5) and incubated for 2 h at 25 °C in the dark. Fresh DDT (375 µL) was added to the mixture, and the samples were shaken for 1 h. The samples were then diluted with 12 mL of 0.1 M Tris-HCl buffer, and a 500 µL aliquot of protease/lipase solution containing 20 mg protease type XIV was added. The samples were incubated in water bath for 20 h at 37 °C. The samples were centrifuged (2.5 g, 10 min), filtered by 0.45 µm, 25 mm syringe filters (Merck Millipore) and freeze-dried.

Standards of CAM-SeCys and CAM-SeMet were prepared by a method described by Jagtap et al [22], adapted from Dernovics and Lobinski [23]. Standards with concentration of 100 mg Se/L in 4 mL of Tris-HCl (0.05 M, pH 8.6) was heated to 37 °C and purged with Ar(g) for 1-2 min, before 100 mg DDT and 224 mg iodoacetamide (both in 4 mL of 0.05 M Tris-HCl, pH 8.6) were added. The standards were incubated in the dark at 37 C for 1 h. The reaction was stopped by addition of 180 mg of DDT dissolved in 4 mL the Tris-HCl buffer, and diluted with Milli-Q water to a final volume of 20 mL. The standards were further diluted with water prior to analysis with reversed-phase HPLC-ICP-MS.

Analysis of selenium species using ion-exchange chromatography

Selenium species were analysed by HPLC (1260 HPLC system) coupled with an ICP-MS 7500cx (both Agilent Technologies, Wilmington, Delaware, USA). An octopole reaction cell was applied to the ICP-MS for Se speciation analysis, and the cell conditions were optimised for the reduction of argon-based polyatomic interferences using H_2 as the reaction gas. The H_2 flow rate was set to 4 mL/min. A Babington nebulizer was used to insert the sample, and the ICP-MS was tuned prior to analysis using a tuning solution, comprising lithium, yttrium, cesium and thallium. Nickel interface cones were used. Isotopes ^{78}Se, ^{80}Se and ^{82}Se were monitored, and the integration time was 0.1 sec. The HPLC was equipped with a quaternary HPLC pump, an autosampler, a vacuum degasser system and a temperature column compartment. The HPLC column outlet and the sample insertion system of the ICP-MS were coupled with 0.125 mm (i.d.) PEEK tubing. The instrumental settings are given in Supplementary data, S-Table 1.

Two ion-exchange chromatographic methods based on previous reports [17, 24-26] were applied for the Se speciation analyses. The chromatographic conditions were optimised by adjusting the buffer, pH, HPLC column and the elution program. Since the chromatographic

resolution of Se species in extracts from food samples is typically difficult to achieve [27, 28], it was not possible to obtain baseline separation of all Se species present in a sample using a single chromatographic principle. Therefore, quantitative determination of Se species was achieved by combining anion-exchange and cation-exchange HPLC-ICP-MS analysis.

The Se species identification in the samples was based on the comparison with the retention times of Se species standards. For Se speciation analysis, a strong cation-exchange column (Ionospher 5C, 150 x 3 mm, 5µm; Agilent Technologies) was used for the analysis of organic Se species, and a strong anion-exchange column (Hamilton PRP-X100, 150 x 4.6 mm, 5µm, VWR, Radnor, PA, USA) was used for the analysis of inorganic Se species. The mobile phases and chromatographic conditions are given in S-Table 1. As differences were observed in the separation efficiency between muscle and diets when analysed by cation-exchange HPLC-ICP-MS, two different gradient elution programs were applied (S-Table 1). The chromatographic peaks were quantified by external calibration. The retention times for Se species were affected by the sample matrix when analysed by cation-exchange HPLC-ICP-MS. For qualitative assignment of the molecular identity of the Se peaks, sample extracts were spiked with Se standards. The limits of detection (LOD) were calculated as three times the baseline noise of a blank sample, and were 0.007 mg/kg for Se(IV) and 0.005 mg/kg for Se(VI) by anion-exchange HPLC-ICP-MS, and 0.01 mg/kg for SeMetSeCys, 0.03 mg/kg for SeMet and 0.02 mg/kg for SeCys2 by cation-exchange HPLC-ICP-MS.

Certified reference materials for speciation analysis of Se in marine samples are not currently available. The reproducibility of analysis was assessed by analysing the reference materials (Selm-1 and ERM-BB442) in all analytical runs. SELM-1 is certified for SeMet and was therefore used for evaluating the accuracy of SeMet determinations.

Analysis of SeCys by SEC-ICP-MS and reversed-phase HPLC-ICP-MS

Chromatographic analysis for SeCys was conducted using the protocol described by Bierla et al [16]. Derivatised samples were injected onto a Size exclusion Chromatographic (SEC) column (TSKgel G3000 SWXL, 7.8 x 300 mm, 5 µm) with guard column (6 x 40 mm, 7 µm, both Tosoh Bioscence, Griesheim, Germany). The SEC column was calibrated for the low molecular weight compounds by SeMet standard, and by analysis of sample extracts prior to the fractionation. A Fraction Collector (Analytical Scale, G1364C to 1260 HPLC, Agilent Technologies) were applied for collection the selenoamino acid fractions from replicate injections (n = 3) of same sample was then pooled, freeze-dried, and suspended in Milli-Q water (270 µL).

The selenoamino fractions were further analysed by ion-pair reagent chromatography using 0.1% HFBA as the ion-pair reagent on a reversed-phase column XBridge C18 (4.6 x 250 mm, 5µm) with guard column (4.6 x 20 mm, 5 µm, Waters, Dublin, Ireland). The presence of SeCys in the sample extracts were determined based on overlap in retention times with CAM-SeCys standard, verified by spike experiments. The mobile phases and chromatographic conditions for the SEC separation and the reversed-phase chromatography are given in S-Table 1.

Data analyses and statistics

The extraction yield (%) was calculated using the following equation;

$$Extraction\ yield\ (\%) = \frac{Total\ Se\ (ng)\ in\ soluble\ extract}{Total\ Se\ (ng)\ in\ sample} x100$$

Retention of Se in muscle of Atlantic salmon (%) was calculated using the following general equation for retention (%);

$$Retention\ (\%) = \left(\frac{(Cmuscle, end\ \times\ biomass\ muscle, end)\_(Cmuscle, start\ \times\ biomass\ muscle, start)}{(Cdiet\ \times\ Feed\ intake)}\right) \times\ 100$$

where C_{muscle} and C_{diet} are the Se concentration in muscle and diet (mg/kg), respectively. The muscle concentration at start was set to 0 mg/kg. The feed intake is the total amount of feed intake for each tank and was adjusted for excess feed. The muscle biomass was estimated based on the weight of whole fish, assuming that 66% of the weight of the whole fish is muscle [29].

The presented data were evaluated statistically using Dell Statistica® (Dell Inc. (2016). Dell Statistica, version 13. software.dell.com). All datasets were tested for normality and homogeneity using Levene's test. Data sets for Se concentrations in muscle were analysed by nested analysis of variance (ANOVA) followed by a Tukey's honest significant difference (HSD) test to identify any significant differences at different time points. To detect significant differences among treatment groups, data set of retention was analysed using one-way ANOVA with post hoc Tukey's HSD test with a level of $p < 0.05$ considered significant. All data are given as mean ± standard deviation.

Results and discussion

Extraction of selenium in feeds and muscle

When using enzymatic cleavage with protease for the extraction of Se, relative high extraction yields were obtained for Se in the muscle samples, ranging from 90 to 110% (Table 2). The extraction yield for Se in the diets were, however, lower and showed a large variation (37 - 114%) compared to the muscle samples. As the extraction efficiency of Se is critically dependent on i.e. the sample matrix, type of extraction solvent and the ratio of enzyme to sample [26, 30, 31], different combinations and levels of protease was applied to the basal high diet to potentially increase the extraction efficiency for Se in the diets. From the use of phosphate buffer for the extraction, it was seen that only minor amounts (5%) of Se was extractable (data not shown). Also, when using aqueous methanol, a polar solvent, the extraction efficiency for Se did not increase, indicating that only minor amounts of Se is water-soluble These results are consistent with previously studies on Se speciation in fish tissue, where only a minor amount of Se (3 - 5%) was reported as being water-soluble [32]. Proteolytic enzymes are extensively used for the extraction of selenoamino acids from tissues, e.g. meat [16] and fish muscle [1, 22] and, therefore, proteolytic enzymes were applied for the extraction of Se. Different levels of protease were added to the diet (10 mg, 20 mg and 30 mg) to study the effect of amount of enzymes. From the total Se measurements of the digests it was seen that 10 mg protease gave an extraction yield of 46 ± 4% (n = 3), 20 mg protease gave 48 ± 6%

(n = 3) and 30 mg protease gave 48 ± 6% (n = 3). Hence, an increased extraction yield for Se was not observed, and 20 mg protease was used for the extraction of samples. As fish feed is a high lipid matrix, typically constituting 11-35% of oil [33, 34], lipase enzymes should potentially increase the release of Se from lipid matrix components. However, the extraction recovery when using lipase in combination with protease was 43 ± 14% (n = 3), and compared to the extraction recovery when using protease alone; 48 ± 6% (n = 3), there were no increase in the extraction yield. Consequently lipase was not added to the extraction solvent when extracting Se from the diets. These results clearly indicate that most of Se in the diet is protein-bound.

Table 2 Selenium concentrations (mg/kg ww, mean ± SD, n = 3) in sample and extracts, extraction yield for Se (%) in the protease extracts and alkaline extracts, the calculated column recovery (%), Selenomethionine (SeMet) and Se(IV) concentration (mg/kg ww, mean SD, n = 3) and the abundance (%) of SeMet and Se(IV) compared to total Se in samples.

Sample type	Sample	Total Se (mg/kg ww)	Total Se in extracts (mg/kg ww)	Extraction yield (%)	Column recovery (%)[a]	SeMet concentration (mg/kg ww)	SeMet abundance (%)	Se(IV) concentration (mg/kg ww)	Se(IV) abundance (%)
Certified reference material	ERM-BB422	1.3 ± 0.1	1.37 ± 0.06[b]	103 ± 4	75	1.4 ± 0.1	107		
	Selm-1	2031 ± 70	1802 ± 48[c]	89 ± 2	117	1271 ± 64	63		
Diets	Basal low	0.35 ± 0.02	0.4 ± 0.1[c]	114 ± 29	33	0.09 ± 0.01	26		
	Inorganic Se low	1.10 ± 0.03	0.52 ± 0.02	47 ± 2	17	0.06 ± 0.02	5		
	Inorganic Se low – alkaline extract		0.9 ± 0.2[c]	82 ± 18[c]				0.23 ± 0.02	21
	Inorganic Se high	15.0 ± 0.5	6.0 ± 1.0	37 ± 8	45	0.12 ± 0.03	0.8		
	Inorganic Se high – alkaline extract		14 ± 4[c]	92 ± 28[c]				13.0 ± 0.03	87
	Organic Se low	2.10 ± 0.05	1.6 ± 0.1	76 ± 5	43	0.59 ± 0.02	28		

	Organic Se high	15.0 ± 0.2	13.2 ± 0.4	88 ± 3	51	6.1 ± 0.2	41
	Basal high	0.89 ± 0.03	0.57 ± 0.03	64 ± 3	49	0.19 ± 0.02	21
Muscle tissue	Basal low	0.12 ± 0.01			116	0.11 ± 0.01	92
	Inorganic Se low	0.14 ± 0.01			121	0.12 ± 0.02	86
	Inorganic Se high	0.46 ± 0.07	0.45 ± 0.02	90 ± 4	67	0.12 ± 0.01	26
	Organic Se low	0.43 ± 0.04			118	0.43 ± 0.02	100
	Organic Se high	3.4 ± 0.2	3.6 ± 0.1	106 ± 3	132	4.0 ± 0.9	118
	Basal high	0.22 ± 0.03[c]				0.20 ± 0.01	91
	Commercial samples[d]	0.38 ± 0.02[c]	0.42 ± 0.04	110 ± 11	109		53

a) Column recovery(%) is calculated as the sum of quantified peaks expressed as a percentage of the total Se concentration of the extract, where the Se peaks are quantified using the Se standard closest in retention time.

b) n = 5

c) n = 2

d) Salmon muscle tissue from the Surveillance program on farmed Atlantic salmon.

The degree of protein degradation in the samples was not measured, but the high extraction yield for Se from the selenised yeast reference material (Selm-1; 89 ± 2 % and ERM-422; 103 ± 4%), diets without inorganic Se supplementation (64 – 114%) and muscle of Atlantic salmon (90 - 110%) indicate that protease type XIV was efficient in extracting protein-bound Se (Table 2). The variable extraction yields seen for Se in the enzymatic digest of the diets, ranging from 37% to 114% (Table 2), was further evaluated by performing a sequential extraction step with protease for selected diets to increase the Se extraction yield (Supplementary data, S-Table 2). The additional extraction steps only slightly increased the extraction yield for Se; 7 - 13% (second extraction step) and 1 - 4% (third extraction step). These results show that the repeated hydrolysis is not effective and not needed. Also, the results indicate that the extraction yield depends on the Se supplementation source, with a lower extraction yield obtained for the inorganic Se diets (37 to 47%), compared to the organic Se diets (76 to 88%) when using enzymatic digestion with protease type XIV (Table 2).

Alkaline solvents have previously been used for the extraction of Se in diets supplemented with inorganic Se [18, 35]. Therefore, a weak alkaline solution (0.1 M NaOH) was applied for the extraction of Se in the inorganic diets. When using the alkaline solution, the extraction yield for Se increased to 82 % and 92 % for the inorganic low diet and the inorganic high diet, respectively (Table 2). These results show that NaOH is more suited for the extraction of inorganic Se species compared to enzymatic cleavage. Further, this shows that two separate methods are required; one for the determination of organic Se species using enzymatic extraction and one for the determination of inorganic Se species using a weak alkaline extraction.

Selenium speciation analysis of feeds

Selenium speciation analysis was performed using different chromatographic methods (Supplementary, S-Table 1), where SeMet was determined by cation-exchange HPLC-ICP-MS (Fig. 1a and 1b) and inorganic Se species were determined by anion-exchange HPLC-ICP-MS (Fig. 1c). In addition, SeCys was analysed for in selected muscle samples by derivatisation and analysis by reversed phase HPLC-ICP-MS (S-Table 1, S-Fig. 1).

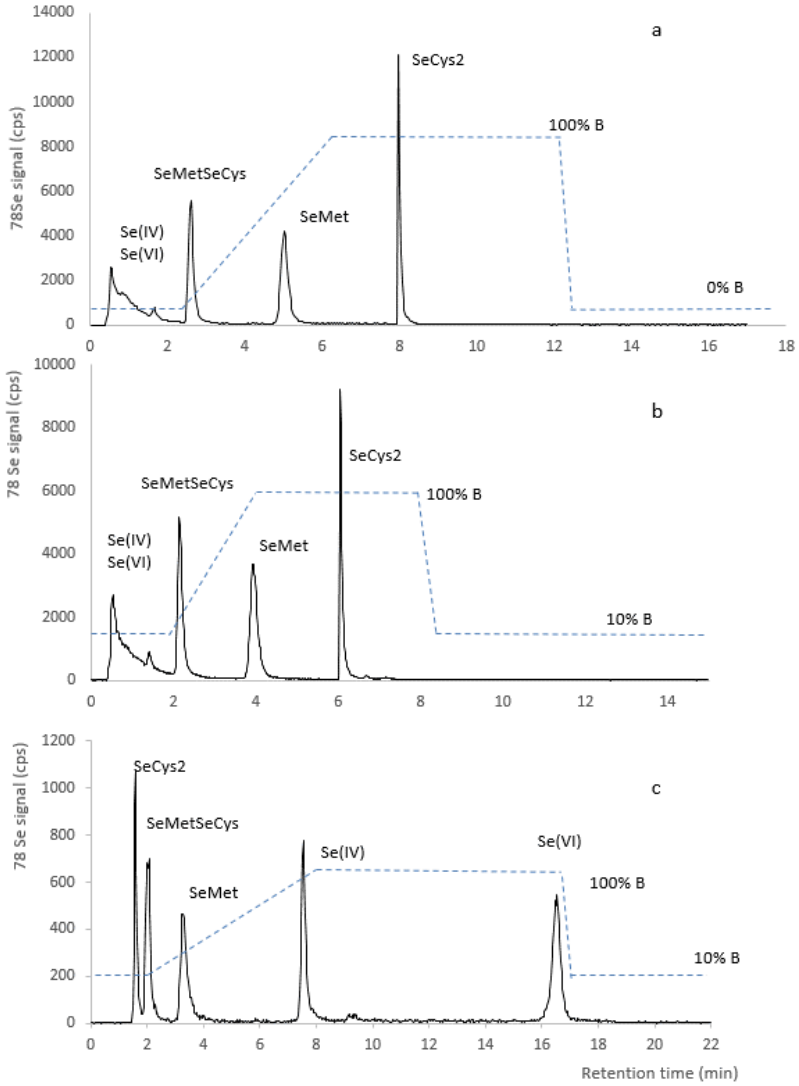

Fig. 1 Chromatograms of a standard mixture (100 µg Se/L) of selenite (Se(IV)), selenate (Se(VI)), Se-methyl-seleno-cysteine (SeMetSeCys), selenomethionine (SeMet) and selenocystine ($SeCys_2$) analysed by cation-exchange HPLC-ICP-MS (a and b) and by anion-exchange HPLC-ICP-MS (c). Different elution gradients were applied for analysis of muscle tissue (a) and diets (b) by cation-exchange HPLC-ICP-MS. The elution gradients are shown for the figures.

No commercial certified reference materials are currently available for Se species in food or feed. For quality control, Selm-1, a certified reference material of selenised yeast, with certified reference values for total Se (2031 ± 70 mg Se/kg dw) and for SeMet (3190 ± 260 mg SeMet/kg dw, i.e. 1268 ± 103 mg Se/kg dw) was used for determination of accuracy and repeatability of the methods. The recovery for SeMet in Selm-1 was in good agreement, 100 ± 5%, (mean ± SD, n = 5), with measured values of 1271 ± 64 mg Se/kg dw (mean ± SD, n = 5) when using the cation-exchange HPLC-ICP-MS method. The recovery for SeMet in Selm-1 was 89 ± 8% (mean ± SD, n = 7) when using the anion-exchange method (measured values of 1131 ± 101 mg/kg dw, mean ± SD, n = 7). The cation-exchange HPLC-ICP-MS method was used for the quantitative analysis of SeMet in feeds and in salmon muscle (Table 2). For the

determination of Se(IV) and Se(VI) in fish muscle and in feed, the accuracy of the method was verified by spiking experiment to the sample extracts. The recovery of the spiking was 110 ± 17% (n = 7) and 107 ± 6% (n = 7) when spiking with standards for Se(IV) and Se(VI), respectively.

From the speciation analysis, it was seen that SeMet was the major Se species in the enzymatic extracts of the un-supplemented basal low diet (low fish meal inclusion) and basal high diet (high fish meal inclusion), where the SeMet accounted for 26% and 21% of the total Se, respectively (Table 2). However, SeMet accounted for 66% and 71% of the Se peaks detected (by area of chromatographic peaks; data not shown), respectively. The relative low column recovery seen for the diets when analysed by cation-exchange HPLC-ICP-MS (Table 2) is reflecting the low proportion of Se species determined in the diets, and could suggest that the column recovery is affected by the sample type. SeMet in these diets is likely to originate from the marine feed ingredients, and mainly fish meal (accounting for 8 and 15% in the basal diet low and high, respectively). Fish meal generally contains high levels of Se [3], and SeMet is considered the major Se species in marine ingredients [26]. SeMet was the major Se species in the diets supplemented with low and high organic Se (as Se yeast), accounting for 28% and 41% of total Se, respectively (Table 2). Similarly, to the basal diets, SeMet was the major Se species detected in the extracts, accounting for 83% and 89% of the total Se peaks detected, respectively (data not shown). Selenised yeast contains SeMet as the major Se species [36], hence, the presence of SeMet in the organic Se diets was expected. Other minor Se peaks detected in this diet is likely to originate from the Se-yeast [37].

In the inorganic Se diets, low levels of SeMet were detected, with concentrations of 0.06 ± 0.02 and 0.12 ± 0.03 mg/kg feed for the low and high diet (Table 2), respectively. In the enzymatic extract of the inorganic Se high diet, several Se peaks were detected when analysed both by anion-exchange and cation-exchange HPLC-ICP-MS (Supplementary S-Fig. 2). However, the SeMet peak accounted for only 5% of the total Se peaks detected in the protease extract (data not shown) and interestingly, Se(IV) was not detected in the enzymatic extract. Subsequent spiking experiments on the extract with standards of Se(IV) and Se(VI) gave a recovery in the range of 98-106% (Supplementary data, S-Fig. 3), suggesting that inorganic Se does not complex with matrix components in the final enzymatic extracts. In contrast to the proteolytic extraction, application of a weak alkaline solution (0.1 M NaOH) for the extraction of the inorganic Se diets, Se(IV) was detected as the major Se species in both the inorganic Se low diet and in the inorganic Se high diet when using anion-exchange HPLC-ICP-MS (Fig. 2). A minor peak for Se(VI) was also detected in the alkaline extracts of the samples (Fig. 2). However, Se(VI) was similarly detected in the blank sample (data not shown), suggesting a minor impurity of Se in the extracting solvent, which was compensated for in the quantitative determinations by subtracting the blank signal. Using an external calibration curve for the quantitative determination of inorganic selenium in the extracts, Se(IV) accounted for and 21% and 87% of the total Se in inorganic Se low and inorganic Se high diet, respectively (Table 2).

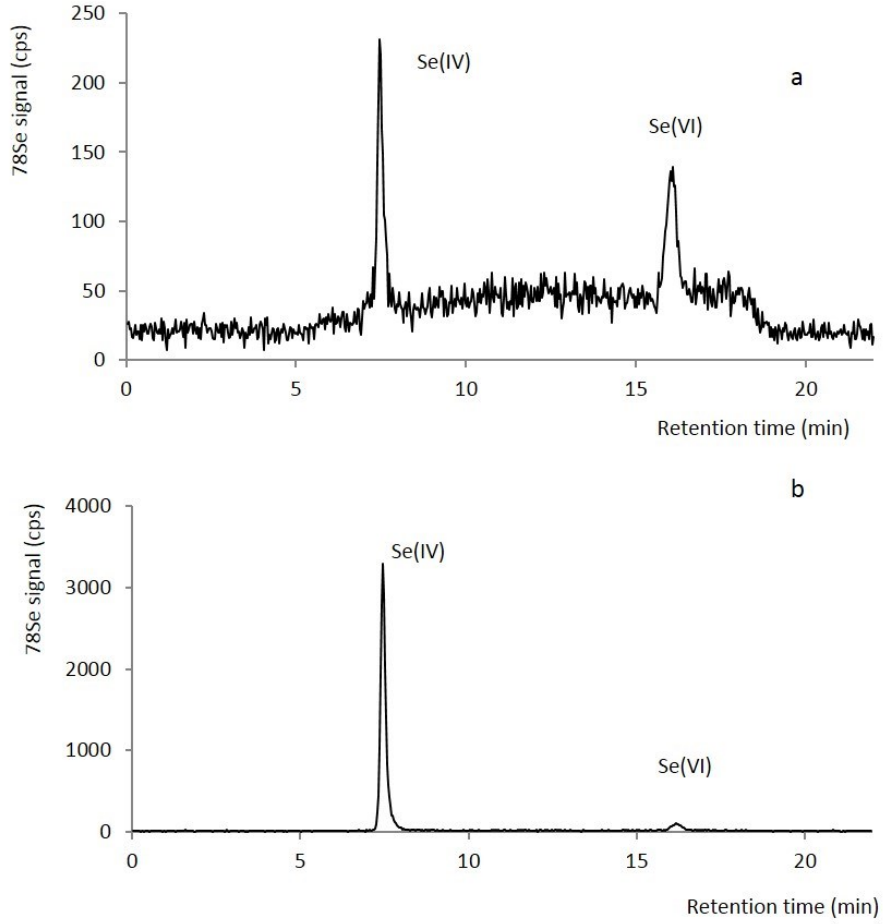

Fig. 2 Chromatograms of the alkaline extracts of inorganic Se low diet (a) and inorganic Se high diet (b) analysed by anion-exchange HPLC-ICP-MS. Please note the different scales on the y-axis

Selenium and Se species in muscle

The accumulation of Se in muscle after 12 weeks of feeding depended on the concentration in feed and on the chemical form of the supplemented Se (Table 1). Higher levels of Se were found in muscle of fish fed diets supplemented with organic Se; 0.43 ± 0.04 (low diet) and 3.4 ± 0.2 (high diet) mg Se/kg ww (n = 3), respectively, compared with fish fed diets supplemented with inorganic Se; 0.14 ± 0.01 (low diet) and 0.46 ± 0.07 (high diet) mg Se/kg ww (n = 3), respectively. In comparison, muscle of fish fed the basal low and basal high diets contained 0.12 ± 0.01 and 0.22 ± 0.03 mg Se/kg ww (n = 3) (Table 1), respectively. A higher retention of Se was seen in muscle of Atlantic salmon fed diets supplemented with organic Se (low: 21.2 ± 1.0 and high: 24.2 ± 1.3 %, n = 3), than in muscle of salmon fed diets supplemented with inorganic Se (13.9 ± 2.6 and 3.7 ± 0.2 %, n = 3) (Table 1). These results suggest that supplemented Se is available to the Atlantic salmon and that organic Se appears to have a higher availability than inorganic Se. The observation is in agreement with earlier findings that SeMet is more readily available than inorganic Se species [6, 18, 38], and that SeMet supplementation with selenised-yeast or SeMet leads to increased levels of Se in fish, e.g.

rainbow trout [18] and Atlantic salmon [39]. In Atlantic salmon fed the basal low and the basal high diets the retention of Se in muscle were 15 ± 1 and 70 ± 4 % (n = 3), respectively. The basal high diet contained more fish meal and krill meal, and less plant protein, than the basal low diet. The higher retention in the salmon fed the basal high diets suggests that Se in marine protein sources is more available than Se in plant protein. This is supported by the work of Godin *et al.* [18], where higher whole body Se levels were found in rainbow trout fry fed a diet based on marine feed ingredients compared to fry fed a plant based diet, indicating a higher bioavailability of Se in marine ingredients.

Muscle of Atlantic salmon fed the basal diets contained SeMet as the major Se species, accounting for 91 and 92% of the total Se in the samples (Table 2). The SeMet concentration in the extracts were 0.11 ± 0.01 and 0.20 ± 0.01 mg/kg ww (mean ± SD, n=3) for the basal low and high, respectively, when using the cation-exchange HPLC-ICP-MS method (Table 2). In comparison, when using the anion-exchange method, the SeMet concentrations were 0.06 ± 0.2 and 0.13 ± 0.05 mg/kg ww (mean ± SD, n=3) for the basal low and high, respectively (data not shown). The lower SeMet concentration detected by the anion-exchange method compared to the cation-exchange HPLC-ICP-MS method for the muscle samples is consistent with the results for the certified reference materials Selm-1 and ERM-BB442. The somewhat lower results for SeMet obtained with the anion-exchange method may be explained by matrix effects that could cause a reduction of the Se signal in the ICP-MS.

SeMet was also the major Se species in muscle of salmon fed diets supplemented with organic Se, with concentrations of 0.43 ± 0.02 and 4.0 ± 0.9 mg/kg ww accounting for 80% and 99% of the Se peaks detected in the fish fed organic low and high diets, respectively (Table 2, Fig. 3a and S-Fig. 4a for organic Se high). Other minor Se species, eluted in the void and in the retention time area of SeMetSeCys (Fig. 3a), suggesting the presence of SeMetSeCys and other Se species with similar ionic properties. SeMet is considered the major Se species in muscle of marine fish, including salmon [1, 22]. In contrast, the muscle of Atlantic salmon fed high inorganic Se diet, SeMet accounted for only 26% of the total Se in the sample (Table 2, Fig. 3b and S-Fig. 4b). The salmon fed inorganic Se diets did not contain elevated levels of SeMet compared to salmon fed the basal diets (Table 2), which is consistent with the suggested metabolic pathway for Se in animals, where SeMet cannot be synthesized from selenite [40]. However, several unidentified peaks were detected in the muscle of Atlantic salmon fed the inorganic Se high diet both when analysed by cation-exchange and anion-exchange HPLC-ICP-MS (Fig. 3b and 3c). Selenite and Se(VI) were not detected in the muscle of the salmon fed the inorganic Se high diet when analysing the protease (Fig. 3c) and alkaline (data not shown) extracts by anion-exchange HPLC-ICP-MS. These results are consistent with the findings of Godin *et al.* [18] where SeMet were detected in whole body rainbow trout fry fed inorganic Se supplemented feed. Furthermore, unidentified Se species were similarly detected in rainbow trout fry fed inorganic Se supplemented feed [18]. However, the trout contained a nearly uniform proportion of unknown Se species (24-38%), irrespectively of being fed inorganic or organic Se supplemented diets [18].

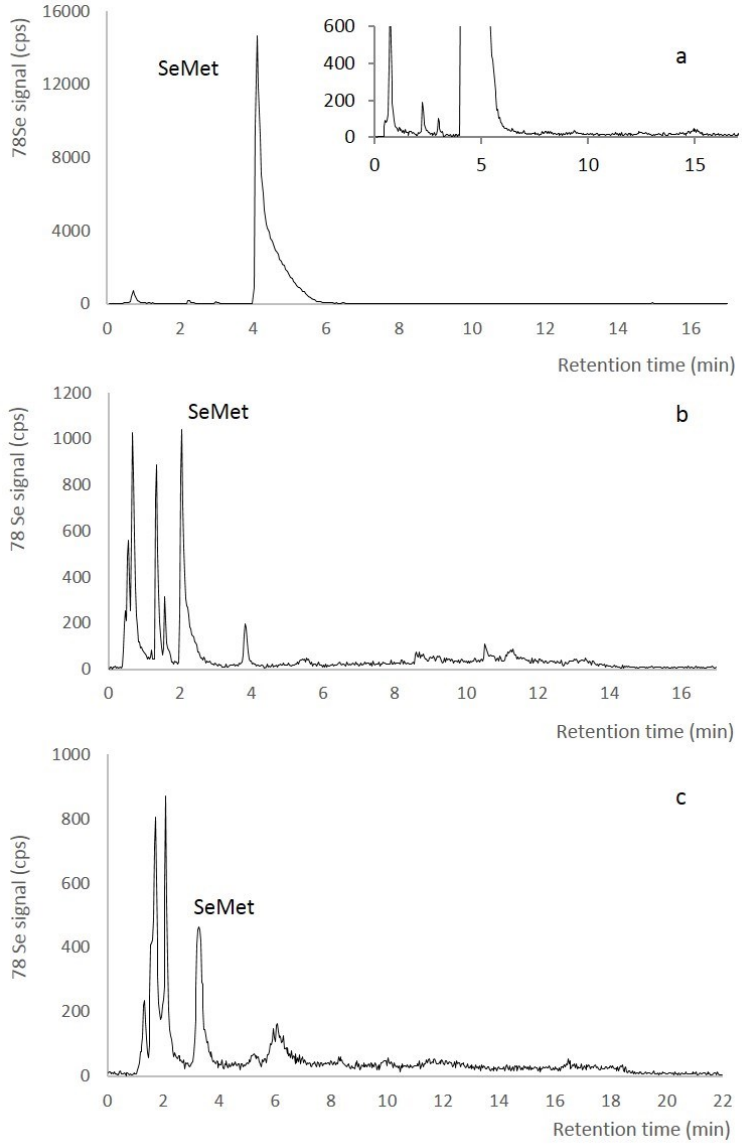

Fig. 3 Chromatogram of protease extract of muscle of Atlantic salmon fed organic Se high diet, analysed by cation-exchange HPLC-ICP-MS (a), the enlargement shows the presence of minor Se peaks in the sample extract, and of muscle of Atlantic salmon fed inorganic Se high diet, analysed by cation-exchange HPLC-ICP-MS (b) and by anion-exchange HPLC-ICP-MS (c).

Selenite is metabolised to hydrogen selenide, and further to SeCys, which is specifically incorporated into selenoproteins, mainly in liver [40].The high dietary level of selenite could lead to the presence of SeCys in the liver, and possibly in the muscle tissue. Analysis of SeCys is challenging due to the highly reactive free selenol group, which cause the species to rarely exist in its free form [41], and requires stabilisation through derivatization prior to analysis [16]. Samples were derivatised and results indicate that SeCys is present in muscle of fish fed high inorganic diet, however, it is a minor Se species (Supplementary, S-Fig. 1). In wild-caught fish, e.g. sand whiting (*Sillago ciliata*), leather jacket (*Monacanthus chinensi*s) and luderick (*Girella*

tricuspidata) low levels of SeCys (from 4 to 17% of total Se) have been found [22]. Similarly, somewhat higher levels of SeCys (from 20 to 30% of total Se) were observed in trout fry, irrespective of whether they were fed diets supplemented with inorganic Se or organic selenised yeast [18]. Other Se species could account for the unidentified Se compounds in muscle of the salmon fed inorganic high diet, such as selenoneine. Selenoneine (2-selenyl-N_α-N_α- N_α-trimethyl-L-histidine) was first identified in blood and muscle of bluefin tuna (*Thunnus orientalis*) [42], and has later been found in muscle of a range of fish [43, 44], often as a minor peak. Yamashita et al. [42] hypothesised that selenite can be metabolised to selenoneine. Hence, selenite, in excess, could lead to formation of selenoneine, which is accumulated in muscle. Further investigations are needed to determine the possible metabolism of selenite to selenoneine in Atlantic salmon.

Conclusion

Se species were analysed using anion-exchange HPLC-ICP-MS for inorganic Se species and cation-exchange HPLC-ICP-MS for organic Se species. SeMet, Se(IV) and Se(VI) were analysed in fish feeds and Atlantic salmon using these methods. Extraction of Se species from fish feed was optimised, and two methods were applied, enzymatic cleavage for organic Se supplemented feeds and weak alkaline solvent for inorganic Se supplemented feeds. In addition, selected muscle samples were analysed for SeCys using extraction procedure including derivatisation and analysis by reversed-phase HPLC-ICP-MS. Supplementation of fish feed with organic and inorganic Se sources have different effects on the retention of Se and the Se species retained in the muscle tissue of Atlantic salmon. Higher retention of Se was seen in Atlantic salmon fed diets supplemented with organic Se than with inorganic Se. Speciation analysis confirmed that SeMet was the major Se species in muscle of Atlantic salmon fed both basal diets and diets supplemented with selenised yeast. Several unidentified Se peaks were detected in muscle of salmon fed high inorganic Se diet, and analysis showed that SeCys is a minor Se species present in this fish muscle tissue.

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Supplementary data

S-Table 1. Instrumental settings for ICP-MS and HPLC settings in selenium speciation analysis.

ICP-MS 7500cx	
RF power	1500 W
Carrier gas flow	0.9 L/min
Plasma gas flow	0.18 L/min
H_2 gas flow (for collision-reaction cell)	4 mL/min
Spray chamber temperature	2 °C
Integration time	0.1 sec
Isotope monitored	78, 80, 82
Anion-exchange HPLC	
Column	Hamilton PRP-X100 (4.6 x 150 mm, 5 µm)
Injection volume	25 µL
Flow rate	1 mL/min
Mobile phase	A: 0.5 mM ammonium acetate, pH 5.2
	B: 100 mM ammonium acetate, pH 5.2
Gradient program	0-2 min: 10% B
	2-8 min: 10-100% B
	8-17 min: 100% B
	17.1-22 min: 10% B
Cation-exchange HPLC	
Column	Ionosphere 5C (3 x 100 mm, 5 µm)
Injection volume	40 µL
Flow rate	1 mL/min
Mobile phase	A: 0.5 mM Pyridine, pH 3
	B: 10 mM Pyridine, pH 3

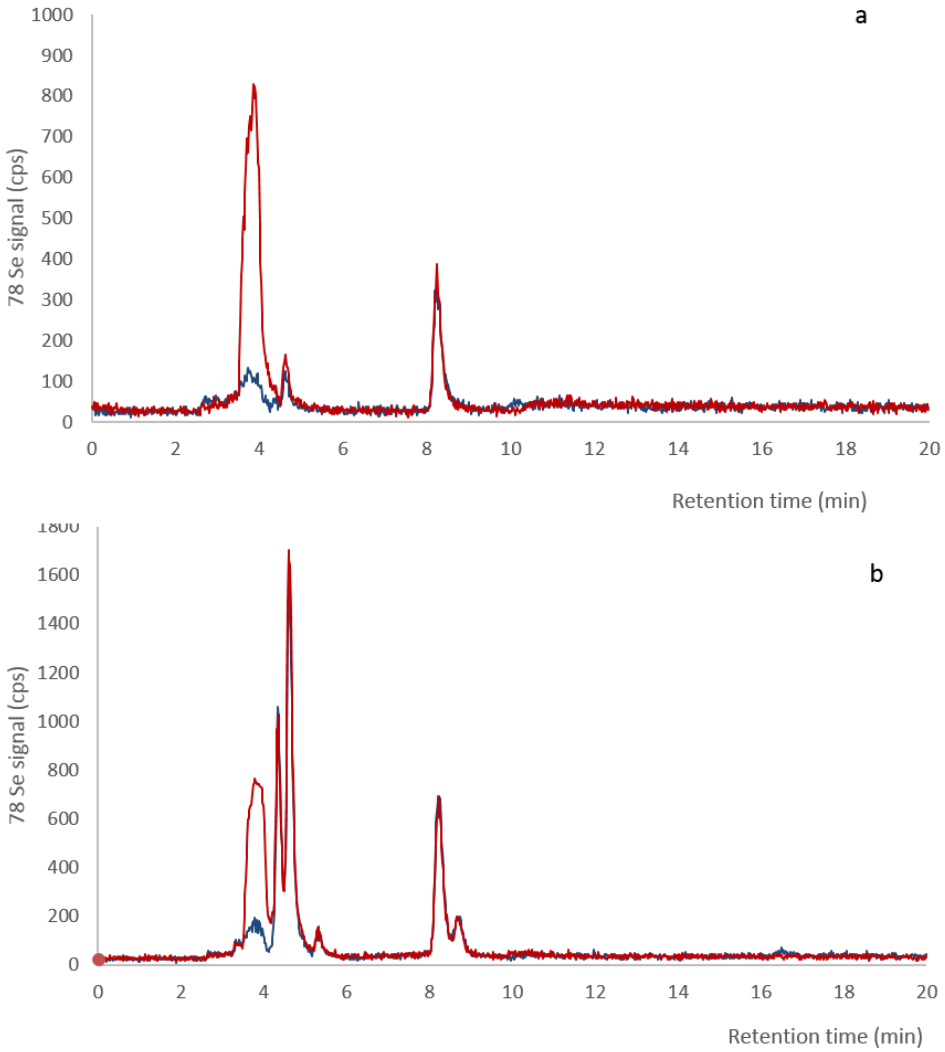
Gradient program 1 (diets)	0-2 min: 10% B
	2-4 min: 10-100% B
	4-8 min: 100% B
	8-8.5 min: 100%B- 10% B
	8.5 – 15 min: 10% B
Gradient program 2 (muscle)	0-2 min: 0% B
	2-6 min: 0-100% B
	6-12 min: 100% B
	12-12.5 min: 100% B-0% B
	12,5 – 17 min: 10% B
Size Exclusion Chromatography	
Column	TSKgel G3000 SWXL (7.8 x 300 mm, 5 µm)
Injection volume	90 µL
Flow rate	0.6 mL/min
Mobile phase	100 mM ammonium acetate, pH 7.5
Reversed-phase HPLC	
Column	XBridge C18 (4.6 x 250 mm, 5µm)
Injection volume	30 µL
Flow rate	0.8 mL/min
Mobile phase	0.1% HFBA in 5% methanol

S-Table 2 Selenium concentration (mg/kg ww, mean ± SD, n = 3) and the extraction yield for Se (%) in the protease extracts of the certified reference material Selm-1 and for the diets after sequential extraction procedures.

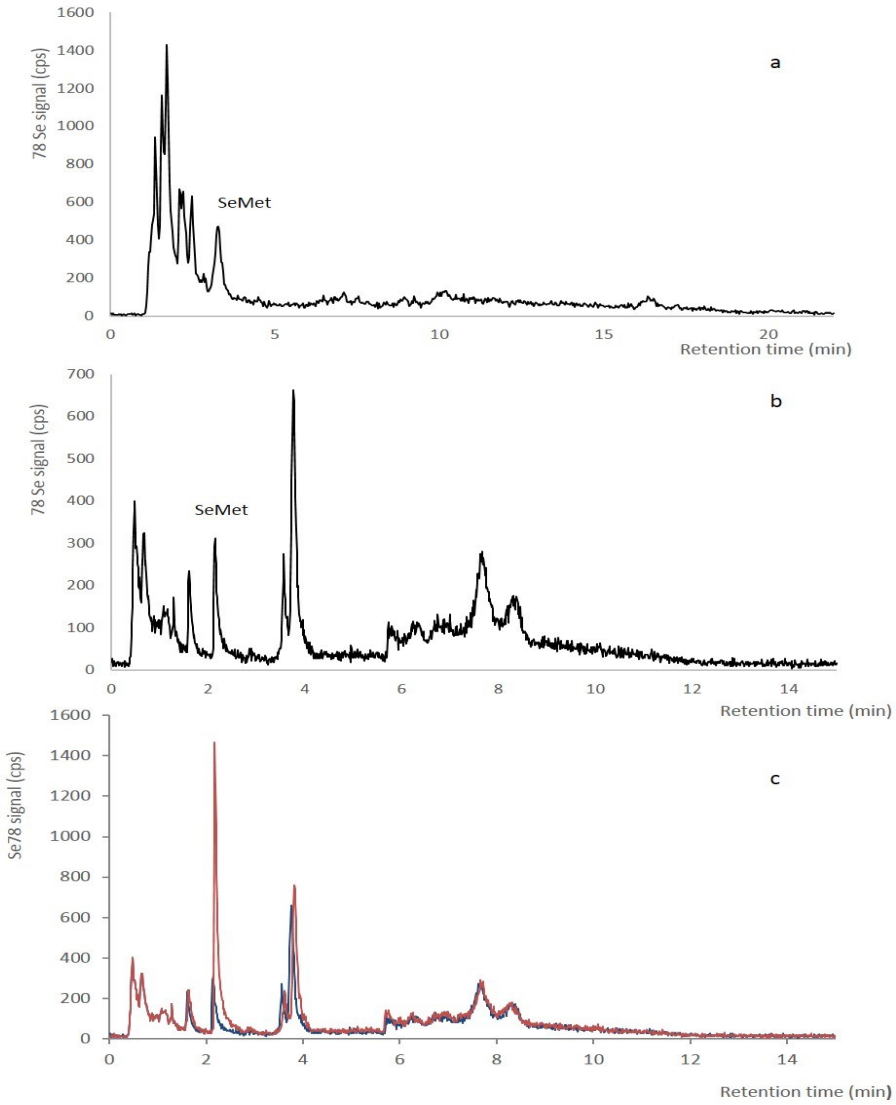
Sample type	Sample	Total Se conc (mg/kg dw)	Se in the 1st enzymatic extract (mg/kg dw)	Se in the 2nd enzymatic extract (mg/kg dw)	Se in the 3rd enzymatic extract (mg/kg dw)	Sum extraction yield (%)
Certified reference material	Selm-1	2059 ± 64	1802 ± 48[a]	49.4[b]	8.9[b]	90
Feed diets	Basal, low	0.35 ± 0.02	0.4 ± 0.1[b]	0.03 ± 0.01	0.02 ± 0.01	129
	Inorganic Se, low	1.10 ± 0.03	0.6 ± 0.2	0.02 ± 0.01	0.02 ± 0.01	58
	Inorganic Se, high	15.0 ± 0.5	6 ± 1	0.23 ± 0.05	0.15 ± 0.04	40
	Organic Se low	2.10 ± 0.05	1.6 ± 0.1	0.05 ± 0.02	0.04 ± 0.01	80
	Organic Se high	15.0 ± 0.2	13.2 ± 0.4	0.29 ± 0.02	0.08 ± 0.01	90
	Basal, high	0.89 ± 0.03	0.57 ± 0.03	0.01 ± 0.01	0.02 ± 0.01	67

a) n = 2

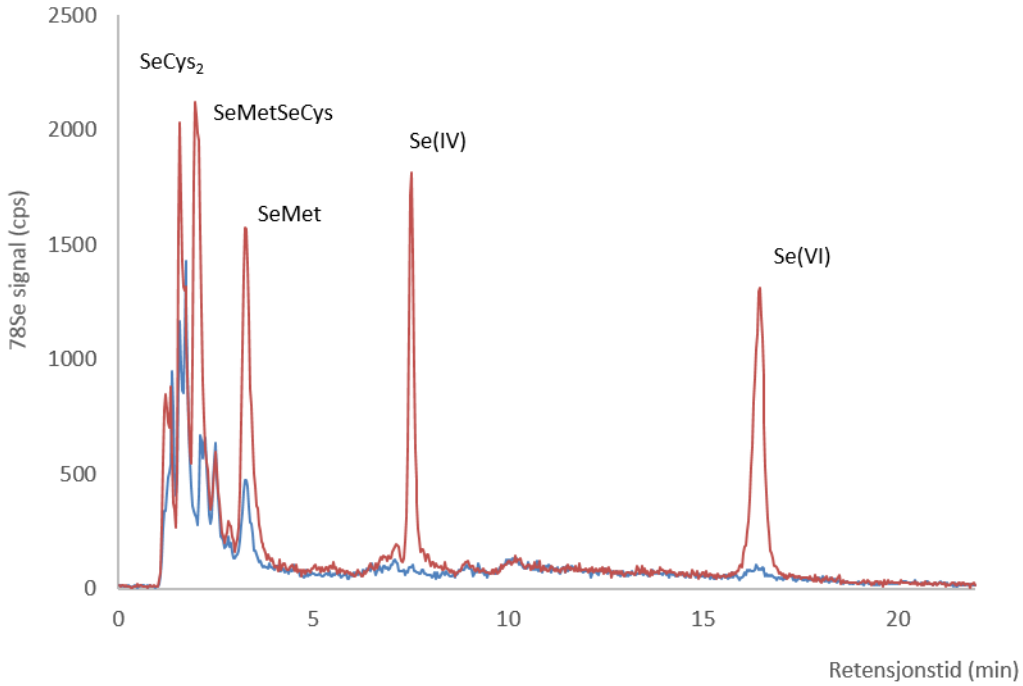
b) n = 1



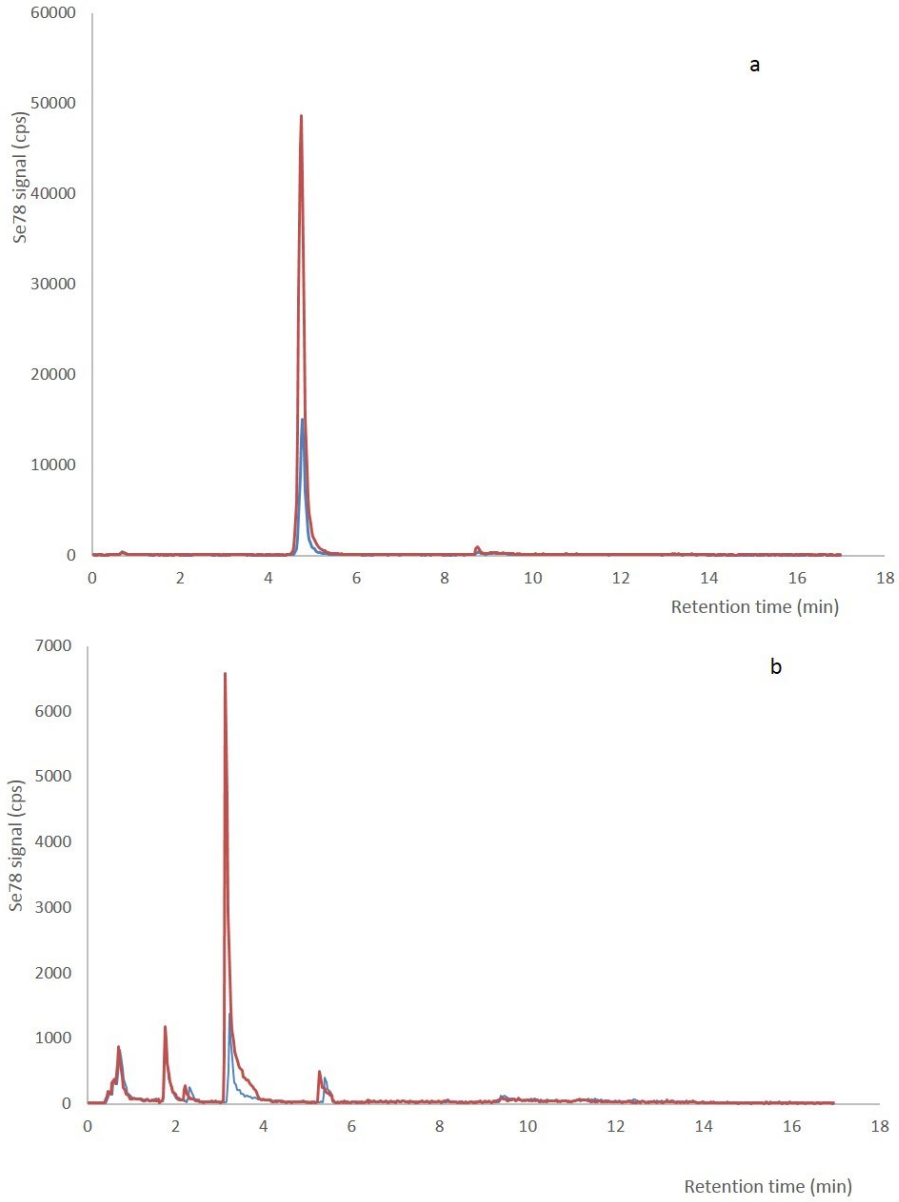
S-Fig. 1 Chromatograms of derivatised muscle tissue of fish fed basal low (a) and inorganic Se high diet (b) when analysed by reversed phase HPLC-ICP-MS. The derivatised sample extracts (blue line) have been spiked with the CAM-SeCys standard (red line) to reach a concentration of CAM-SeCys of 50 µg Se/L (r.t. 3.9 min). The Se peak at retention time 8.1 min corresponds to CAM-SeMet. Please note the different scales on the y-axis for the chromatograms.



S-Fig.2. Chromatogram of the protease extract of inorganic Se high diet when analysed by anion-exchange HPLC-ICP-MS (a), cation-exchange HPLC-ICP-MS (b) and when extract is spiked with SeMet to reach a concentration of 10 µg/L in cation-exchange HLPC-ICP-MS (c). The unassigned Se peaks are unknown Se species



S-Fig. 3 Chromatogram of inorganic Se high diet (blue line) spiked with a standard mixture (red line) of selenocystine (SeCys2), Se-methyl-seleno-cysteine (SeMetSeCys), selenomethionine (SeMet), selenite (Se(IV)) and selenate (Se(VI)) analysed by anion-exchange HPLC-ICP-MS. The standard mixture is spiked to the protease extract of the diet to reach a concentration of 20 µg Se/L in the extract.



S-Fig. 4 Chromatogram of protease extract of muscle of Atlantic salmon fed organic Se high diet (diluted 1:20, blue line) spiked with a standard of SeMet (red line), and protease extract of muscle of fish fed inorganic Se high diet (diluted 1:5; blue line) spiked with SeMet (red line) (b) when analysed by cation-exchange HPLC-ICP-MS. The standard is spiked to the protease extract to reach a concentration of 100 µg Se/L (a) and 20 µg/L (b) in the extract.

4 Toxic modes of action

Sensitivity and toxic mode of action of dietary organic and inorganic selenium in Atlantic salmon (*Salmo salar*)

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Sensitivity and toxic mode of action of dietary organic and inorganic selenium in Atlantic salmon (Salmo salar)

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Abstract

Depending on its chemical form, selenium (Se) is a trace element with a narrow range between requirement and toxicity for most vertebrates. Traditional endpoints of Se toxicity include reduced growth, feed intake, and oxidative stress, while more recent finding describe disturbance in fatty acid synthesis as underlying toxic mechanism. To investigate overall metabolic mode of toxic action, with emphasis on lipid metabolism, a wide scope metabolomics pathway profiling was performed on Atlantic salmon (*Salmo salar*) (572 g) that were fed organic and inorganic Se fortified diets. Atlantic salmon were fed a low natural background organic Se diet (0.35 mg Se kg^{-1}, wet weight (WW)) fortified with inorganic sodium selenite or organic selenomethionine-yeast (SeMet-yeast) at two levels (~ 1-2 or 15 mg kg^{-1}, WW), in triplicate for 3 months. Apparent adverse effects were assessed by growth, feed intake, oxidative stress as production of thiobarbituric acid-reactive substances (TBARS) and levels of tocopherols, as well as an overall metabolomic pathway assessment. Fish fed 15 mg kg^{-1} selenite, but not 15 mg kg^{-1} SeMet-yeast, showed reduced feed intake, reduced growth, increased liver TBARS and reduced liver tocopherol. Main metabolic pathways significantly affected by 15 mg kg^{-1} selenite, and to a lesser extent 15 mg kg^{-1} SeMet-yeast, were lipid catabolism, endocannabinoids synthesis, and oxidant/glutathione metabolism. Disturbance in lipid metabolism was reflected by depressed levels of free fatty acids, monoacylglycerols and diacylglycerols as well as endocannabinoids. Specific for selenite was the significant reduction of metabolites in the S-Adenosylmethionine (SAM) pathway, indicating a use of methyl donors that could be allied with excess Se excretion. Dietary Se levels to respectively 1.1 and 2.1 mg kg^{-1} selenite and SeMet-yeast did not affect any of the above mentioned parameters. Apparent toxic mechanisms at higher Se levels (15 mg kg^{-1}) included oxidative stress and altered lipid metabolism for both inorganic and organic Se, with higher toxicity for inorganic Se.

Keywords: feed, selenite, organic selenium, Atlantic salmon, toxic mode of action, lipid, oxidative stress

Introduction

Selenium (Se) is a well-known essential trace element active as part of functional selenoproteins (Kryukov and Gladyshev, 2000) involved in physiological processes such as antioxidant defense (glutathione peroxidases)(Toppo et al., 2008) and thyroid homeostasis (deiodinases)(Schweizer and Steegborn, 2015). However, Se has a narrow range between its toxic and its beneficial effects (Han et al., 2010; Lee et al., 2016; Wang and Lovell, 1997). In the aquatic system, Se contamination occurs as result of natural geological processes and/or anthropogenic pollution such as mining, irrigation runoff, and fossil fuel waste (Janz, 2012; Lemly, 2002). Inorganic Se is biotransformed to organoselenides including selenomethionine (SeMet), which accumulates along the aquatic food chain causing dietary exposure to fish (Maier and Knight, 1994). The organic SeMet form has a higher ability to raise tissue Se levels than inorganic selenite, which is attributed to the difference in metabolism of SeMet compared to selenite (Fontagne-Dicharry et al., 2015; Godin et al., 2015; Rider et al., 2009). Selenomethionine can be directly and nonspecifically incorporated in any protein containing methionine, while selenite needs to react with glutathione (GSH) to form hydrogen selenide (H2Se) before being incorporated in specific selenoproteins (Godin et al., 2015; Suzuki, 2005). At excess intake, both SeMet and hydrogen selenite can be excreted into urine after methylation in the liver by using s-Adenosylmethionine (SAM) as methyl donor (Suzuki, 2005).

In general, inorganic Se is considered more toxic than organic Se forms (Thiry et al., 2012). However, organic SeMet has a narrow window of requirement and toxicity has been observed for Nile tilapia (Oreochromis niloticus) (Lee et al., 2016).Juvenile rainbow trout (Oncorhynchus mykiss) appears to have threshold levels for chronic dietary SeMet toxicity that is in the same range as for dietary selenite (Hamilton, 2004; Vidal et al., 2005). In fish in general, one of the major Se toxicity symptoms is embryo/larval teratogenic deformities in wild and laboratory studies (Janz, 2012; Lemly, 2002), although in a chronically dietary SeMet exposure study with cutthroat trout (Oncorhynchus clarki bouvieri) no change in reproductive performance was seen and offspring did not show appreciable deformities (Hardy et al., 2010). Other adverse effects include reduced reproduction in fathead minnow (Pmephales-promelas) (Schultz and Hermanutz, 1990) and altered sex steroid hormone production in female rainbow trout (Wiseman et al., 2011a), impaired growth in channel catfish (Ictalurus punctatus) (Wang and Lovell, 1997) and energy storage capacity in juvenile fathead minnow (McPhee and Janz, 2014), reduced feed intake in white sturgeon (Acipenser transmontanus) (Zee et al., 2016a), altered swimming performance in fathead minnow (McPhee and Janz, 2014; Thomas et al., 2013), induction of stress hormones in goldfish (Carassius auratus) and rainbow trout, respectively (Choi et al., 2015; Wiseman et al., 2011b), or reduced neurological and immunological functions in gold fish (Choi et al., 2015).

Oxidative stress has been suggested as one of the main causes of Se toxicity (Lee et al., 2015) for both inorganic Se (Choi et al., 2015; Hauser-Davis et al., 2016; Miller et al., 2007) and organic Se forms (Han et al., 2010; Hursky and Pietrock, 2015; Palace et al., 2004). Selenium mediated oxidative stress has been attributed to Se's ability to oxidize thiols (Spallholz, 1994), removing (SH) sulfhydryl groups in protein formation that are essential to cellular oxidative processes (Maier and Knight, 1994), and/or metabolism of inorganic and organic Se that create Se-metabolites that form reactive oxygen species (Misra et al., 2012; Misra and Niyogi, 2009; Palace et al., 2004). In spite of these mechanisms, which are probably common for vertebrates, oxidative stress was not believed to be a main driver of dietary SeMet toxicity in white sturgeon (Acipenser transmontanus) (Zee et al., 2016a; Zee et al., 2016b). In least killifish (Heterandria formosa) dietary selenite and SeMet reduced rather than increased oxidative stress, but at the same time inhibited osmoregulatory enzymes, (Xie et al., 2016). Furthermore, in juvenile rainbow trout exposed to selenized-yeast worms, no oxidative stress response were seen,

however, growth and liver triglyceride (TAG) levels were reduced (Knight et al., 2016). Recently, several wide-scope pathway assessments have shown that disturbed lipid synthesis and metabolism could be one of the main drivers of organic Se toxicity in rainbow trout (Knight et al., 2016; Pacitti et al., 2016a). Transcriptomic approaches to determine the underlying mechanisms associated with dietary Se toxicity in juvenile rainbow trout, showed that organic Se increases the expression of networks for growth-related signaling cascades in addition to those related to fatty acid synthesis and metabolism (Knight et al., 2016). The disruption of metabolites related to triglyceride processing and storage, as well as gene networks for epidermal growth factor and Notch signaling in the liver, were suggested to represent key molecular initiating events for adverse outcomes related to growth and Se toxicity in fish (Knight et al., 2016). Other transcriptomic assessments of rainbow trout fed Se-yeast, showed that in liver, lipid metabolism was the main pathway altered by Se exposures (Pacitti et al., 2016a). The use of wide-scope "omic" tools have been proven to be valuable tools in identifying new pathways of toxic exposures, identifying possible new biomarkers of toxicity in addition to already identified classic end-point of toxicity (Shaw, 2006). In addition to transcriptomics, proteomics (profiling of proteins) and metabolomics (profiling of biochemicals or metabolites) are used to identify possible biomarkers of toxicity (Shaw, 2006). Metabolomics have been used to identify pathways of toxicity for several contaminants in in vitro (Olsvik et al., 2015; Softeland et al., 2014) and in vivo fish trials (Berntssen et al., 2016), and have been used to assess Se toxicity in yeast (Kitajima et al., 2012) and mammals (Garcia-Sevillano et al., 2013).

In Atlantic salmon, little is known about the toxic mode of action of Se. Thus, the purpose of the present study was to assess the underlying toxic mechanisms and sensitivity of both dietary selenite and SeMet-yeast in Atlantic salmon by using classic endpoints of Se toxicity such as growth, oxidative stress as well as overall metabolomics profiling approaches to assess non target end-points of toxicity. Establishing the sensitivity of Atlantic salmon to dietary selenite and SeMet-yeast dose has implications for setting safe limits in aquatic environments such as threshold body and tissue levels.

Materials and methods

Experimental conditions and sampling

The feeding trial was carried out at EWOS Innovation located in Dirdal, Norway, during the period October 24, 2014 – February 25, 2015. The experiment was approved by the Norwegian Animal Research Authority (now the Norwegian Food Safety Authority; approval number 2309; date of approval: 4 January 2010) and performed according to national and international ethical standards. A total of 540 Atlantic salmon (Salmobreed, 18 months, both genders) were randomly distributed into 18 fiberglass tanks (V = 0.49 m3), with 30 fish per tank. Initial weight of the fish was 572 ± 7 g (mean ± SD, n=540). Prior to the experiment, all fish were fed a control diet during a 2-week acclimation to holding facilities. Thereafter, randomly selected tanks received one of five experimental diets for 3 months, in triplicate. Fish were reared under a 12 hours light: 12 hours dark regime and fed by automatic feeders in three meals a day to a level approximating 0.90 % of body weight per day. The feeding rate was adjusted for the number of fish and growth biomass increase, which was assessed by measuring average weight gain of the sampled fish per sampling time point. Uneaten pellets were collected in a flow-over system and registered daily thus providing exact feed intake values daily. Environmental parameters in tanks were measured five times a week, showing a salinity of 28.4 ± 0.3 ‰, temperature of 8.9 ± 0.3 °C, and oxygen levels of 87 ± 6 % at the outlet. Three fish per tank (n = 9 per dietary group) were sampled at 1.5 months and 3 months of exposure

for tissue sampling. All sampled fish were carefully terminally anaesthetised with an overdose of 3-amino benzoic acid ethylester (∼1 g l−1, pH 8.0). Liver and skeletal muscle samples were taken from individual fish. The liver was dissected and weighed and care was taken to remove the gall bladder in order to avoid contamination of the liver. The liver was divided into four parts in the same manner for each fish and the parts were always taken from the same region of the liver for each analyses (for analyses of Se content, metabolomics, and oxidative stress as tocopherol, ascorbic acid and TBARS, see sections under). Liver sections were immediately frozen in liquid nitrogen followed by storage at −80◦C until biochemical analyses. Standardized Norwegian quality cut (QC) skeletal muscle samples without skin were taken and frozen immediately at -20◦C until Se analysis. In addition, five fish from each tank were randomly sampled, in the same manner as the fish sampled for tissue analyses, for whole-body fish analyses, they were homogenized as whole fish, frozen at -20 °C and analyzed for minerals and total lipid content. Fish analyzed for whole fish analyses were starved 24 hours prior to sampling to avoid Se contribution from feces. Fork-tail lengths and weight were registered for all sampled fish, and for the remaining fish at the end of the trial.

Calculations

Growth and nutritional indices were calculated as follows:

Hepato Somatic Index (HSI) = liver weight (g) x body weight (g)-1 x 100

Food Conversion Ratio (FCR) = feed intake (g) x fish weight gain (g)

Specific Growth Rate (SGR) = (ln(W2)-ln(W1))*(t2-t1)-1; where W2 and W1 are weights on day t2 and t1, respectively.

Feed production

Selenium was added as part of the mineral premixture to the basal diets with low natural Se content. The experimental feeds were produced by BioMar A/S (Brande, Denmark), and were formulated based on commercial diets that fulfilled the nutritional requirements of salmonids (NRC, 2011), using standard commercially available feed materials. The general basal diet had the following composition: fish meal (8%), krill meal (2%), plant meal blend (59%), fish oil (8 %), rape seed oil (16%) and micro-nutrient mixture (7 %). In addition to a relatively low inclusion level of fish meal, which is the main natural source of Se (Sissener et al., 2013), care was taken to select ingredients with low levels of Se, as levels in plant material can vary depending on the soil (Alfthan et al., 2015). The basal diets with natural low levels of organic selenium were supplemented with either high (15 mg kg-1) or low (1 and 2 mg kg-1, respectively) inorganic Se (sodium selenite, Na2SeO3, DSM, Heerlen, Netherland) or organic Se (>98% of total Se) as inactivated whole cell yeast (Saccharomyces cerevisiae) containing >70% L-selenomethionine (AlkoSel®, Lallemand, Malvern link, England) (SeMet-yeast). The selenite and SeMet-yeast levels were chosen as to match the levels found in commercial Atlantic salmon feed (~1.4 mg kg-1 wet weight (WW); Sissener et al., 2013) and levels that are known to provoke chronic toxicity for organic Se (~20 organic Se as Se-yeast fed worms mg kg-1 dry weight (DW); Knight et al., 2016) or inorganic Se,(~9-13 mg Se kg-1 WW; Hamilton, 2004) in adult salmonids. The analyzed Se level in the basal diet was 0.35 ± 0.02 mg kg-1 (control) and 1.1 ± 0.03 (low) and 15.0 ± 0.5 (high) mg kg-1 WW for the low and high selenite

supplemented diets respectively, and 2.1 ± 0.1 (low) and 15.0 ± 0.2 mg kg-1 (high) for respectively the low and high SeMet-yeast supplemented diets (n=3, mean±SD)

Selenium analyses

Diets and tissues of fish were digested using microwave-acid decomposition based on the method described by Julshamn et al. (2007). Samples were accurately weighted (0.20 – 0.25 g) into 15 mL quartz digestion vessels (Milestone Srl, Sorisole, BG, Italy), containing deionized water (0.5 mL; Milli-Q, Merck Millipore, Oslo, Norway). Concentrated nitric acid (HNO3,2 mL, Fluka, Sigma-Aldrich, Oslo, Norway) were added to all samples. The vessels were capped and placed in the Ultrawave (UW, SRC, Milestone, Shelton, CT, USA), with a container of 30 mL Milli-Q water and 5 mL hydrogen peroxide (H2O2, Emsure ACS, ISO, 32% w/w; VWR, Oslo, Norway). The gas pressure in the UW was set to 40 bar and the temperature increased incrementally to 260 °C. The muscle samples were diluted to 25 mL and 10 mL, respectively, with Milli-Q water. Total Se concentration were determined in the digests using ICP-MS (iCAP-Q and FAST SC-4Q DX auto sampler, both Thermo Fisher Scientific Inc, Waltham, Massachusetts, USA). An external calibration curve was made from freshly prepared multi element standard diluted to appropriate concentrations by 5% (v/v) HNO3. The instrument was used with a collision cell gas, using He KED (kinetic energy discrimination) mode for interference removal. A solution of internal standard (Ge, Rh and Tm, Thermo Fisher Scientific Inc) was added on-line for correction of instrumental drift during the analysis. The instrument was optimized using a tuning solution (1 ppb tuning solution B, Thermo Fisher Inc, in 2% HNO3 and 0.5% HCl (puriss. p.a, Sigma-Aldrich) prior to analysis. Plasma power was set to 1550 W, carrier/nebulizer gas flow to 1.05 L/min, the plasma/auxilliary gas flow to 0.8 L/min, and He gas (CCT1) flow was 4.6 mL/min. Isotope 78Se was monitored, and the integration time was 0.1 sec. Oyster Tissue (OT, CRM 1566 b, National Institute of Standards and Technology, Gaithersburg, MD, USA) and Lobster Hepatopancreas (TORT-3, National Research Council Canada, NRC, Ontario, Canada) were used as reference materials for the analysis.

Anti-oxidant, vitamin E

Vitamin E refers to the lipid-soluble antioxidants tocopherols and tocotrienols (van Meeteren et al., 2005). The method determines α-, β-, γ- and δ-tocopherol isomers and α-, β-, γ- and δ-tocotrienol by high performance liquid chromatography (HPLC), and was performed according to the method described by Hamre et al. (2010). In short, the homogenized liver samples of individual fish were saponified (20 minutes at 100 °C) using ethanol, potassium hydroxide, pyrogallol, ascorbic acid and EDTA, before the samples were extracted three times with hexane. The solvent was subsequently evaporated under nitrogen and the samples were diluted with a standard volume of hexane before injection into the HPLC and detection by fluorescence detector.

Thiobarbituric acid-reactive substances (TBARS) and total body fat

Thiobarbituric acid-reactive substances (TBARS) are products of lipid peroxidation and were determined by the method described by Hamre et al. (2001a; 2001b). Using Bligh and Dyer extraction (Bligh and Dyer, 1959), fat and water-soluble components in the liver samples were

separated, and the aldehydes were extracted from the sample in the methanol:water phase. Thiobarbituric acid (TBA) were added in excess to an aliquot of the methanol:water phase and then heated to form a colored complex between aldehydes in the sample and TBA. The absorption was measured at 532 nm, and the concentration of TBARS were quantified using a standard curve. Total amount of whole body fat in intact fish was determined gravimetrically after extraction of homogenized and freeze-dried material with ethyl acetate.

Metabolomics

Global biochemical profiles were determined as described by Olsvik et al. (2015) and Berntssen et al. (2016), and performed by Metabolon, Durham, USA. Briefly, samples were extracted and prepared for analysis using Metabolon's standard solvent extraction method. The extracted samples were split into equal parts for analysis on gas chromatography coupled to mass spectrometry (GC/MS) and liquid chromatography coupled to tandem mass spectrometry (LC/MS/MS) platforms. Water-soluble metabolites were separated on a LC (UPLC ACQUITY, Waters, Milford, MA 01757 USA) coupled to a LTQ MS (linear ion-trap (LIT) mass spectrometer, Thermo-Finnigan Rockwood, USA) with an electrospray ionisation (ESI) source. The samples were analysed in acidic positive ion optimised conditions and basic negative ion optimised conditions in two independent injections using separate dedicated HPLC columns. For analysis in acidic positive ion mode a gradient elution program with water/methanol, both containing 0.1% formic acid, was used. For analysis in basic negative ion mode a gradient with water/methanol, containing 6.5 mM ammonium bicarbonate, was used. The samples destined for GC/MS analysis were re-dried under vacuum (24 h) and derivatised under dried nitrogen using bistrimethyl-silyl-triflouroacetamide (BSTFA). The GC compatible metabolites were analysed on a Trace DSQ fast-scanning single-quadrupole mass spectrometer using electron impact ionisation (Thermo-Finnigan Rockwood, USA). The GC column was 5% phenyl and the temperature ramp ranged from 40° to 300° C in a 16 minute period. Fragmentation spectra (MS/MS) were typically generated in data dependent manner (a fixed number of precursor ions whose m/z values were recorded in a survey scan were subsequently subjected to a second stage of mass selection in a MS/MS analysis), but if necessary, targeted MS/MS could be employed, such as in the case of lower level signals. Instrument variability was 4% for internal standards and total process variability for endogenous metabolites was 12%. Identification of known chemical entities was based on comparison to metabolomic library entries of purified standards.

Statistics and bioinformatics analysis

To account for the variance among experimental tanks within a dietary treatment, as well as variance among fish within an experimental tank, nested ANOVA, followed by Tukey's HSD post hoc test were used. Significant differences among group was set at $p<0.05$. All statistics were performed using the program Statistica (Statsoft Inc., Tulsa,USA). Metabolomics data were subjected to multivariate statistical and bioinformatics analyses as described in Rasinger et al. (Rasinger et al., 2017). In short, the data were screened for treatment induced changes in relative metabolite abundances using the Qlucore omics explorer version 2.3 (Qlucore AB, Lund, Sweden). Metabolites displaying significant ($p < 0.05$, Qlucore) differences in relative abundance were subjected to biological network analysis within the Ingenuity Pathway Analysis software suite (IPA, Qiagen, Redwood City, CA, USA) using an IPA Metabolomics Core Analysis with default settings.

Results

Growth, feed intake, feed conversion, and whole body lipid deposition

No mortality was observed in any of the experimental dietary groups. Fish fed the high selenite diet (15 mg kg^{-1} WW) had significantly reduced final body weights (p=0.012) and fork-tail length (p=0.032) compared to the control groups (Table 1). None of the other fish fed Se fortified diets had significantly different final body weights. Whole body lipid content was reduced in fish fed the high selenite diet, however not significantly (p>0.05) compared to the control group. The specific growth rate, feed intake and feed conversion are given in two periods; the first 1.5 months of experimental period (GP1; growth period 1) and the last 1.5 months of the experimental period (GP2; growth period 2). During the GP1, the high selenite group (15 mg kg^{-1}WW) had a significantly (p=0.023) lower feed intake (% bodyweight day^{-1}) compared to the other experimental groups. Concurrent with reduced feed intake, the specific growth rate (%) was also significantly (p=0.031) lower in the high selenite group compared to the other groups. No significant differences in feed conversion was observed among the dietary groups, although the high selenite group had elevated feed conversion rate (FCR) compared to the other groups. During the GP2, the feed intake and growth in the selenite group while lower, were not significant different from the other groups.

Table 1. Final weight and length (g and cm, respectively, n=45), whole body lipid content (% WW, n=3) and mean tank feed intake (% body weight day^{-1}, n=3), specific growth rate (SGR, %, n=3), and feed conversion rate (FCR; kg feed eaten kg^{-1} weight increase, n=3) in Atlantic salmon (*Salmo salar*) fed on control diet, or diets fortified with low (1.1 and 2.1 mg kg^{-1} WW, respectively) or high (15 mg kg^{-1} WW) selenite or selenomethionine (SeMet)-yeast -yeast during the first growth period (0-1.5 months; GP1) and second growth period (1.5- 3 months; GP2)(mean±SD). Values in rows with the same superscripts are not significantly different (P<0.05, one-way ANOVA, Tukey's t-test).

	Control	Low selenite	High selenite	Low SeMet-yeast	High SeMet-yeast
Final weight	1211±32[a]	1226±54[a]	1008±51[b]	1242±76[a]	1242±74[a]
Final lenght	44.56±1.44[a]	44.7±2.62[a]	42.5±1.43[b]	44.1±1.55[a]	45.2±2.57[a]
Lipid content	16.5±0.73	16.6±0.50	15.5±0.73	17.4±0.61	16.2±0.98
GP1					
Feed intake	0.87±0.012[a]	0.92±0.052[a]	0.75±0.035[b]	0.91±0.026[a]	0.89±0.038[a]
SGR	1.15± 0.04[a]	1.18±0.04[a]	0.88±0.04[b]	1.18±0.05[a]	1.16±0.12[a]
FCR	0.77±0.03	0.78±0.03	0.86±0.01	0.77± 0.04	0.76±0.05
GP2					
Feed intake	0.80±0.24	0.93±0.46	0.62±0.15	0.76±0.12	0.71±0.13
SGR	0.68±0.05	0.58±0.03	0.48±0.12	0.62±0.15	0.59±0.05
FCR	1.18±0.37	1.60±0.73	1.35±0.47	1.26±0.28	1.19±0.12

Whole body, muscle and liver selenium levels

Whole body Se levels (mg kg^{-1} DW) were significantly higher in fish fed the two SeMet-yeast diets (2.1 and 15 mg kg^{-1}, p=0.0012 and 0.0013, respectively) and the highest selenite diet (15 mg kg^{-1}, p=0.0021, compared to control (Figure 1A). For fish fed the low selenite diet, no

significant elevated whole body Se level were seen when compared to the fish fed control diet. Both the SeMet-yeastgroups had significantly (p=0.0041 and 0.0032, respectively) higher whole body Se levels compared to the corresponding selenite groups (Figure 1A). Muscle Se levels (WW) were not significantly elevated in fish fed the low selenite diet compared to control diet (Figure 1B). The high selenite group had elevated Se levels in muscle, significantly (p=0.007) higher than the low SeMet-yeast group. Highest muscle Se levels were observed in the high SeMet-yeast group with significantly higher levels compared to all other groups (Figure 1B). Liver Se levels (mg kg^{-1} WW) were not significantly different in fish fed low selenite and SeMet-yeast compared to the control (Figure 1C). Both high selenite and SeMet-yeast fed fish had significantly (p=0.0081 and 0.007, respectively) higher liver Se compared to control, but higher Se levels were seen in the liver of fish fed the high selenite diet compared to the high SeMet-yeast diet (Figure 1C).

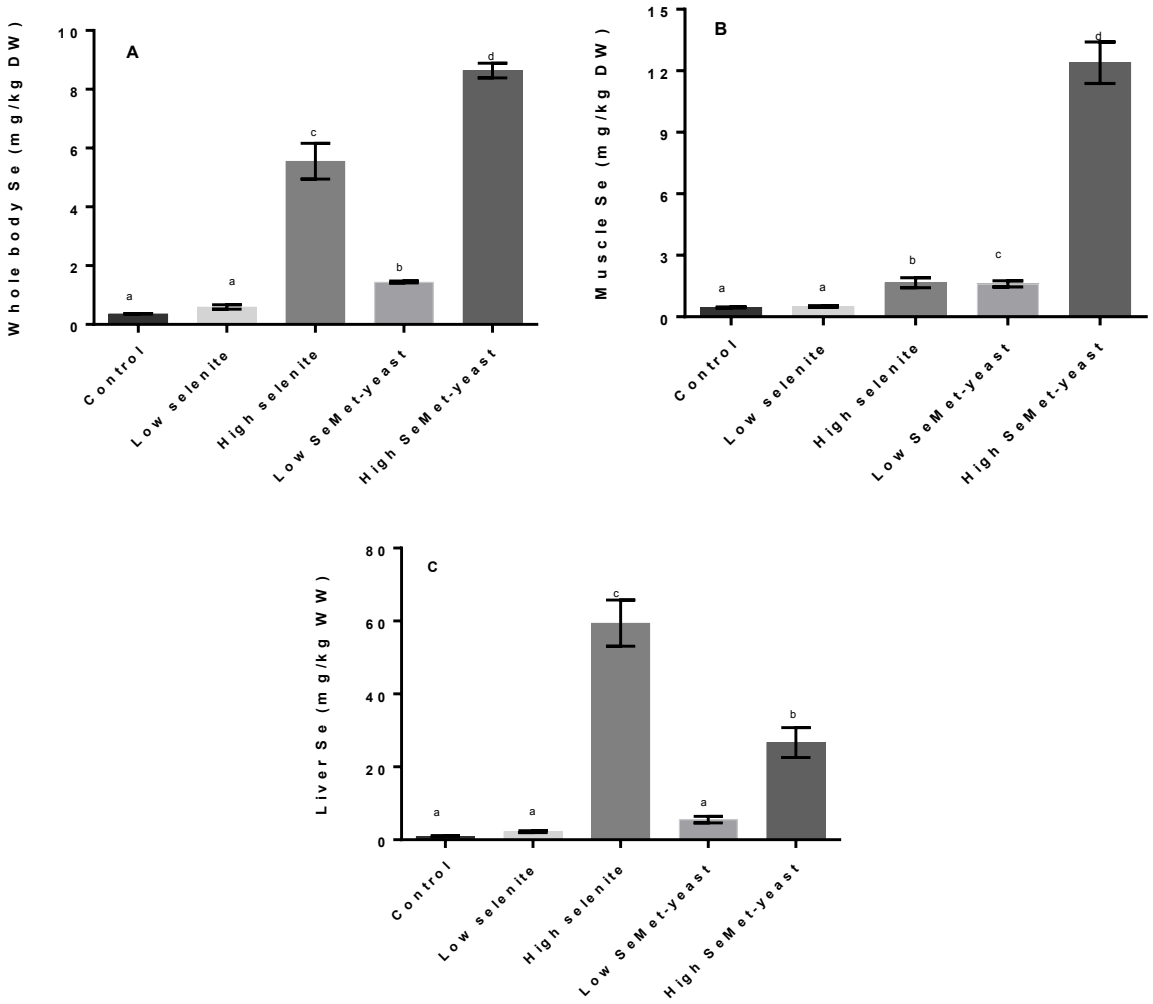

Figure 1. Selenium (Se) concentrations (mg kg^{-1} dry weight (DW) or wet weight (WW)) in whole fish (A), muscle (B) and liver (C) in Atlantic salmon (*Salmo salar*) fed control, low or high selenite fortified diets (1.1 and 15.0 mg kg^{-1} WW, respectively), or low or high selenomethionine (SeMet)-yeast (2.1 and 15.0 mg kg^{-1} WW, as activated Se-yeast/selenomethionine, respectively) for 3 months (mean ± SD, N=3 for whole fish and n=9 for muscle and liver). Bars with the same letters are significantly different from each other (P<0.05, one-way ANOVA, post hoc Tukey's t-test).

Antioxidant vitamins and TBARS.

No significant differences in hepatic somatic (HSI) was seen among the dietary groups. Fish fed the high selenite diet showed significantly (p=0.021) reduced liver levels of the fat soluble antioxidant tocopherol in liver compared to control (Table 2). The liver level of TBARS was elevated in the group fed the high selenite diet, however no significant differences were observed among the other exposure groups.

Table 2. Liver hepatic somatic index (HSI, %, n=45) α, β, and γ tocopherol (mg kg^{-1} wet weight (WW)) and TBARS (nmol g^{-1} WW) in Atlantic salmon (*Salmo salar*) fed control diet, or diets fortified with low (1.1 and 2.1 mg kg^{-1} WW, respectively) or high (15 mg kg^{-1} WW) selenite or selenomethionine (SeMet)-yeast for 3 months exposure (mean±SD, n=9). Values in rows with different superscripts are significantly different from each other (P<0.05, one-way ANOVA, Tukey's t-test).

	Control	Low selenite	High selenite	Low SeMet-yeast	High SeMet-yeast
HSI	1.31±0.15	1.35±0.5	1.38±0.16	1.33±0.21	1.26±0.11
α-tocopherol	1613±240^{a}	1557±287^{a}	753±181^{b}	1453±176^{a}	1413±21^{a}
β-tocopherol	1.5±0.3^{a}	1.4±0.4^{a}	0.3±0.4^{b}	0.8±0.7^{a,b}	1.1±0.2^{a,b}
γ-tocopherol	75±12^{a}	67±7^{a}	32±7^{b}	60±19^{ab}	64±7^{a}
TBARS	4.0±0^{a}	4.0±0.1^{a}	5.2±0.4^{b}	4.3±0.3^{a}	4.4±0.6^{a,b}

Metabolomics

A total of 217 liver metabolites were significantly (p<0.05, supplementary data on individual p-values) affected in the selenite and SeMet-yeast exposure groups compared to control. The high selenite group (15 mg kg^{-1} WW) had the highest abundance of significantly affected metabolites with 79 metabolites that were only affected by this exposure. In contrast, the low selenite (1.1 mg kg^{-1}WW) group had no metabolites that were only significantly affected by this exposure (Figure 2). The s-Adenosylmethionine (SAM) pathway was one of the main pathways affected by high selenite, followed by the cysteine-glutathione pathway (Figure 3A). The latter was also significantly affected by the high SeMet-yeast group. In the SAM pathway, the high selenite caused reduced liver methionine, S-adenosylhomocysteine, homocysteine and cystathionine, compared to control fish. Both high selenite and SeMet-yeast caused a reduction in cysteine as well as the amount of the reduced form of glutathione, with stronger reduction in the high selenite group (5 and 7 fold reduction of cysteine and reduced glutathione, respectively) compared to the high SeMet-yeast group (2.5 and 2 fold reduction compared to control, respectively) (Figure 3A). The other main pathway affected by the high selenite Se group, and to a lesser degree in the high SeMet-yeast group, that was related to lipid metabolism, with reduced liver monoacylglycerols and diacylglycerols, long-chain free fatty acids (LCFAs), and polyunsaturated fatty acids (PUFAs) (Figure 3B) (Table 3). The high selenite group caused a significant reduction of 11 LCFAs, while the high SeMet-yeast group reduced 3 of these LCFAs. The common 3 LCFAs (myristoleate, myristatate, palmitoleate) affected by both selenite and SeMet-yeast had a higher reduction in the high selenite group compared to the high SeMet-yeast group (~1.5 versus ~0.5 fold, respectively) (Table 3). Coenzyme A was significantly lower (2.1-fold reduction compared to control) in fish fed high selenite, while no significant differences were observed in any of the other dietary exposure

groups. High selenite caused a reduction in 4 PUFAs related to the linoleate metabolism, while in the high SeMet-yeast group one PUFAs in linoleate metabolism was reduced. Concurrent with the reduction in LCFAs and PUFAs, 13 monoacylglycerols metabolites and 3 diacylglycerol were reduced in the high selenite group compared to only 2 similar monoacylglycerols and one diacylglycerol in the high SeMet-yeast group (Table 3). Other pathways that were significantly affected by high selenite, and to a lesser degree high SeMet-yeast, are pathways for endocannabinoids production. Fish fed high selenite showed a reduction in six endocannabinoids, namely linoleoyl ethanolamide, N-stearoyltaurine, oleoyl ethanolamide, palmitoyl ethanolamide, N-oleoyltaurine, and N-palmitoyltaurine (2.6, 1.5, 2.1, 1.3, 4.1, and 1.7 fold decrease compared to control, respectively) (Figure 4 A-F). The endocannabinoids N-oleoyltaurine N-palmitoyltaurine were significantly reduced in both high selenite and SeMet-yeast group (2.2 and 1.7 fold decrease compared to control, respectively) (Table 3) (Figure 4 E-F). The high selenite group also had significantly reduced antioxidants such as ascorbate (vitamin C, 1.6 fold reduction), the α-tocopherol intermediate α-tocopherol acetate (1.3 fold reduction), and reduced GSH (4 fold reduction). The high SeMet-yeast group had also significantly reduced GSH, but to a lesser degree as selenite (2 fold reduction) (Figures 5 A-D).

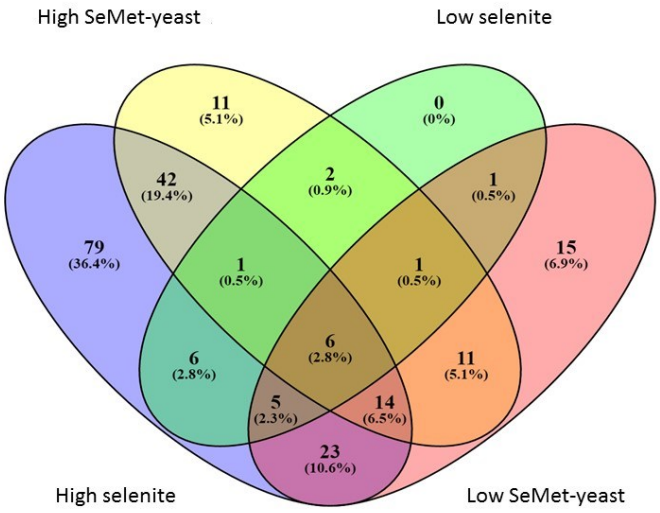

Figure 2. Selenium (Se) concentrations (mg kg^{-1} dry weight (DW) or wet weight (WW)) in whole fish (A), muscle (B) and liver (C) in Atlantic salmon (*Salmo salar*) fed control, low or high selenite fortified diets (1.1 and 15.0 mg kg^{-1} WW, respectively), or low or high selenomethionine (SeMet)-yeast (2.1 and 15.0 mg kg^{-1} WW, as activated Se-yeast/selenomethionine, respectively) for 3 months (mean ± SD, N=3 for whole fish and n=9 for muscle and liver). Bars with the same letters are significantly different from each other ($P<0.05$, one-way ANOVA, post hoc Tukey's t-test).

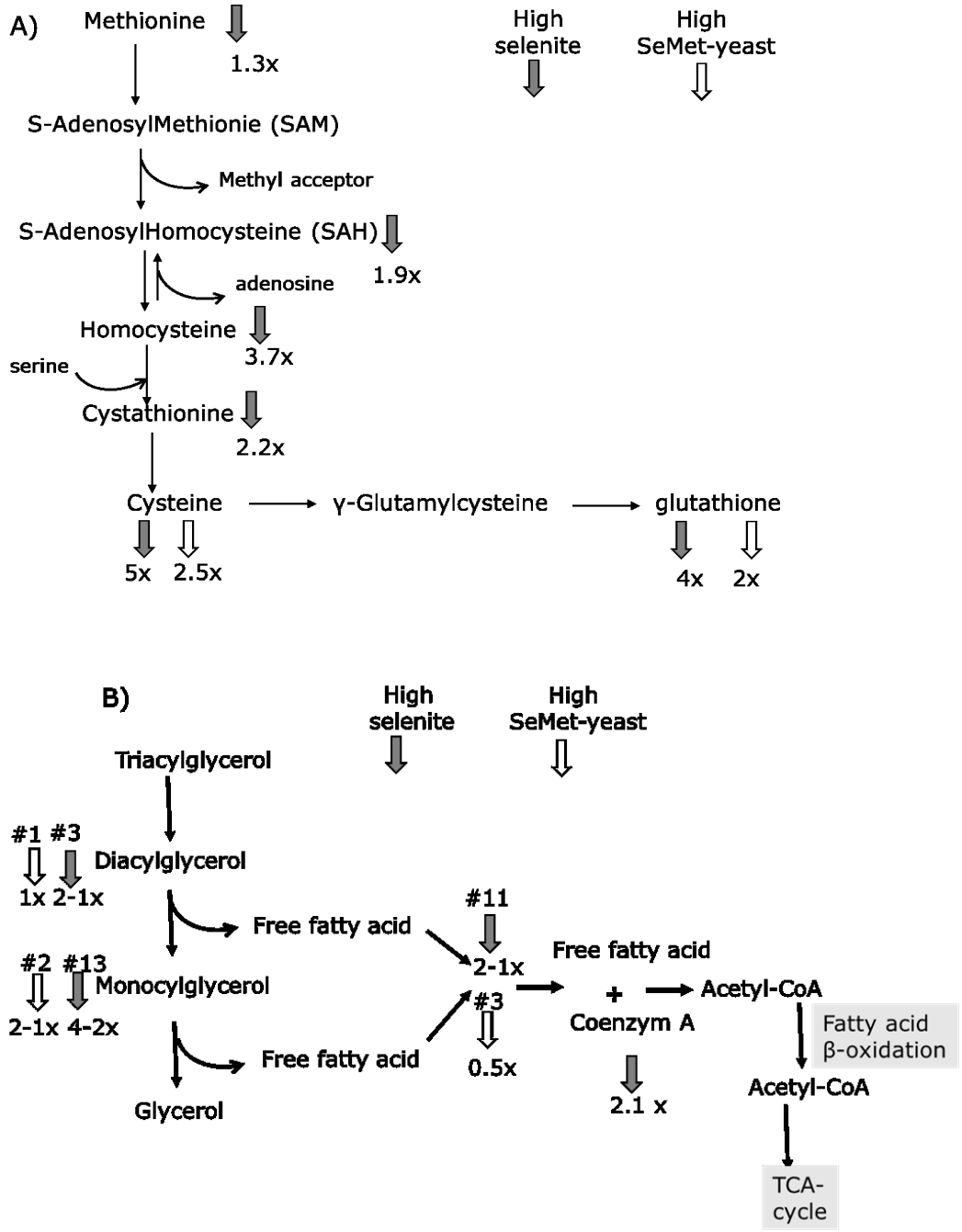

Figure 3 A and B. Overview of liver metabolites in the predicted s-Adenosylmethionine(SAM) (A) and lipid metabolism (B) pathways in Atlantic salmon (*Salmo salar*) that are significantly ($p<0.05$) affected by high (15 mg kg^{-1} WW) selenite (grey arrow) or high (15 mg kg^{-1} WW) selenomethionine (SeMet)-yeast (open arrow) fortified diets for 3 months. Arrow indicated significant ($p<0.005$) increase or decrease of metabolite compared to control. Times fold decrease range is given under the arrow, and when more metabolites per metabolite class were significantly affected (B) the total number of metabolite affected are given above the arrow as # number of metabolites.

Table 3. Number of metabolites in main pathways that were significantly affected in the liver of Atlantic salmon (*Salmo salar*) fed low or high selenite fortified diets (1.1 and 15.0 mg kg^{-1} WW, respectively), or low or high selenomethionine (SeMet)-yeast (2.1 and 15.0 mg kg^{-1}WW, as activated selenium yeast, respectively) for 3 months

Pathway	Treatment	Number of metabolites	Pathway	Treatment	Number of metabolites
Monoacylglycerol	low selenite	0	Methionine, Cysteine, SAM Metabolism	low selenite	0
Monoacylglycerol	low SeMet-yeast	0	Methionine, Cysteine, SAM Metabolism	low SeMet-yeast	0
Monoacylglycerol	high selenite	13	Methionine, Cysteine, SAM Metabolism	high selenite	8
Monoacylglycerol	high SeMet-yeast	2	Methionine, Cysteine, SAM Metabolism	high SeMet-yeast	5
Diacylglycerol	low selenite	0	Endocannabinoid	low selenite	0
Diacylglycerol	low SeMet-yeast	0	Endocannabinoid	low SeMet-yeast	0
Diacylglycerol	high selenite	3	Endocannabinoid	high selenite	6
Diacylglycerol	high SeMet-yeast	1	Endocannabinoid	high SeMet-yeast	2
Long Chain Fatty Acid	low selenite	0	antioxidants vitamins	low selenite	0
Long Chain Fatty Acid	low SeMet-yeast	1	antioxidants vitamins	low SeMet-yeast	0
Long Chain Fatty Acid	high selenite	11	antioxidants vitamins	high selenite	3
Long Chain Fatty Acid	high SeMet-yeast	3	antioxidants vitamins	high SeMet-yeast	0
Polyunsaturated Fatty Acid	low selenite	0	Glutathione Metabolism	low selenite	0
Polyunsaturated Fatty Acid	low SeMet-yeast	0	Glutathione Metabolism	low SeMet-yeast	0
Polyunsaturated Fatty Acid	high selenite	4	Glutathione Metabolism	high selenite	2
Polyunsaturated Fatty Acid	high SeMet-yeast	1	Glutathione Metabolism	high SeMet-yeast	1

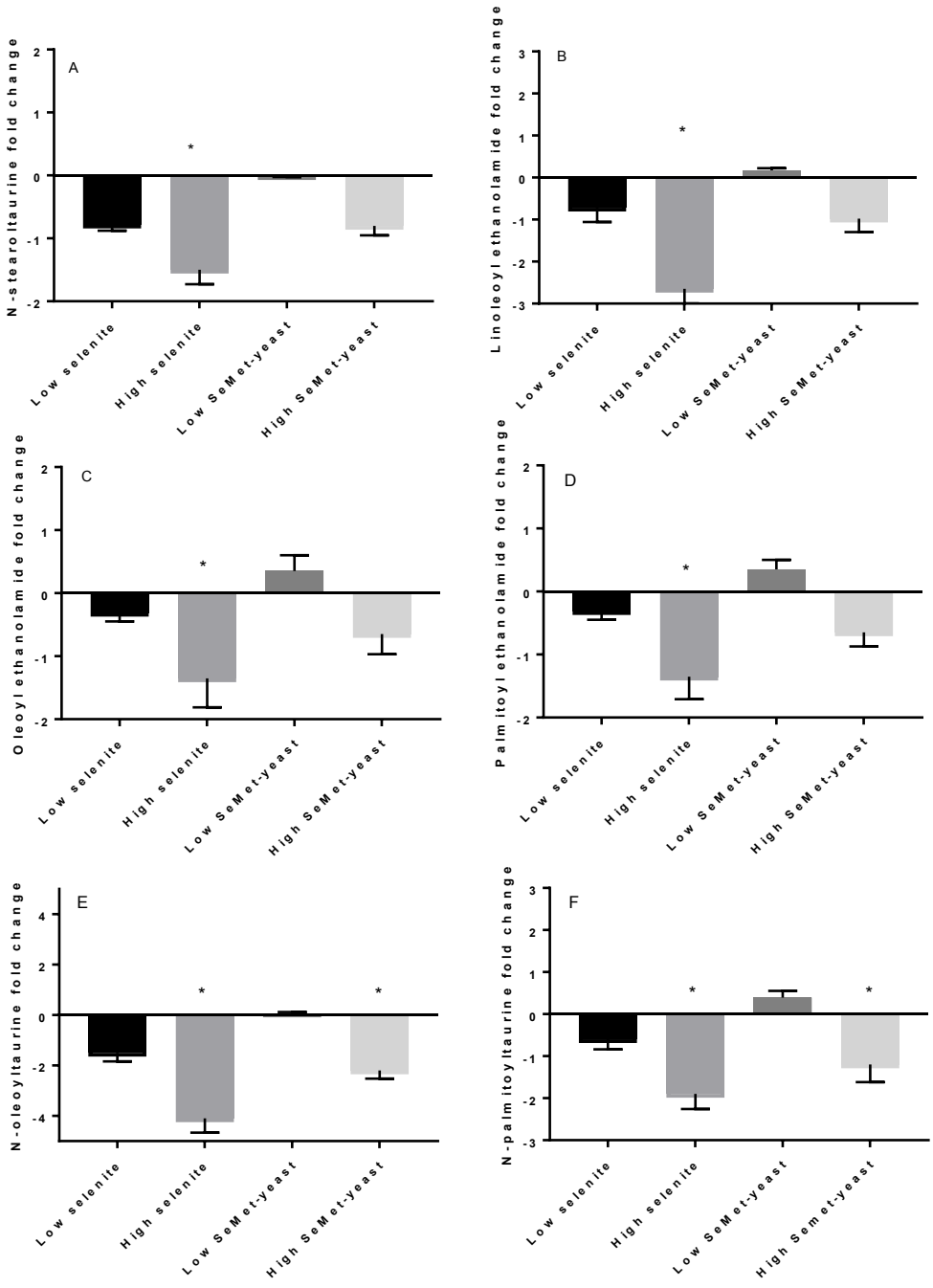

Figure 4 (A-F) Relative fold change compared to control of the liver endocannabinoids, N-stearoyltaurine (A), linoleoyl ethanolamide (B), oleoyl ethanolamide (C), palmitoyl ethanolamide (D), N-oleoyltaurine (E), and N-palmitoyltaurine (F) in Atlantic salmon (*Salmo salar*) fed control, low or high selenite fortified diets (1.1 and 15.0 mg kg^{-1} WW, respectively), or low or high selenomethionine (SeMet)-yeast (2.1 and 15.0 mg kg^{-1} WW, as activated SeMet-yeast, respectively) for 3 months (mean ± SD, n=9). Bars with asterisk (*) are significantly ($p<0.05$) different from control.

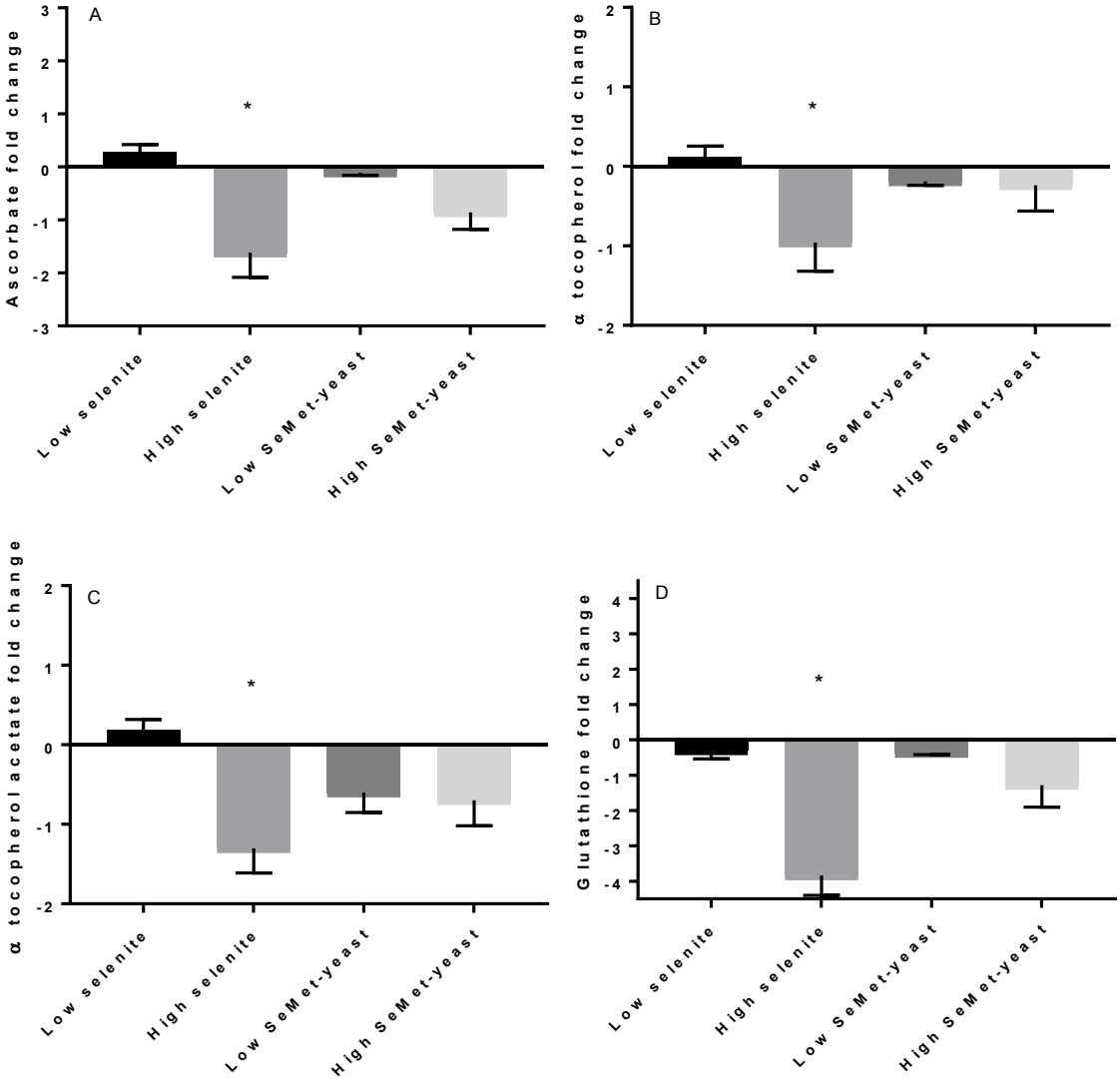

Figure 5 (A-D). Relative fold change compared to control of the liver antioxidant metabolites, ascorbate (vitamin C) (A), α-tocopherol (vitamin E) (B), α-tocopherol acetate (C), glutathione (D) in Atlantic salmon (*Salmo salar*) fed control, low or high selenite fortified diets (1.1 and 15.0 mg kg^{-1} WW, respectively), or low or high organic Se (2.1 and 15.0 mg kg^{-1} WW, as activated SeMet-yeast, respectively) for 3 months (mean ± SD, n=9). Bars with asterisk (*) are significantly ($p<0.05$) different from control.

Discussion

In the present study, no significant difference in growth or feed conversion was observed between the control group and fish fed the low selenite (1.1 mg kg^{-1}WW) or the low SeMet-yeast(2.1 mg kg^{-1}) diets. In earlier toxicological studies, the no observed adverse effect level (NOAEL) was set at higher levels. Studies with adult rainbow trout also showed no adverse effects (unchanged growth or liver lipid peroxidation) when fed 3.9 mg kg^{-1} selenite or 7.4 mg kg^{-1} Se yeast WW (Rider et al., 2009), and cutthroat trout (*Oncorhynchus clarkii*) tolerated SeMet dietary levels of 11.2 mg kg^{-1} WW (Hardy et al., 2010). In contrast, initial-feeding rainbow trout fed 4.6 mg kg^{-1} DW SeMet showed reduced growth (Vidal et al., 2005), juvenile chinook salmon (*Oncorhynchus tshawytscha*) fed 9.6 mg kg^{-1} WW SeMet showed increased mortality (Hamilton, 2004), and juvenile rainbow trout chronically fed 9 mg kg^{-1} selenite showed increased mortality (Hamilton, 2004). Although there were some significant differences between the control group and the low selenite and low SeMet-yeast groups in terms of affected metabolites, no signs of adverse effects could be seen for these groups. Thus, the control and the two lowest levels of Se seemed to be within the requirement and tolerance range of Se.

In the present study, liver Se levels were highest in the fish given diets fortified with high (15 mg kg^{-1} WW) selenite whereas whole-body Se concentrations were highest in the fish given diets supplemented with high (15 mg kg^{-1} WW) SeMet-yeast . Similar results in liver and whole-body Se levels were observed in earlier studies where Atlantic salmon was fed non-toxic 1 and 2 mg Se kg^{-1} WW as selenite or SeMet, respectively (Lorentzen et al., 1994), In the present study, Atlantic salmon fed high selenite showed reduced feed intake and growth, i.e., apparent toxicity, while those fed of SeMet-yeast were free of such changes. The reduced growth was only seen in the first 1.5 months of the exposure, while the last 1.5 months of exposure showed a recovery in growth. Such compensatory growth is the unusually fast growth that follows a period of reduced growth as a result from restricted food intake or external stress (Sevgili et al., 2012). The observed reduced feed intake at 15 mg kg^{-1} WW selenite concurs with studies on juvenile rainbow trout showing a reduced feed intake and growth at 13 mg kg^{-1} selenite WW (Hamilton, 2004; Hilton et al., 1980). Reduced feed intake or food avoidance has also been reported for SeMet fortified feed when given at levels of 104 mg kg^{-1} DW to white sturgeon, while no alteration in feed behavior was observed in fish fed 22 mg kg^{-1} DW (Zee et al., 2016a). In the present study, no changes in feed intake or growth were seen at lower exposure levels of 15 mg kg^{-1} WW SeMet-yeast. As the high selenite group was the only group with reduced feed intake, it is unclear whether metabolic changes, such as altered liver lipid metabolism, are the cause of reduced feed intake only or direct organ damage due to Se accumulation. In addition, food aversion in fish fed Se-spiked diets could be due to the odor. However, despite reduced feed intake during the first 1.5 months of the experiment, Se accumulated in a dose-dependent matter with higher whole fish and liver Se levels for fish fed high selenite compared to low selenite group.

Although the high SeMet-yeast fed fish showed no reduced feed intake, similar changes in lipid metabolism were seen as in the high selenite fed fish, although to a lesser extent. Interestingly in the present study, metabolic assessment showed a strong significant reduction in liver endocannabinoids in the high selenite group. Endocannabinoids play a role in many biological processes including pain-sensation, memory (Ruhl et al., 2015), stress (Wolkers et al., 2015), immune function (Witkamp and Meijerink, 2014), and appetite (Hansen and Artmann, 2008). The reduction of endocannabinoids might be due to a blocking of enzymes in endocannabinoid (e.g. anandamide) synthesis, which are typically blocked by SH blocking agents (Wang and Ueda, 2009), e.g excess selenium. In addition, selenite is a sulfur substitute and selenite is incorporated into selenocysteine, a constituent part of enzymes active in the synthesis of endocannabinoid (Wang and Ueda, 2009). Further liver lipid metabolomics

responses in the high selenite fed fish included a decrease in LCFA, monoacylglycerols (MAG) and diacylglycerols (DAG). Fish fed high SeMet-yeast also showed reduced LCFA and MAG, and DAG, but to a lesser extent than for selenite. Recently, also rainbow trout fed lower levels of Se-yeast (4 mg kg^{-1}) showed an upregulation of genes involved in lipid metabolism (Pacitti et al., 2016b). Rainbow trout fed sub-lethal levels of SeMet (20-40 mg kg^{-1} WW) showed an upregulation on genes involved in long chain fatty acid transport and oxidation pathways that indicated increased mitochondrial use of long fatty acids as energy source, as was also reflected in reduced liver triglyceride (TAGs) levels (Knight et al., 2016). In contrast, female rainbow trout fed 8.4 mg SeMet kg^{-1} WW had no changes in liver triglyceride, while muscle triglycerides increased rather than decreased (Wiseman et al., 2011b). In the present study neither high selenite nor high SeMet-yeast affected liver TAGs, but a clear reduction in MAG and DAG was seen in the high selenite group, and a reduction in only MAG in the high SeMet-yeast group. Increased energy expenditure of lipids could have caused a reduced LCFA and mono- and diacylglycerol, as coenzyme A was also reduced in the present study as a possible result of increased β-oxidation. Alternatively, high selenite has affected the synthesis of free fatty acids. The increased lipid metabolism in the fish fed high selenite and to a lesser extent in the fish fed high SeMet-yeast diet, was also suggested by the Ingenuity Pathway Analyses (IPA), with 11 metabolites predicting inhibited lipid deposition in the selenite group and 5 in the high SeMet-yeast group (Figure 6A and B, respectively). In the present study, however, the increased lipid metabolism did not lead to significant differences in whole body lipid in the high selenite group, and tissue specific (e.g. liver) lipid deposition assessment is recommended in future dietary Se toxicity assessments.

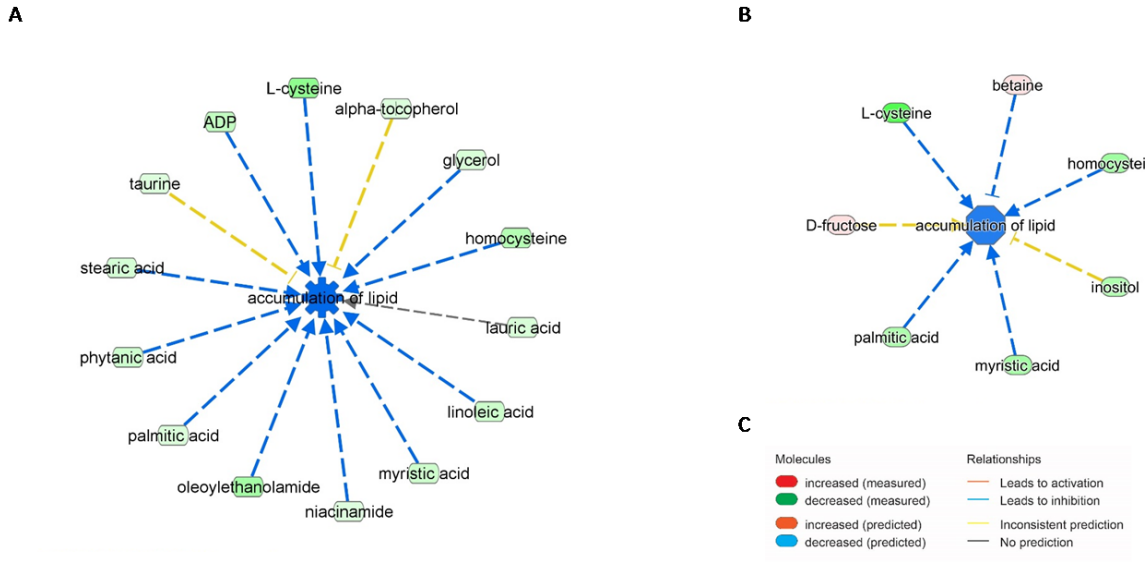

Figure 6 (A-C). Ingenuity Pathway Analysis (IPA) of “upstream regulators” and “biological functions” of the lists of significantly regulated proteins ($p < 0.05$, one-way ANOVA). Biological functions analysis of IPA highlighted “accumulation lipids” to be significantly inhibited by 11 metabolites in high selenite (15 mg kg^{-1} WW) fed Atlantic salmon (*Salmo salar*) (A), while high selenomethionine (SeMet)-yeast (15 mg kg^{-1} WW, as activated SeMet-yeast) significantly inhibited "accumulation of lipids" with 5 metabolites (B). Activation (displayed in green) and inhibition (displayed in blue) of regulators and functions are based on IPA activation z-scores, which combine directional information encoded in the protein expression results with knowledge from the literature to make predictions about likely adverse outcome pathways. Up-regulated proteins are coloured red, down-regulated proteins are coloured green (C). The full data-sets of the upstream regulator analysis and the diseases and biological functions are presented in Table S1

Concurrent with reduced growth and lipid metabolic changes, oxidative stress as seen from TBARS and reduced tocopherol was observed in the high selenite group. Metabolomic assessment further indicated oxidative stress in the high selenite, with lowered vitamin C, α-tocopherol intermediates and GSH. Fish fed high SeMet-yeast had no differences in TBARS or tocopherol levels or altered anti-oxidant metabolomics (e.g. vitamin C or α-tocopherol intermediates) as observed for the high selenite group, however, high SeMet-yeast did have lowered levels of GSH. The higher oxidative damage of selenite compared to SeMet-yeast is further corroborated from differences in IPA predicted activation of the formation of reactive oxygen species (ROS) between the two groups, with more metabolites related to pathways leading to formation of reactive oxygen species significantly affected in fish fed high selenite compared to high SeMet-yeast fed fish (Figure 7A and B, respectively).

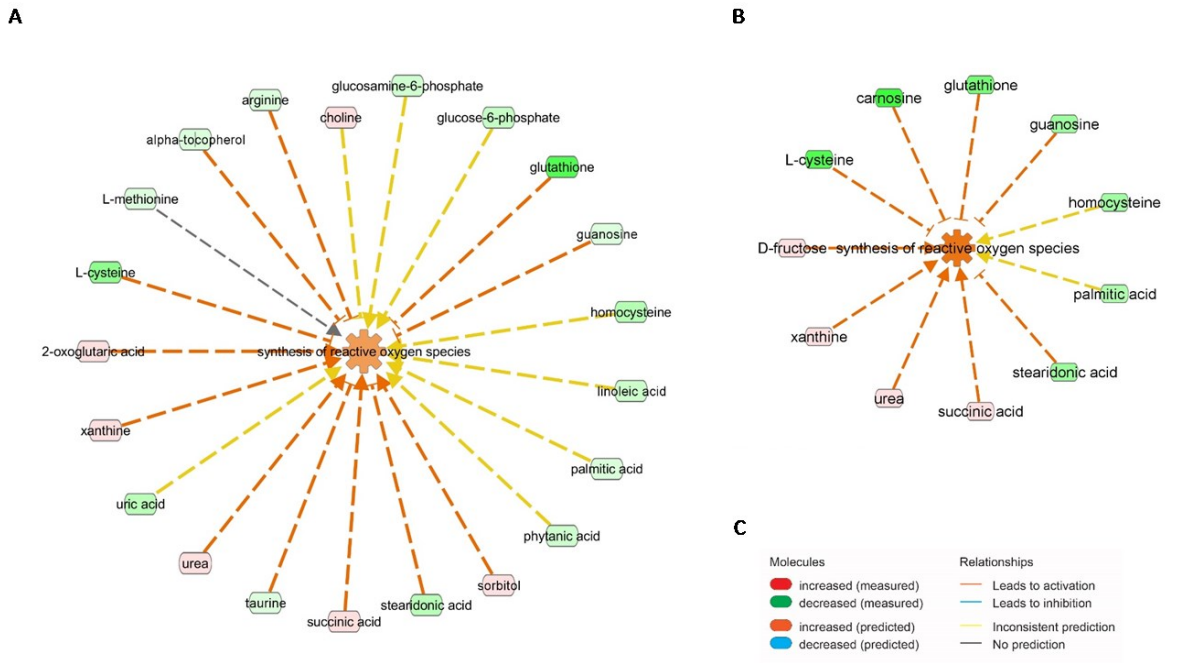

Figure 7 (A-C). Ingenuity Pathway Analysis (IPA) of "upstream regulators" and " disease and functions" of the lists of significantly regulated proteins ($p < 0.05$, one-way ANOVA). Biological functions analysis of IPA highlighted "synthesis of active oxygen species" to be significantly activated by 12 metabolites in high selenite (15 mg kg^{-1}) fed Atlantic salmon (*Salmo salar*)(A), while high selenomethionine (SeMet)-yeast (15 mg kg^{-1} WW, as activated Se-yeast) significantly inhibited " synthesis of active oxygen species " with 8 metabolites (B). Activation (displayed in green) and inhibition (displayed in blue) of regulators and functions are based on IPA activation z-scores, which combine directional information encoded in the protein expression results with knowledge from the literature to make predictions about likely adverse outcome pathways. Up-regulated proteins are coloured red, down-regulated proteins are coloured green. The full data-sets of the upstream regulator analysis and the diseases and biological functions are presented in Table S1.

Oxidative stress has been suggested as a central mechanism in selenite and SeMet mediated toxicity in fish (Hursky and Pietrock, 2015; Lee et al., 2015; Miller et al., 2007; Misra et al., 2012; Misra and Niyogi, 2009), although recent studies report dietary SeMet induced toxicity without clear oxidative stress (Knight et al., 2016; Zee et al., 2016b). Two mechanisms of Se mediated redox cycling and GSH consumption have been identified; one is production of

superoxide anion by Se compounds (Spallholz et al., 2004), where selenite is much more potent than SeMet (Goswami et al., 2012), and the other is oxidation by flavin containing monooxygenase to selenoxides, which are potent oxidizers of GSH and protein thiols (Lavado et al., 2012). Both the high selenite and SeMet-yeast groups had reduced GSH and cysteine, with less reduction in the SeMet-yeast group compared to the selenite group. As cysteine is part of the GSH tripeptide, depletion of both cysteine and GSH indicate use of GSH during high selenite and SeMet-yeast exposures. Lowered GSH and a shift from reduced to oxidized glutathione (GSH:GSSG) together with increased anti-oxidant enzymes was observed in selenite and SeMet exposed rainbow trout hepatocytes (Misra and Niyogi, 2009). The lowered GSH at excess selenite and SeMet intake could be due to GSH's direct role as antioxidant or substrate to antioxidant enzymes such as glutathione peroxidase (GPx), but also due to GSHs involvement in selenite and SeMet cellular metabolism. Selenite reacts with GSH and produces hydrogen selenide, which can form ROS (Lin and Spallholz, 1993) that further increases the demand for antioxidants such as GSH. SeMet is methylated to methylselenol forms for further excretion, if not directly incorporated in general body proteins (Suzuki, 2005). The redox cycling of these methylselenols in the presence of GSH has been suggested to produce oxidative stress (Palace et al., 2004).

In addition to reduced GSH and cysteine, the high selenite group also showed a reduction in metabolites of the methionine and SAM pathway. The SAM pathway is involved in the formation of methionine to homocysteine which is further metabolized to cysteine, and depletion of SAM metabolites can at least partly be explained by increased GSH and cysteine expenditure as seen in both high selenite and SeMet-yeast fed fish. In addition to cysteine production, the SAM pathway is also essential as methyl donor in the excretion of excess selenite and SeMet (Suzuki, 2005). Hydrogen selenide from selenite is methylated by SAM into methylselenol (MMSe), which is further stepwise methylated into dimethylselenide (DMSe) and trimethylselenonium (TMSe) for excretion (Suzuki, 2005). The need to excrete excess selenite through methylation could also explain the depletion of the SAM metabolites. The high SeMet-yeast group showed no significant difference in metabolites of the SAM pathway, despite excess level in feed and reduced GSH and cysteine levels. Excess SeMet is, like hydrogen selenide from selenite, directly methylated into methylselenol forms for excretion (Thiry et al., 2012). However, as opposed to selenite, SeMet can be non-specifically be incorporated in general proteins in place of methionine, while part can be included into specific selenoproteins after first being metabolized into selenocysteine (SeCys) (Suzuki, 2005). Selenite, after being metabolized into hydrogen selenide and further to SeCys, only becomes part of selenoproteins (Suzuki, 2005). The use of SeMet as a substitute for methionine in the general protein pool, might explain the absence of SAM depletion in high SeMet-yeast fed fish compared to selenite fed fish and consequently the lower ability to induce oxidative stress. In the present study, oxidative stress concurred with other toxic responses such as reduced growth and altered lipid metabolism, and oxidative stress seemed to be one of the mechanisms of dietary selenite induced toxicity. For SeMet-yeast supplemented feeds, oxidative stress was less profound but still causing a metabolic depletion in GSH and GSH precursors. The depletion of SAM pathway intermediates by selenite and not SeMet-yeast is likely to reflect the direct methylation and the larger general SeMet incorporation in proteins with less need for excretion.

The non-specific incorporation of SeMet in the general protein pool as opposed to the specific incorporation of selenite in selenoproteins as SeCys, is reflected by the higher muscle Se levels in fish fed SeMet-yeast compared to selenite in the present study. Rainbow trout fry fed Se-yeast and selenite showed elevated whole body SeMet levels in Se-yeast fed fish, while SeCys which is as part of the specific selenoproteins was similar (Godin et al., 2015). Several guidelines have been proposed for relating Se concentrations in the whole body of fish with

adverse effects. Deforest et al. (1999) suggested a whole body threshold of 9 mg kg^{-1} DW for warm water fish and 6 mg kg^{-1} DW for larval cold-water anadromous fish.. A. In the present trial whole body levels of 15 mg kg^{-1} SeMet-yeast fed fish was 8.6 mg kg^{-1} DW, which is slightly above the proposed USEPA (2016) Se criterion of 8.5 mg kg^{-1} DW Se. The 15 mg kg^{-1} selenite fed fish had whole body levels of 5.5 mg kg^{-1} DW, which is below the proposed USEPA (2016) criterion. However, several toxic responses such as reduced feed intake, growth and lipid peroxidation, were seen for the selenite fed fish compared to the SeMet-yeast fish, suggesting that a Se specific assessment for tissue and whole body criteria is necessary.

In conclusion, selenite at a level of 1 mg kg^{-1} and SeMet-yeast at a level of 2.1 mg kg^{-1} WW did not cause adverse effects in adult Atlantic salmon. Sub-lethal toxic levels of 15 mg kg^{-1} WW selenite caused reduced feed intake and growth, while SeMet-yeast at the same level had no effect on growth. The high selenite exposure, and to a lesser degree the SeMet-yeast exposure, caused disturbance in lipid metabolism as seen from depressed levels of free fatty acids, monoacylglycerols and diacylglycerols as well as endocannabinoids. Selenite, but not SeMet-yeast, affected the metabolites in the S-Adenosylmethionine (SAM) pathway, indicating a use of methyl donors that could be allied with excess Se excretion. Together with disturbance in lipid metabolism, oxidative stress was one of the modes of toxic action for both high selenite and SeMet-yeast fed Atlantic salmon, with a higher toxicity for selenite compared to SeMet-yeast. The identification of biochemical pathways after elevated Se exposure is an essential step in identifying possible adverse outcome pathways that biochemical findings to organism and/or population level (Ankley et al., 2010). The Se induced oxidative stress observed in the present study is likely to affect the nutritional status of the fish as seen from lowered vitamin E levels, which can eventually lead to vitamin deficiency, while the observed liver lipid peroxidation can cause tissue damage. Future more detailed studies on liver toxicity as determined by specific histological and biochemical end-points are necessary to set safe limits. The observed alteration in endocannabinoid can disturb immunological function, while general loss of lipid synthesis can affect the build-up of energy stores and impact seasonal energy reserves in a fish population. Further research is needed to assess the role of biochemical pathways towards well-known endpoint of Se toxicity such as embryo/larval teratogenic deformities.

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5 Fish welfare

Safe limits of selenomethionine and selenite supplementation to plant-based Atlantic salmon feeds.

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Abstract
The use of plant based feeds may warrant the supplementation with selenium to cover the requirement for Atlantic salmon. Depending on its chemical form, selenium (Se) is a trace element with a narrow range between requirement and toxicity for most vertebrates. Information on safe upper limit for Atlantic salmon feed supplementation is lacking. Atlantic salmon (147 g) were fed a low natural background organic Se diet (0.45 mg Se kg^{-1}, wet weight (WW)) fortified with 5 graded levels of inorganic sodium selenite (0.45, 5.4, 11.0, 29.4, or 60.0 mg kg^{-1} ww) or organic selenomethionine (SeMet) at 5 levels (0.45, 6.2, 16.2, 21, 39 mg kg^{-1} ww), in triplicate for 3 months. Excess Se supplementation was assessed by targeted biomarkers of Se toxicity pathways (e.g. markers of oxidative stress and lipid metabolism), as well as general adverse effect parameters (plasma biochemistry, hematology, liver histopathology, and growth). Safe limit of intake were set by model-fitting the dose-response effect data in a dose-response (lower bound) bench mark dose (BMD) evaluation. Fish fed the two highest selenite levels showed mortality while fish fed SeMet had no mortality. Fish fed selenite 5.4-11 mg kg-1 showed significantly (ANOVA, Tukey's t-test, $p<0.05$) increased liver oxidative stress, as seen from altered GSH and vitamin E, and liver damage as seen from increased plasma ASAT and liver histopathology such as degeneration and focal necrosis. Fish fed SeMet mainly showed liver pathology and kidney dysfunction as seen from altered plasma creatinine and total protein in fish fed ≥ 21 mg kg^{-1}, compared to control. For selenite exposed fish, a safe feed limit were set at 1-2 mg kg^{-1} ww feed (daily dose 0.01-0.02 mg kg BW^{-1} day^{-1}), based on plasma ASAT increase, liver vitamin E depletion, and liver histopathology. For SeMet fed fish, the safe feed limit was higher than for selenite with a 2.8 mg kg-1 ww (dose 0.03 mg kg BW^{-1} day^{-1}), based on liver histopathology and plasma creatinine. In conclusion, with regards to fish welfare, Atlantic salmon seemed to tolerate the supplementation of selenite or SeMet to a level of total selenium of respectively 1-2 or 3 mg kg^{-1} feed (daily dose ~0.01-0.02, or ~0.03 mg kg BW^{-1} day^{-1}), respectively, in a high plant-based salmon feed with background levels of 0.45 mg Se kg^{-1}.

Key words: Atlantic salmon, selenite, selenomethionine, toxicity, bench mark dose

Introduction

Due to a rapid growth in aquaculture and limited access to marine resources, fish oil and fish meal in feeds for carnivorous marine species such as Atlantic salmon (Salmo salar) have been replaced with plant ingredients the last decade (Ytrestoyl et al., 2015). The change from marine to plant feed ingredients will alter the nutritional composition of salmon feeds, reducing essential micro-nutrients that are naturally high in fish meal and oil such as vitamins and minerals (Sissener et al., 2013). Selenium (Se) is one of the essential minerals that is known to be higher in fish meal than plant ingredients (Betancor et al., 2016), although plant products can differ largely in Se content according to Se concentration in soil (Alfthan et al., 2015). A decline of selenium in Norwegian produced commercial salmon feed during the last decade has been attributed to the decreased use of fish meal (Sissener et al., 2013). Furthermore, the use of plant ingredients may reduce the bioavailability of minerals due to present of phytates (Denstadli et al., 2006). Selenium concentration in Atlantic salmon (*Salmo salar*) flesh were lower when fed on plant protein replacement feeds compared to marine protein feeds (Betancor et al., 2016). Several studies have indicated the need for supplementation of Se in plant based feed to marine carnivorous fish (Fontagne-Dicharry et al., 2015; Godin et al., 2015; Pacitti et al., 2015; Ilham et al., 2016a; Ilham et al., 2016b). Of the mineral supplements used, the organic form, seleno-methione (SeMet) or in Se-yeast forms have a higher bioavailability than inorganic selenite forms (Rider et al., 2009; Fontagne-Dicharry et al., 2015). Studies on Atlantic salmon indicate that the natural Se levels in plant based diets cover requirement (Hamre et al., 2016). Higher Se requirements, and hence need for possible supplementation, might occur during the early life stages of fish (Bell et al., 1985) or during handling stress (Rider et al., 2009). In the EU, feeds can be supplemented with organic (e.g. selenized yeasts) Se to a maximum authorised level of 0·2 mg/kg (Regulations (EU) No 427/2013; 445/2013; 121/2014; 847/2014 and 2015/489). The maximum limit for total Se in animal feeds including fish feed has been set at 0.5 mg/kg feed ((EC) No 1831/2003 and amendments).

Selenium has a narrow range between its toxic and its beneficial effects (Wang and Lovell, 1997; Teh et al., 2004; Han et al., 2011; Lee et al., 2016). Supplementation of aquafeeds with SeMet or selenite hence require toxicological assessment to set safe upper limits that protect fish welfare (Berntssen et al., 2017). Several studies have given an overview adverse effect levels in several fish species exposed to both excess dietary inorganic and organic Se (Lemly, 1993a; Hamilton, 2004; Zee et al., 2016a). A wide range of effect concentrations have been reported that differ between fish species and life stages, hence species specific possible adverse effects of Se supplementation is important to consider. Several studies have performed graded dose response with organic or inorganic selenium for salmonids, including chinook salmon (*Oncorhynchus tshawytscha)* (Hamilton et al., 1990), cutthroat trout (Oncorhynchus clarkii) (Hardy et al., 2010), and rainbow trout (*Oncorhynchus mykiss*) (Hilton et al., 1980; Hamilton et al., 1990; Hamilton, 2004; Palace et al., 2004; Rider et al., 2009; Hunt et al., 2011; Wiseman et al., 2011a; Wiseman et al., 2011b; Knight et al., 2016; Pacitti et al., 2016b). However, few studies have assessed selenite and SeMet supplementation in Atlantic salmon (Lorentzen et al., 1994; Berntssen et al., 2017), which is one of the main farmed salmonids.

Several modes of toxic action (MOA) for both inorganic and organic selenium have been reported. Oxidative stress has been suggested as one of the main causes of Se toxicity (Lee et al., 2015) for both inorganic Se (Miller et al., 2007; Choi et al., 2015; Hauser-Davis et al., 2016) and organic Se forms (Palace et al., 2004; Han et al., 2010; Hursky and Pietrock, 2015). However, oxidative stress was not believed to be a main driver of dietary SeMet toxicity in white sturgeon (*Acipenser transmontanus*) (Zee et al., 2016a; Zee et al., 2016b), and juvenile rainbow trout exposed to selenized-yeast worms showed reduced growth and liver triglyceride

(TAG) while no oxidative stress response were seen (Knight et al., 2016). Recent wide-scope pathway assessments by use of metabolomics have shown that oxidative stress is one of the main underlying toxic mechanism of both dietary selenite and SeMet in Atlantic salmon (Berntssen et al., 2017). Further wide-scope pathway assessments by transcriptomics have shown that disturbed lipid synthesis and metabolism could be one of the main drivers of organic Se toxicity in rainbow trout (Knight et al., 2016; Pacitti et al., 2016a), which was confirmed by use of metabolomic screening in Atlantic salmon fed excess selenite and SeMet (Berntssen et al., 2017). Assessment of biochemical markers in the central pathways of dietary selenite and SeMet toxicity can be used as early biomarkers of excess selenite and SeMet exposures. Atlantic salmon fed sublethal selenite and SeMet levels (15 mg kg-1), showed reduced vitamin E production of peroxidative products and reduction in glutathione as markers of oxidative stress, and altered lipid class production as markers of disturbed lipid metabolism (Berntssen et al., 2017). The use of biomarkers of central pathways of toxicity is valuable in sub-chronic studies (10% of life cycle) were chronic whole body adverse effects are expected to occur only after prolonged exposure. Final adverse effect outcomes of dietary selenite and SeMet in fish, include decreased egg viability (Schultz and Hermanutz, 1990), reduced neurological and immunological functions (Choi et al., 2015), reduced growth (De Riu et al., 2014; Zee et al., 2016a; Berntssen et al., 2017) and energy stores (De Riu et al., 2014; Zee et al., 2016a) pathological effects on kidney, liver (Hicks et al., 1984; Teh et al., 2004; Tashjian et al., 2006; Zee et al., 2016a), pathological effects on heart and ovaries, and skeleton/cranial deformation (Lemly, 2002; Hamilton, 2003, 2004).

Traditionally, animal welfare safe dietary levels of feed supplements in toxicological studies are assessed by establishing a no observed adverse effect level (NOAEL) based on a (sub)-chronic dose-response study with graded levels of the supplement (Teh et al., 2004). The European food safety agency (EFSA), recently evaluated the methods to assess safe feed levels, and advised to use bench mark dose (BMD) models instead of NOAEL to address the adverse effect to excess supplements or level of contaminants (EFSA, 2017b). In addition a guidance document was published in which the difference between adverse effect, biomarkers of exposure or effect, and mode of action (MOA) were defined (EFSA, 2017a). In general, dose response effects of direct adverse effects (i.e. reduced growth, histopathology) are weighed in the BMD with a benchmark response (BMR) of 5%, while for biomarker of effect or exposure (i.e. plasma enzymes and organ oxidative stress) a higher (20%) BMR is used. For histopathology data (i.e. degeneration and focal necrosis) a BMR of 10% is used with an extra risk factor assessment (EFSA 2017b). The present study assessed the safe limits of selenite and SeMet supplementation to plant based feed with regards to the welfare of Atlantic salmon by assessment of targeted biomarkers of Se toxicity pathways (e.g. markers of oxidative stress and lipid metabolism) and general adverse effect parameters (plasma biochemistry, hematology, histopathology, and growth) in an EFSA dose-response bench mark dose assessment.

Material and methods

Ethic statement

The experiment was approved by the Norwegian National Animal Research Authority (Mattilsynet; FOTS ID: 9003) and performed in compliance with national and international ethical standards.

Experimental conditions

The feeding trial was carried out at NOFIMA (Sunndalsøra, Norway) between November, 15 2016-March 3 2017. A total of 1890 Atlantic salmon smolt (*Salmo salar*, L., Salmobreed, 6 months, both genders) were randomly distributed into 27 tanks (1.4 m^2 and ca 840 l volume) with 70 fish in each tank with an initial weight of 147 ± 4 g (mean ± SD, n=30). Prior to the experiment, all fish were fed a control diet during a 2-week acclimation to holding facilities. Thereafter, randomly selected tanks received one of nine experimental diets for 3 months, in triplicate. The feeding regime was based on automatic feeders under a photoperiod regime with 24h light. Six daily meals were provided with 4 hours between the meals, to a level approximating 1 % of body weight per day. The feeding rate was adjusted for growth biomass increase, which was assessed by measured average weight gain of the sampled fish per sampling time point. Fish were routinely monitored for nutritional performance and appetite throughout the experiment. Unconsumed feed pellets were collected and weighed once per day, and feed intake, feed conversion and Se exposure were calculated. To avoid possible leakage from feces or pellets to the water, a relative high water flow-through was maintained of 12 L min−1 per tank. Water Se levels were monitored by routine water samples of 50 ml, which were taken from each tank and acidified with nitric acid 65% HNO3 (Suprapur, Merck, Germany) in a final concentration of 5.2% for Se analysis. Environmental parameters in tanks were measured five times a week, showing a salinity of 27 ± 0.3 ‰, temperature of 8.0 ± 0.3 °C, and oxygen levels of 85 ± 4 % at the outlet.

Five fish per tank (n = 15 per dietary group) were sampled at 90 days of exposure for tissue sampling. Fish were randomly collected from the tanks, anesthetized in a bath of tricaine methanesulfonate (FINQUEL MS-222; ~ 60mg L^{-1}). The fish were sacrificed by a blow on the head and blood samples were taken from the caudal vein quickly following the initial anesthetization, using a heparinized VACUETTE® blood collection tube with 21G x 1' needle. Whole blood was divided into two aliquots, one of which was used for immediate on-site analyses of haematocrit and the other aliquot (~200 mL) was kept on ice for erythrocyte count and haemoglobin determination, which were performed within two days after sampling. For plasma samples, the remaining whole blood were centrifuged at 3500*g* for 10 min, and the plasma was snap-frozen in liquid nitrogen and stored at -80℃ until further analysis (see below). Body weight and length of each fish were recorded, and liver, heart and spleen was sampled and weighed. Liver samples were divided into three parts (for analyses of Se content, oxidative stress as tocopherol and TBARS, and glutathione, see sections under) and immediately frozen in liquid nitrogen followed by storage at −80◦C until biochemical analyses. In addition, a liver section was taken for histological assessment of the first 3 sampled fish per tank (see under). Liver, heart and spleen of the five sampled fish per tank was weighed as well to assess further organ indexes. Lengths and weight of the remaining fish at the end of the trial were recorded.

Experimental diets

Selenium was added as part of the mineral premixture to the basal diets with low natural Se content. The experimental feeds were produced by Biomar (Denmark), and were formulated based on commercial diets that fulfilled the nutritional requirements of salmonids (NRC, 2011), using standard commercially available feed materials. The general basal diet had the following composition: fish meal (10%), soya SPC (10%), wheat gluten (17%), maize gluten (10%), pea protein 50 (5%), pea protein >72 (5%), wheat (10.5 %), fish oil (12.2 %), rape seed oil (12.2%) and micro-nutrient mixture (8.1 %).

Care was taken to select ingredients with low levels of Se, as levels in plant material can vary depending on the soil (Alfthan et al., 2015) and a relative low fish meal inclusion was used as this is the main source of Se in salmon diets. The basal diets were supplemented with either inorganic Se (sodium selenite, Na_2SeO_3, DSM, Heerlen, Netherland) or organic Se (>98% L-selenomethionine (Excential Se4000 Minsups, Winsford England) at a nominal concentration of 0, 5, 15, 25 and 50 mg kg-1. The inorganic and organic Se levels were chosen to give a dose-response gradient (see bench mark dose description below), that was expected to provoke mild sub lethal biomarker effects of inorganic and organic Se (1-5 mg kg-1(Berntssen et al., 2017)), more sever chronic sub-lethal toxicity for organic Se (~20 organic Se as Se-yeast fed worms mg kg^{-1} dry matter, (Knight et al., 2016; Berntssen et al., 2017) or inorganic Se,(~9-15 mg Se kg^{-1} (Hamilton, 2004; Berntssen et al., 2017), and or sever chronic toxicity (positive control) (>20 SeMet mg kg-1(Hardy et al., 2010), >15 mg selenite kg-1 (Berntssen et al., 2017)) in adult salmonids. The analyzed Se level in the basal diet was 0.45 ± 0.04 mg kg^{-1} (control), 5.4 ± 0.09 or 6.2 ± 0.2 (low), 11.0 ± 0.3 or 16.2 ± 0.3 (low-medium), 29.4±0.9 or 21± 0.3 (medium), 60.0 ± 2 or 39± 0.4 (high) mg kg^{-1} for respectively the inorganic Se and organic Se supplemented diets (n=3, mean±SD)

Se analyses

Diets and tissues of fish were digested using the microwave-acid decomposition method based on the method described by Berntssen et al. (2017), modified after Julshamn et al. (2007). Briefly, samples were weighted (0.20 – 0.25 g) into 15 mL quartz digestion vessel (Milestone Srl, Sorisole, BG, Italy), containing deionized water (0.5 mL; Milli-Q, Merck Millipore, Oslo, Norway). Concentrated nitric acid (HNO_3,2 mL, Fluka, Sigma-Aldrich, Oslo, Norway) were added to all samples. The vessels were capped and placed in the Ultrawave (UW, SRC, Milestone, Shelton, CT, USA), with a container of 30 mL Milli-Q water and 5 mL hydrogen peroxide (H_2O_2, Emsure ACS, ISO, 32% w/w; VWR, Oslo, Norway). The gas pressure in the UW was set to 40 bar and the temperature increased incrementally to 260 °C. The muscle samples were diluted to 25 mL and 10 mL, respectively, with Milli-Q water. Total Se concentration were determined in the digests using ICP-MS (iCAP-Q and FAST SC-4Q DX auto sampler, both Thermo Fisher Scientific Inc, Waltham, Massachusetts, USA). An external calibration curve was made from freshly prepared multi element standard diluted to appropriate concentrations by 5% (v/v) HNO_3. The instrument was used with a collision cell gas, using He KED (kinetic energy discrimination) mode for interference removal. A solution of internal standard (Ge, Rh and Tm, Thermo Fisher Scientific Inc) was added on-line for correction of instrumental drift during the analysis. The instrument was optimized using a tuning solution (1 ppb tuning solution B, Thermo Fisher Inc, in 2% HNO_3 and 0.5% HCl (puriss. p.a, Sigma-Aldrich) prior to analysis. Plasma power was set to 1550 W, carrier/nebulizer gas flow to 1.05 L/min, the plasma/auxilliary gas flow to 0.8 L/min, and He gas (CCT1) flow was 4.6 mL/min. Isotope ^{78}Se was monitored, and the integration time was 0.1 sec. Oyster Tissue (OT, CRM 1566 b, National Institute of Standards and Technology, Gaithersburg, MD, USA) and Lobster Hepatopancreas (TORT-3, National Research Council Canada, NRC, Ontario, Canada) were used as reference materials for the analysis. ***Liver redox-homeostasis***

Earlier wide-scope metabolic screening of Atlantic salmon fed inorganic and organic Se showed liver oxidative stress as one of the main modes of toxic actions(Berntssen et al., 2017). In order to assess liver oxidative stress, reduced and oxidized glutathione (GSH and GSSG, respectively), the fat soluble antioxidant vitamin E, and the lipid peroxidative products were analysed for individual sampled fish. For GSH and GSSG, frozen liver tissue samples were weighed and homogenized in either 4x volume of ice-cold 0.9% saline buffer (9 g/L NaCl in ddH_2O) for GSH analyses, or 2x volume of ice-cold thiol scavenger (*N*-ethylmaleimide pyridine derivative solution, Cat. No. GT35c; Oxford Biomedical Research, MI, USA) diluted 3:7 in 0.9% saline buffer for GSSG analyses, using a ball mill (25 rpm for 1-2 min; Retsch MM301 ball mill, Haan, Germany). The homogenates were then centrifuged (5 min, 1500*g,* 4 °C), and the supernatant was transferred to new tubes. The samples were further prepared using the Cuvette Assay kit for GSH/GSSG (Cat. No. GT35; Oxford Biomedical Research, MI, USA) following the manufacturer's instructions, and GSH and GSSG were analyzed spectrophotometrically for absorbance at 405 nm in a Wallac VICTOR (TM) X5 2030 Multilabel Reader (PerkinElmer Life Sciences, MA, USA).

Vitamin E was analysed as α-, β-, γ- and δ-tocopherol isomers and α-, β-, γ- and δ- tocotrienol by high performance liquid chromatography (HPLC) according to the method described by Hamre et al. (Hamre et al., 2010a; 2010b). In short, the homogenized liver samples were saponified (20 minutes at 100 °C) using ethanol, potassium hydroxide, pyrogallol, ascorbic acid and EDTA, before the samples were extracted three times with hexane. The solvent was subsequently evaporated under nitrogen and the samples were diluted with a standard volume of hexane before injection into the HPLC and detection by fluorescence detector.

Lipid peroxidative products were analysed as thiobarbituric acid-reactive substances (TBARS) and were determined by the method described by Hamre et al. (2001a; Hamre et al., 2001b). Using Bligh and Dyer extraction, fat and water-soluble components in the liver samples were separated, and the aldehydes were extracted from the sample in the methanol:water phase. Thiobarbituric acid (TBA) were added in excess to an aliquot of the methanol:water phase and then heated to form a colored complex between aldehydes in the sample and TBA. The absorption was measured at 532 nm, and the concentration of TBARS were quantified using a standard curve.

Lipid classes

Earlier wide-scope metabolic screening of Atlantic salmon fed inorganic and organic Se showed altered lipid metabolism as one of the main modes of toxic actions (Berntssen et al., 2017). Lipids from pooled liver samples (five fish per tank, hence N=3 per diet) were extracted in a mixture of chloroform–methanol 2:1 (Merck) with 1 % 2,6-di-tert-butyl-4-methylphenol (Sigma-Aldrich) as described by Torstensen *et al* (2004). Briefly, chloroform:methanol (at approximately twenty times the weight of the sample) was added to the samples and lipids extracted overnight at -20 °C. After the extraction of lipids as described above, the samples were filtered and the quantification of lipid class composition was carried out by HPTLC as described by Torstensen *et al.* (2011). Lipid classes were quantified by scanning densitometry using a CAMAG TLC Scanner 3 and calculated using an integrator (WinCATS-Planar Chromatography, version 1.2.0; CAMAG, Berlin, Germany). Quantitative determination (mg lipid class/g tissue) of lipid classes was performed by establishing standard equations for each lipid class within a linear area, in addition to including a standard mixture of all the lipid classes at each high-performance TLC plate for corrections between plate variations. After the extraction of lipids as described above, neutral lipids (NLs) and polar lipids (PLs) were separated and an aliquot of 10 mg lipids (solved in 200 µL chloroform) was applied to a solid-phase extraction column (Isolute; Biotage). NLs were eluted with 10 mL chloroform–methanol (98:2, v/v) and PLs were eluted with 20 mL methanol. For analysis of FAs, the two lipid extracts were filtered and the remaining samples were saponified and methylated using 12% boron trifluoride (BF_3) in methanol. FA composition was analysed where the methyl esters were separated using a Trace gas chromatograph 2000 (Fison, Elmer, USA) equipped with a 50-m CP-sil 88 (Chromopack) fused silica capillary column (id: 0.32 mm) (Lie and Lambertsen, 1991; Torstensen et al., 2004). The FAs were identified by retention time using standard mixtures of methylesters (Nu-Chek, Elyian, USA), and the FA composition (area %) was determined. All samples were integrated using the software Chromeleon® version 6.8 connected to the Gas liquid chromatography (GLC). Amount of FA per gram sample was calculated using 19:0 methyl-ester as internal standard.

Plasma biochemistry

Blood samples were centrifuged at 3500*g* for 10 min to obtain the plasma fraction. The plasma was separated into aliquots, snap-frozen in liquid nitrogen and stored at -80℃ until further analysis. Plasma concentrations of albumin and total protein, alanine aminotransferase (ALT), aspartate aminotransferase (AST), bile acids, bilirubin, creatinine and lysozyme were measured on a PL multipurpose diagnostic analyzer (Maxmat S.A., Montpellier, France) using DIALAB diagnostic kits (Vienna, Austria). Osmolality was assessed by freezing point determination, using a Fiske One-Ten osmometer (Fiske, VT, USA). Sodium, potassium, chloride and free calcium in plasma were determined using the Radiometer ABL-77 Blood gas and electrolyte analyzer (Radiometer, Copenhagen, Denmark).

Hematology

Hematocrit (Hct) was determined immediately from individual sampled blood using Vitex Pari microhematocrit capillary tubes (Vitrex Medical A/S, Denmark) and a microhematocrit centrifuge (Haematofuge, Heraeus-Christ GmbH, Germany). The number of red blood cells (RBC) and amount of hemoglobin (Hb) in full blood were measured in a Cell Dyn 400 Hematological Analyzer (Sequoia- Turner) according to the manufacturer's instructions, using Para 12 Extend control blood (Streck, MedMark Ref:218777) for calibration. Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were calculated from Hct, RBC and Hb as described in Sandnes et al. (1988).

Histology

Liver samples were fixated in 4% formaldehyde overnight, washed in phosphate buffered saline (PBS) and then stored in 70% ethanol until further processing. The fixed tissues were further dehydrated through graded alcohols and xylene, and finally embedded in paraffin. Tissue sections of 5 µm were then stained with hematoxylin and eosin (H&E) and periodic acid-Schiff stain (PAS) for histopathological evaluation. Sections were scanned with a ZEISS Axio Scan.Z1 (Carl Zeiss A/S, Birkeroed, Denmark). After a first screening of the slides, the main histopathological changes were considered for evaluation as these were constantly present in most of the samples. These histopathological changes were graded giving scores from 0-2 or 0-3 (see supplementary data table 1 for description of scoring). All analyses were performed in a double-blinded format

Statistics

No observed adverse effect levels (NOAEL) and lowest observed adverse effect levels (LOAEL) were assessed by addressing significant differences among the dietary treatments by one-way ANOVA. To account for the variance among experimental tanks within a dietary treatment, as well as variance among fish within an experimental tank, nested ANOVA, followed by Tukey's HSD post hoc test were used. All statistics were performed using the program Statistica (Statsoft Inc., Tulsa,USA). In addition to NOAEL and LOAEL assessment, a Benchmark dose (BMD) analysis was conducted on the responses of the graded dietary exposures according to the EFSA's benchmark dose technical guidance (EFSA, 2017b). The use of the 90% lower confidence interval of the BMD (BMDL) is used as alternative to NOAEL for assessing the feed concentration that is safe to use with regards to animal welfare. For continuous data (whole body, organ indices, plasma and blood parameters), two families of (nested) models: the exponential and Hill models were fit on individual data, using the EFSA BMD platform (Proast, version 64.9 https://shiny-efsa.openanalytics.eu/app/bmd). For quantal data (histology), seven models were assessed (logistic, probit, log-logidtic, log probit, Weibull, gamma, and LMS (two-stage) on individual data in the EFSA BMD platform. Selection of models (significantly better model fit) was based on the Akaike information criterion (AIC). A default value of 2 units difference between AICs is considered as the critical value by the EFSA (EFSA, 2017b). BMD models were accepted when the AIC of the model was lower than the AIC of the null model (no dose response) -2 (AIC<AICnull-2), and the model with lowest AIC (AICmin) was lower than the AIC of the full model +2 (AICmin<AICfull+2) (EFSA, 2017b). As model averaging

is recommended as the preferred method for calculating the BMD confidence interval, model averaging was performed for those data sets (quantal: histology parameters) where this option was available in the current version of Proast. For data sets were no averaging option was available (continuous data: whole body, organ indices, plasma and blood parameters) best model based on AIC was used as described by the EFSA (EFSA, 2017b). The 90 % lower and upper confidence intervals for the BMD (BMDL and BMDU, respectively) were estimated including bootstrap with standard 200 Bootstraps. The BMD is defined as the dose that corresponds with a specified estimated change in response compared with the modelled background response. The BMDL is defined as the dose not expected to give an adverse effect. The BMR (benchmark response) is the estimated response corresponding with the BMDL of interest. A default of BMR of 5% change was used as starting point for analyses for continuous data ($BMDL_{05}$) of apparent adverse effects such as reduced growth, altered organ indices or disturbance in haematology. For markers of liver function and osmoregulation (plasma enzymes and electrolytes) or markers of lipid peroxidative stress (vitamin E, GSSSG and GSH), the BMR was expanded as described in the EFSA technical guidance document (EFSA, 2017b), and the BMDL for BMRs of 20% changes were considered ($BMDL_{20}$). For quantal and ordinal data (histology) the default BMR was defined as a specified increase in incidence over background, and BMR of 10% (extra risk; $BMDL_{10}$) was used as described by the EFSA (EFSA, 2017b). Condition factor, specific growth rate, feed intake and feed conversion rate were calculated with the following equations:

$$\text{Condition factor (CF)} = \left(\frac{\text{Final body weight (g)}}{\text{Final body length (cm)}^3}\right) * 100$$

$$\text{Specific growth rate (SGR)} = \left(\frac{\ln(\text{Final body weight (g)}) - \ln(\text{Mean initial body weight(g)})}{\text{90 days of feeding experiment}}\right) * 100$$

$$\text{Daily feed intake} * \text{fish}^{-1}\text{(FI)} = \frac{\text{Recorded feed intake } * \text{tank}^{-1} * \text{day}^{-1}\text{(g)}}{\text{Number of fish } * \text{tank}^{-1}}$$

$$\text{Feed conversion rate (FC)} = \left(\frac{\text{Total feed intake} * \text{fish}^{-1}\text{(g)}}{\text{Body weight gain (g)}}\right)$$

Organ somatic indices were calculated as the ratio of organ- to body weight

Results

Mortality and water quality

Fish fed dietary SeMet had no mortality in any of the dietary groups. In contrast, fish fed selenite showed mortality when fed 29 and 60 mg kg^{-1}, these groups were therefore excluded from subsequent analyses. Mortality in the highest selenite group started after 19-20 days of feeding while for the second highest exposure group (29 mg kg-1), mortality started after 29-31 days of exposure. The dietary groups experiencing mortality were terminated when mortalities reached a pre-defined cut-off described in the animal research approval, after 35

days of exposure. Model predicted mortality curves gave best fitted (logistic) lethal time for 50% of the population (LT50) of 34 (31-37.2, min-max.) days and 27 (27.2-29.6 min.-max.) days for fish fed 29 and 60 mg kg^{-1} selenite fed fish respectively. Apparent leaching of Se from feces or pellets to the water seemed to be minimal (including the two highest dietary selenite groups with mortality), as none of the exposure groups had significantly higher ($p<0.05$) waterborne Se levels than the control group (ANOVA, tukey's t-test, n=3).The waterborne Se levels (mean ±SD) in the experimental groups were 18.8±11.7, 18.1±8.1, 11.8±2.3, 21.4±1.0, 33.3±6.2, 12.7±2.7, 15.3±2.4, 22.4±11.3, 13.2±2.2 (µg L^{-1}), for the control, 5, 11, 35, and 60 mg kg-1 selenite groups and 6, 16, 21, and 39 mg kg^{-1} SeMet groups, respectively.

Liver Se levels

Fish from all exposure groups had significantly elevated liver Se levels compared to the control groups. Besides fish fed 16 and 21 mg kg^{-1} SeMet, fish fed graded levels of selenite or SeMet showed a significant ($p<0.05$) increase in liver Se levels with increased dietary levels. Despite the higher dietary SeMet levels compared to dietary selenite levels, fish fed selenite had significantly higher liver Se levels than fish fed SeMet. Fish fed 5.4 and 11 mg kg^{-1} selenite had significantly ($p<0.05$) higher liver Se levels compared to fish fed respectively 6.2 and 16 mg kg^{-1} SeMet. Even fish fed 11 mg kg-1 selenite had significantly higher liver Se levels compared to fish fed much higher dietary SeMet levels of 39 mg kg^{-1}.

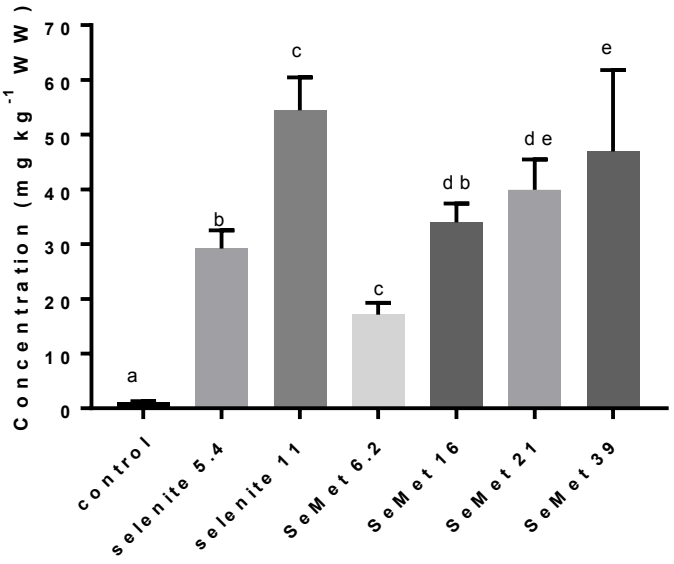

Figure 1. Selenium (Se) concentrations (mg kg^{-1} wet weight (WW)) in liver of Atlantic salmon (*Salmo salar*) fed control, graded levels of selenite fortified diets (5.4 and 11 mg kg^{-1} WW, respectively), or graded levels of seleno-methionine (SeMet) fortified diets (6.2, 16, 21, and 39 mg kg^{-1} WW, respectively) for 3 months (mean ± SD, n=15). Bars with the same letters are not significantly different from each other ($P<0.05$, one-way ANOVA, post hoc Tukey's t-test).

Weight, length, growth and organ index.

Fish fed selenite levels of 11 mg kg^{-1} had significantly reduced individual weights and lengths at the end of the 3 month trial. Despite the highest Se accumulation and apparent liver histopathology (see under) in this group, no significant differences ($p<0.05$) in liver somatic index (LSI%) was seen compared to control (table 1). In contrast, the relative spleen somatic index (SSI%) decreased with increased dietary selenite levels, with significantly reduced SSI% in fish fed 11 mg kg^{-1} selenite compared to the control fish. No significant dietary differences were observed in among the tank assessed specific growth rate (SGR) or feed conversion (FCR) in fish fed any of the dietary selenite groups (table 1).

Fish fed the highest dietary SeMet level (39 mg kg^{-1}) had significantly reduced final individual weights and lengths, compared to fish fed the second highest SeMet level (6.2 mg kg^{-1}), but not comparted to the control fish. No significant differences were observed in fish fed dietary 6.2 mg kg^{-1} SeMet compared to the control fish. No significant differences were seen in any of the relative organ indexes among the dietary SeMet groups. The SGR was significantly lower in fish fed the highest SeMet group compared to all other SeMet groups, including control. The FCR significantly increased with increasing dietary SeMet levels, with significantly increased FCR in fish fed 39 mg kg-1, compared to fish fed control, 6.2 and 16 mg kg^{-1} SeMet, respectively.

Table 1. Final individual length (cm), weight (gr), condition factor, relative liver somatic index (LSI, %), heart somatic index (HSI%), spleen somatic index (SSI, %) (mean±SD, n=15), as well as tank specific growth rate (SGR), feed conversion ratio (FCR), and daily feed intake (FI) (mean±SD, N=3), in Atlantic salmon (*Salmon salar*) fed graded levels of selenite (5.4 and 11 mg kg^{-1}) and seleno-methione (SeMet) (6.2, 16, 21, and 39 mg kg^{-1}) for 3 months (triplicate tanks per diet). Values with different superscripts are significantly different from each other (one-way ANOVA, Tukey's HSD test, p-values, $p<0.001$, $p<0.01$, $p<0.05$).

	control	selenite 5.4	selenite 11	p-value
Final length	32.9±1.8^{ab}	33.7±1.7^{a}	31.8±1.3b	**p<0.001**
Final weight	445±102^{ab}	469±92^{a}	411±61b	**p<0.05**
Final CF	1.23±0.10	1.21±0.08	1.28±0.09	0.33
Final LSI	1.01±0.16	0.92±0.12	0.99±0.09	0.12
Final HSI	0.093±0.013	0.11±0.019	0.10±0.012	0.19
Final SSI 0-90 days	0.099±0.028^{a}	0.077±0.014^{ab}	0.075±0.018^{b}	**p<0.01**
SGR	1.24±0.10	1.26±0.10	1.14±0.03	0.21
FCR	0.80±0.07	0.77±0.07	0.89±0.01	0.085
FI	0.99±0.02	0.97±0.02	1.01±0.01	0.06

	control	SeMet 6,2	SeMet 16	SeMet 21	SeMet 39	p-value
Final length	32.9±1.8^{ab}	33.3±2.0^{a}	33.1±1.8^{ab}	32.2±2.2^{ab}	31.0±1.6^{b}	**p<0.01**
Final weight	445±102^{a}	467±101^{a}	458±91^{a}	405±110^{a}	352±60^{b}	**p<0.05**
Final CF	1.23±0.10	1.25±0.10	1.25±0.09	1.18±0.10	1.17±0.08	0.06
Final LSI	1.01±0.16	1.01±0.08	0.94±0.14	1.06±0.12	1.01±0.15	0.23
Final HSI	0.093±0.013	0.096±0.014	0.084±0.019	0.098±0.014	0.092±0.019	0.41
Final SSI 0-90 days	0.099±0.028	0.084±0.019	0.090±0.023	0.11±0.034	0.12±0.067	0.07

SGR	1.24±0.10[a]	1.28±0.09[a]	1.25±0.03[a]	1.13±0.08[a]	0.97±0.07[b]	**p<0.001**
FCR	0.80±0.07[a]	0.76±0.08[a]	0.79±0.01[a]	0.90±0.06[ab]	1.13±0.08[b]	**p<0.001**
FI	0.99±0.02a	0.97±0.03a	0.98±0.02a	1.01±0.02a	1.1±0.02b	**p<0.001**

Blood and plasma parameters

A number of hematological indices were monitored at the end of the exposure (table 2). Significant differences were only observed for MCHC and MCV for both selenite and SeMet exposed fish. Fish fed 11 mg kg^{-1} selenite had significantly reduced MCHC compared to control. For SeMet exposed fish fish fed 39 mg kg^{-1} SeMet had significantly lower MCHC and MCV than the 16 mg kg-1 SeMet exposed group, however no significant differences were observed compared to the control group.

Table 2. Blood hematocrit (Hct, %), red blood cell count (RBC, number*10^{-12} L^{-1}), hemoglobin (HGB g 100 mL^{-1}), mean corpuscular volume (MCV, 10^{-5} L^{-1}), Mean corpuscular hemoglobin concentration (MCHC, g L-1), mean corpuscular hemoglobin (MCH µg) in Atlantic salmon (*Salmon salar*) fed graded levels of selenite (5.4 and 11 mg kg-1) and seleno-methione (SeMet) (6.2, 16, 21, and 39 mg kg-1) for 3 months (triplicate tanks per diet). Values with different superscripts are significantly different from each other (one-way ANOVA, Tukey's HSD test, p<0.001, p<0.01, p<0.05).

	control	selenite 5.4	selenite 11	p-values		
Hct	43.5±3.52	46.0±2.55	46.9±2.66	0.10		
RBC	1.34±0.129	1.42±0.106	1.43±0.087	0.29		
HGB	9.88±0.900	10.2±0.61	10.3±0.54	0.71		
MCV	324±9.3	324±17.8	328±13.9	0.87		
MCHC	22.7±0.85[a]	22.1±0.62[ab]	21.9±0.74[b]	**p<0.05**		
MCH	73.7±2.54	71.6±3.39	72.1±3.72	0.30		
	control	SeMet 6.2	SeMet 16	SeMet 21	SeMet 39	p-values
Hct	43.5±3.52	44.9±2.21	45.0±3.37	45.1±3.50	43.6±4.14	0.098
RBC	1.34±0.129	1.39±0.077	1.39±0.092	1.42±0.118	1.38±0.11	0.55
HGB	9.88±0.900	10.3±0.52	10.2±0.70	10.4±0.80	10.1±0.99	0.29
MCV	324±9.3[ab]	323±13.7[ab]	325±15.7[b]	318±13.4[ab]	315±18.4[a]	**p<0.01**
MCHC	22.7±0.85[ab]	22.8±0.78[ab]	22.6±0.81[b]	23.0±0.48[ab]	23.1±0.59[a]	**p<0.05**
MCH	73.7±2.54	73.8±3.06	73.5±3.33	73.3±2.21	72.8±4.20	0.43

For plasma parameters, fish fed 11 mg kg^{-1} selenite had only elevated alkaline phosphatase, and fish fed 5.4 mg kg^{-1} reduced ALT, compared to the control group, as a marker for liver injury. For fish exposed to SeMet, plasma AP and ASAT showed a significant decrease in fish feed 39 mg kg^{-1} SeMet. Of the plasma ions, only Ca was significantly reduced in fish fed 39 mg kg^{-1} SeMet, while plasma osmolality, Na, Cl, and K were not significantly affected by dietary SeMet exposures. Plasma markers of kidney function and protein and energy metabolism, such as creatinine and total protein were also significantly reduced in fed the highest SeMet level (39 mg kg^{-1}), compared to control fish. Selenite had no significant effects on these

parameters. Plasma ureic acid was reduced in both SeMet and selenite fish, however, not significantly (p=0.06, for SeMet exposed fish) (table 3)

Table 3. Plasma biochemistry alkaline phosphate (AP, Units L^{-1}), Alanine aminotransferase (ALT, U L^{-1}), aspartate aminotransferase (ASAT, U L^{-1}), glucose (mmol), albumin (µmol), creatinine (µmol), total protein (g L^{-1}), ureic acid (µmol), and ions Na, K, Cl (mmmol) and osmolality (Osm, mOsm) in Atlantic salmon (*Salmon salar*) fed graded levels of selenite (5.4 and 11 mg kg^{-1}) and seleno-methione (SeMet) (6.2, 16, 21, and 39 mg kg^{-1}) for 3 months (triplicate tanks per diet). Values with different superscripts are significantly different from each other (one-way ANOVA, Tukey's HSD test, p<0.001, p<0.01, p<0.05).

	control	selenite 5.4	selenite 11	p-values		
AP	448±168^{a}	624±143^{ab}	720±204^{b}	***p<0.01***		
ALT	13.5±3.14^{a}	21.2±5.6^{b}	19.4±1.42^{b}	***P<0.05***		
ASAT	339±104	332±52	320±84	*0.13*		
glucose	5.19±0.79	5.77±1.05	4.79±0.56	*0.36*		
albumine	301±43	315±24	321±29	0.26		
creatinine	17.7±6.47	15.7±5.26	14.8±4.99	0.37		
tot prot	41.4±7.31	42.5±5.12	42.6±3.72	0.83		
Ureic acid	45.1±12.1	30.6±11.9	28.6±15.2	0.51		
Osm	322±4.64	322±5.17	322±4.04	0.89		
Ca	2.67±0.18	2.77±0.13	2.72±0.92	0.12		
Cl	139±8.78	134±10.6	137±10.0	0.94		
Na	169±2.55	169±2.41	169±2.89	0.61		
K	1.53±0.45	1.10±0.37	0.86±0.28	0.43		
	control	SeMet 6,2	SeMet 16	SeMet 21	SeMet 39	p-values
AP	448±168^{a}	601±155^{a}	566±153^{ab}	512±196^{ab}	422±17^{b}	**p<0.01**
ALT	19.5±7.14	18.3±10.9	15.1±9.86	12.3±7.66	15.5±10.0	0.12
ASAT	339±104^{a}	384±105^{a}	330±81^{a}	333±110^{a}	253±91^{b}	**p<0.05**
glucose	5.19±0.79	5.17±0.53	5.33±0.85	5.72±1.39	5.63±1.33	0.31
albumine	301±43	325±30	309±36	287±35	284±39	0.085
creatininee	17.7±6.47^{a}	20.2±7.17^{a}	15.3±6.33^{a}	13.4±6.78^{a}	10.8±4.61^{b}	**p<0.01**
tot prot	41.4±7.31^{a}	44.9±5.40^{a}	42.59±5.56^{a}	40.18±7.58a	37.3±6.05^{b}	**p<0.01**
Ureic acid	45.1±12.1	51.1±15.4	43.3±18.1	52.7±21.9	35.4±19.1	0.06
Osm	322±4.64	322±3.64	322±4.78	323±4.17	321±5.92	86
Ca	2.67±0.18^{a}	2.74±0.14^{a}	2.68±0.19a	2.68±0.18a	2.53±0.12^{b}	**p<0.01**
Cl	139±8.78	139±10.6	140±8.28	136±13.5	140±10.1	0.94
Na	169±2.55	170±3.09	169±3.03	168±2.24	169±2.37	0.43
K	1.53±0.45	1.26±0.45	1.06±0.50	1.24±0.73	1.52±0.46	0.46

Markers of lipid metabolism

As markers of altered lipid metabolism, sum total lipid was significantly ($p<0.05$) reduced compared to control group in fish fed 11 mg kg^{-1} selenite and 39 mg kg^{-1} SeMet. The relative (% of sum lipid) distribution of lipid classes was significantly affected in the 11 mg kg^{-1} selenite group with reduced Triacylglycerol compared to control groups. Although fish fed graded levels of SeMet also showed decreased TAG, these differences were not significant ($p=0.08$, one-way ANOVA, Tukey's t-test)

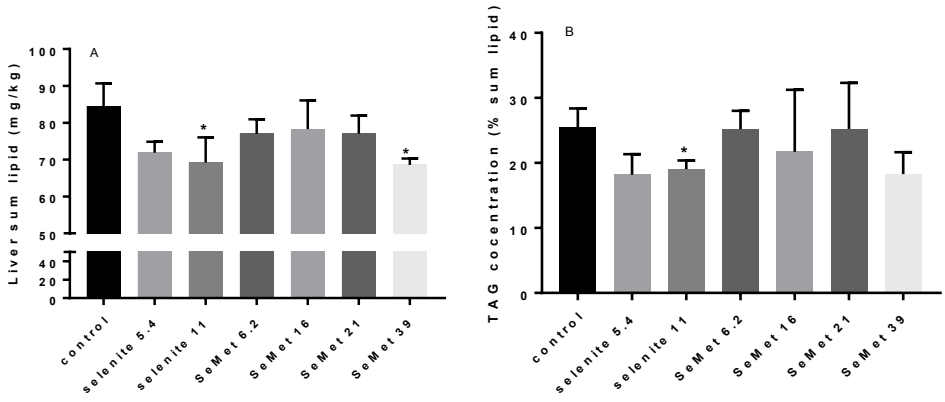

Figure 2. Liver sum lipid (mg kg-1 ww)(A), relative triacylglycerol (TAG, % sum lipid) (B), in Atlantic salmon (*Salmo salar*) fed control, graded levels of selenite fortified diets (5.4 and 11 mg kg^{-1} WW, respectively), or graded levels of seleno-methionine (SeMet) fortified diets (6.2, 16, 21, and 39 mg kg^{-1} WW, respectively) for 3 months (mean ± SD, n=3). Bars with * are significantly different from control ($P<0.05$, one-way ANOVA, post hoc Tukey's t-test).

Markers of oxidative stress

As markers of oxidative stress, reduced glutathione (GSH) was significantly reduced compared to control group in fish fed 5.4 and 11 mg kg^{-1} selenite and 16, 21, and 39 mg kg^{-1} SeMet. The oxidized glutathione (GSSG) was also significantly reduced in the same exposure groups compared to the control fish, causing the ration GSG: GSSG (data not shown) not to alter significantly among any of the exposure groups compared to control fish.

Fish fed the highest level of selenite (11 mg kg^{-1}) and SeMet (39 mg kg^{-1}) had significantly reduced vitamin E levels compared to control fish. No significant differences in liver TBARS levels, an indicator for lipid peroxidative stress were observed in any of the exposure groups compared to control fish.

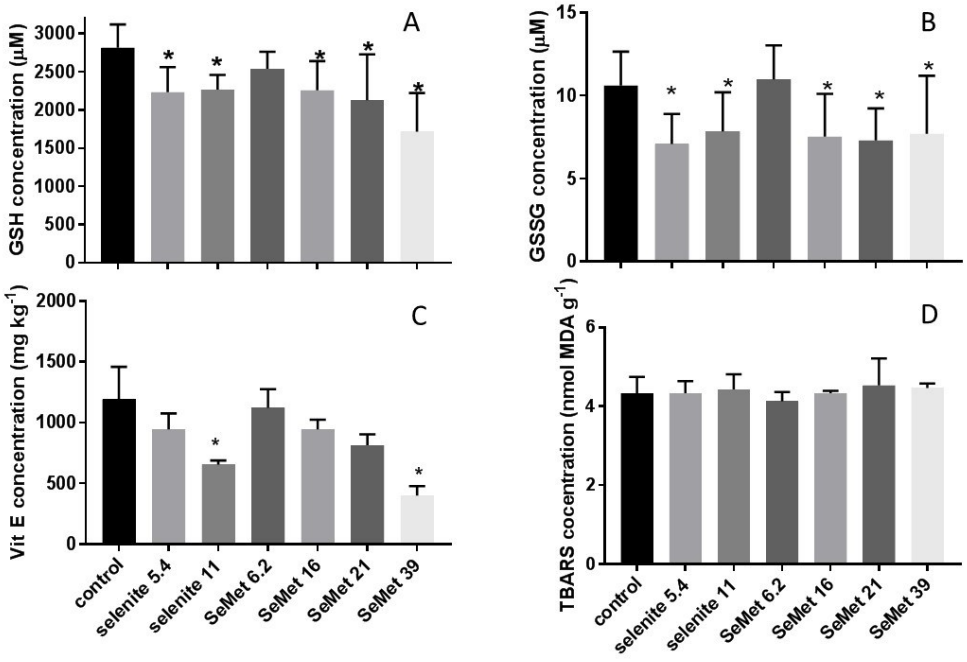

Figure 3. Liver reduced glutathione (GSH) (A), oxidised glutathione (GSSG (B), vitamin E (as alfa-tocopherol) (C) and thiobarbituric acid reactive substances (TBARS) (D) in Atlantic salmon (*Salmo salar*) fed control, graded levels of selenite fortified diets (5.4 and 11 mg kg^{-1} WW, respectively), or graded levels of seleno-methionine (SeMet) fortified diets (6.2, 16, 21, and 39 mg kg^{-1} WW, respectively) for 3 months (mean ± SD, n=15). Bars with * are significantly different from control ($P<0.05$, one-way ANOVA, post hoc Tukey's t-test).

Histopathology

Hepatocyte lipid intracytoplasmatic vacuolization, degeneration and focal necrosis, and inflammation were the main histopathological changes that were constantly present in most of the samples and these histopathological changes were scored for semi-quantitative evaluation. Significant differences among the dietary treatments were observed in two of the evaluated parameters, liver hepatocyte vacuolization ($p = 0.040$) and degeneration and focal necrosis ($p = 0.000$). Although an increasing trend was seen in inflammation in fish fed SeMet and selenite, no significant differences were observed compared to control fish ($p=0.09$ and 0.12, respectively)(Table 4). Fish fed both 5.4 and 11 mg kg^{-1} selenite had significantly higher degeneration and focal necrosis compared to the control fish. Only fish fed the next highest SeMet level (21 mg kg^{-1}) had significantly increased focal necrosis compared to control fish. Fish fed the highest selenite levels (11 mg kg^{-1}) had significantly reduced liver lipid intracytoplasmatic vacuolization compared to control fish (Table 4).

For degeneration and focal necrosis, several patterns of lesions could be observed. A common finding was the presence of basophilic foci (Figure 4a), which have been demonstrated to some degree to be precursors of primary hepatocellular neoplasms. Diffuse presence of hepatocyte hyalinization, characterized by enlarged hepatocytes that contain discrete or pancytoplasmatic inclusions of refractile, eosinophilic material was occasionally observed (Figure 4b). Degenerated hepatocytes presented a dark nucleus with condensed chromatin, piknotic nuclei and were frequently surrounded by red blood cells and oedematous areas

(Figure 4c). No inflammatory reaction was observed surrounding the lesions. A mononuclear cell infiltrate was often observed surrounding blood vessels (Figures 5 A & B) and bile ducts (Figures 5 C & D), although it could also be observed in the hepatic parenchyma. Other histological alterations were observed in some of the fish fed the dietary treatments. One of the fish fed the diet Selenite 5.4 mg kg^{-1} showed a granuloma consistent of concentric layers of macrophages and inflammatory cells adjacent to blood vessels with discrete inflammatory reaction (Figure 6a). Another fish fed the diet Selenite 11 mg kg-1 presented a cluster of well-differentiated bile ducts that formed an expansive wall with discrete borders and surrounded by stromal proliferation (Figure 6b). The histological appearance of this finding resembles a cholangioma or bile duct adenoma. A finding that was commonly observed among fish fed selenite 5.4 mg kg^{-1} and selenite 11 mg kg^{-1} was bile duct hyperplasia characterized by proliferation of small scattered well-differentiated bile ducts without compression of or expansion into adjacent parenchyma (Figure 7A-D). Variable periductular mononuclear inflammation and fibrosis was also observed. The prevalence of this alteration was 66.7 % of the fish fed selenite 11 mg kg-1 whereas 22.2 % of the fish fed selenite 5.4 mg kg-1 showed this alteration

Table 4. Score for the main histopathological changes (liver lipid for hepatocyte vacuolization, degeneration and focal necrosis and inflammation) in liver of Atlantic salmon (*Salmo salar*) fed control, graded levels of selenite fortified diets (5.4 and 11 mg kg^{-1} WW, respectively), or graded levels of seleno-methionine (SeMet) fortified diets (6.2, 16, 21, and 39 mg kg^{-1} WW, respectively) for 3 months (mean ± SD, n=9). Different superscript letters denote significant differences among the dietary treatments ($p < 0.05$).

	vacuolization	degeneration focal necrosis	inflammation
Control	1.4 ± 0.9^{a}	0.3 ± 0.2^{c}	0.2±0.4
SeMet 6.2	0.9 ± 0.9^{ab}	0.5 ± 0.2^{bc}	0.6±0.5
SeMet 16	0.8 ± 0.4^{ab}	0.7 ± 0.4^{abc}	0.6±0.5
SeMet 21	0.8 ± 0.7^{ab}	0.8 ± 0.5^{ab}	0.2±0.3
SeMet 39	0.9 ± 0.6^{ab}	1.1 ± 0.2^{a}	0.4±0.3
Selenite 5.4	0.7 ± 0.8^{ab}	1.0 ± 0.5^{ab}	0.5±0.4
Selenite 11	0.3 ± 0.5^{b}	1.0 ± 0.4^{a}	0.6±0.8

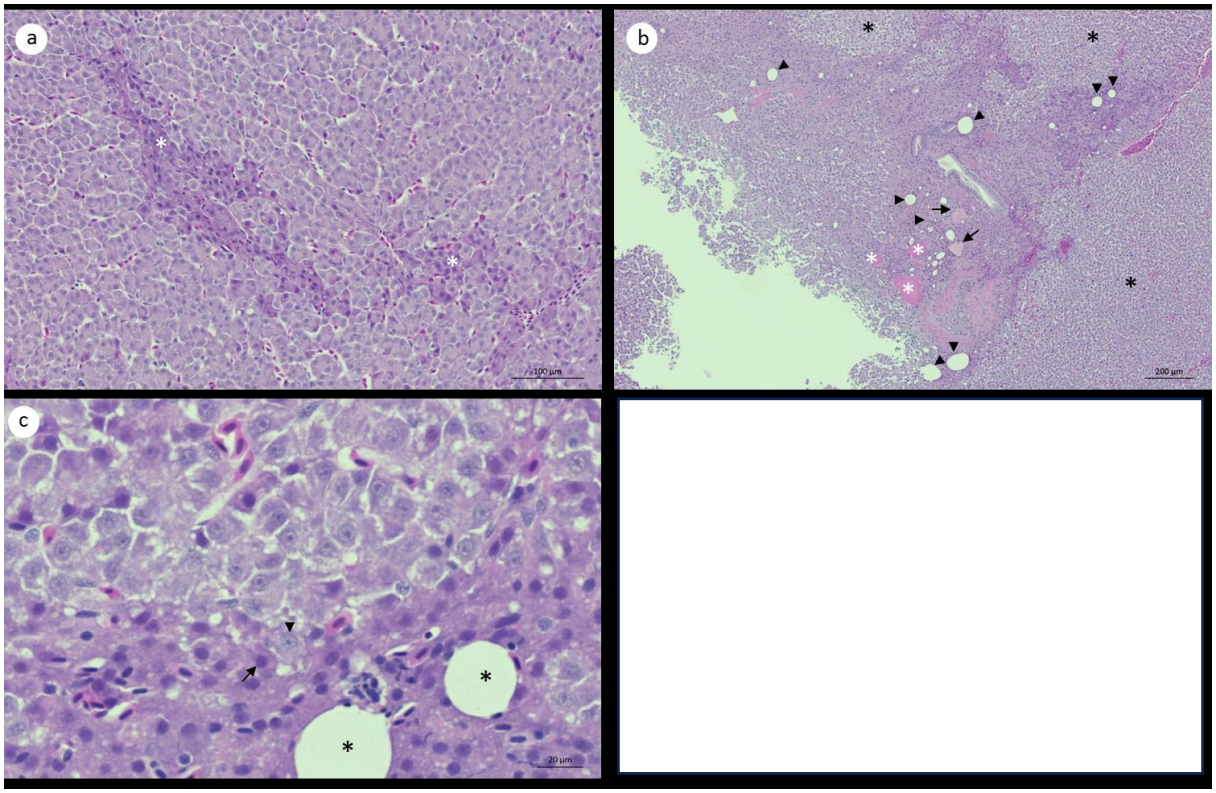

Figure 4 (A-C).- Several degenerative and necrosis associated lesions observed in sections (5 µm) from the liver of Atlantic salmon fed Selenite 11 mg kg^{-1} (a), SeMet 21 mg kg^{-1}(b) and Selenite 5.4 mg kg^{-1} (c). (a) Basophilic focus (asterisk) of degeneration showing smaller cells than normal hepatocytes arranged in cords. (b) Focal degeneration and necrosis clearly differentiated from a healthy area (dark asterisk). In the degenerated area the presence of hyaline substance can be clearly observed (white asterisk) and oedema (arrow) together with fat vacuoles (arrowhead) resulting from the rupture of hepatocytes and not directly due to vacuolization of the cells. (c) Higher magnification of degenerated area where a normal hepatocyte (arrowhead) with vacuolated cytoplasm and characteristic nucleus can be observed next to degenerated hepatocytes with contracted cytoplasm and basophilic nucleus. Fat vacuoles resulting from the rupture of degenerated cells can also be observed (asterisks). Haematoxylin and Eosin staining.

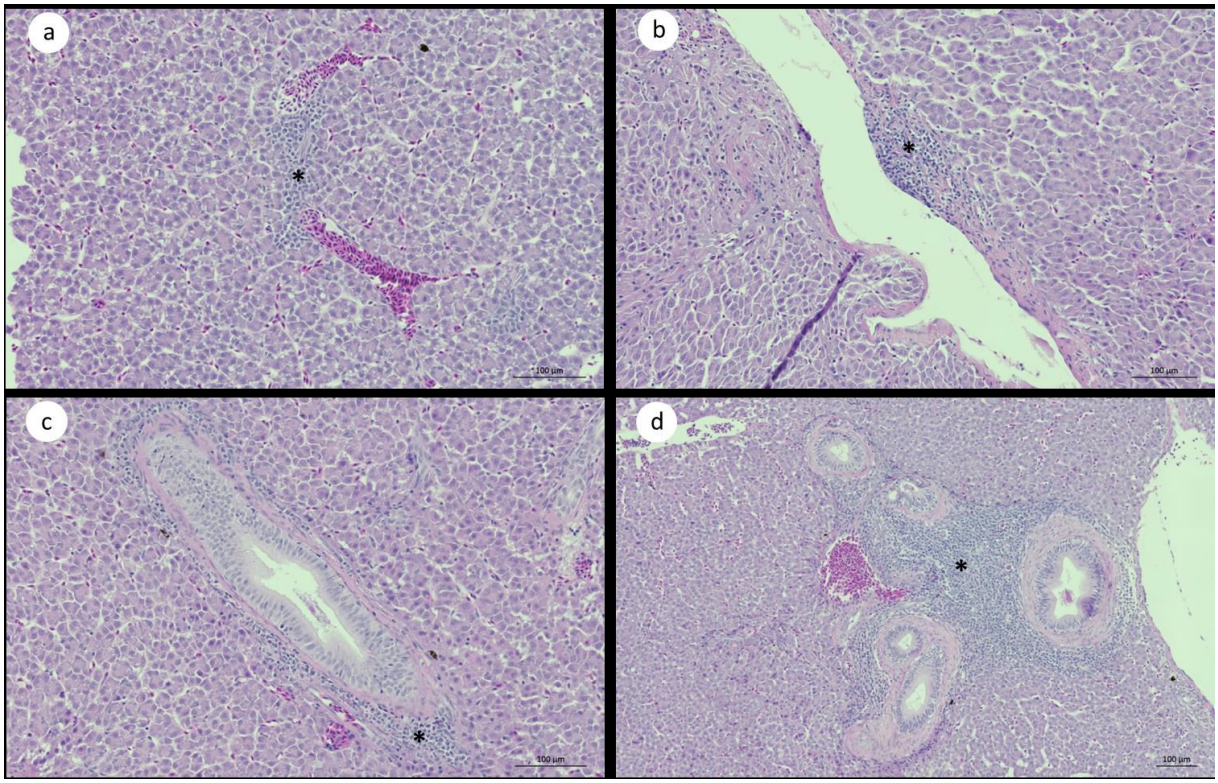

Figure 5.(A-D)- Sections (5 µm) from Atlantic salmon liver fed the diets Selenite 5.4 mg kg-1 (a), Selenite 11 mg kg-1 (b & d) and SeMet 16 mg kg-1(c). A mononuclear and lymphocytic inflammatory reaction could be observed (asterisk) surrounding the blood vessels (a & b) and the bile ducts (c & d). Moderate periductular fibrosis can be observed in (d) (arrow). Haematoxylin and Eosin staining.

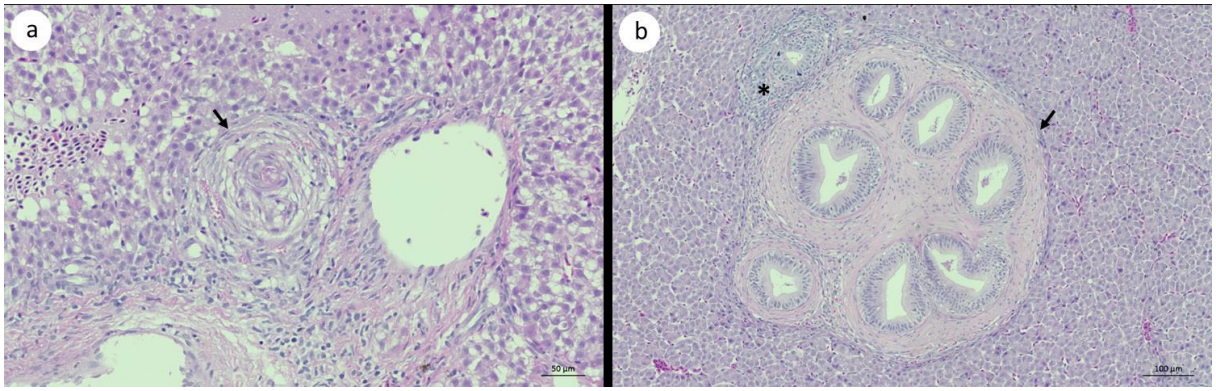

Figure 6. Liver sections (5 µm) of fish fed diets Selenite 5.4 mg kg-1 (a) and Selenite 11 mg kg-1 (b). (a) Presence of a granuloma formed by concentric layers of macrophages and inflammatory in the liver parenchyma (arrow). (b) Well circumscribed structure formed by a collection of bile ducts surrounded by stroma (arrow). Discrete mononuclear inflammation is also observed (asterisk). Haematoxylin and Eosin staining.

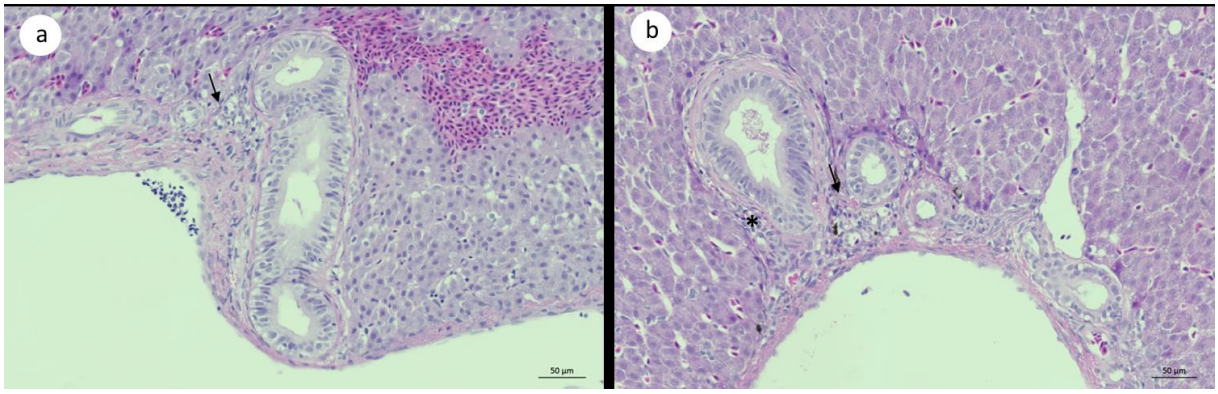

Figure 7.- Liver sections (5 µm) from Atlantic salmon fed Selenite 5.4 mg kg-1 (a) and selenite 11 mg kg-1 (b) showing proliferation of the bile ducts (arrow) as well as inflammatory cell infiltrate and fibrosis (asterisk). Haematoxylin and Eosin staining.

Benchmark dose analyses

Parameter BMDL analyses as well as NOAEL and LOAEL assessment are given in Table 6. For SeMet exposed fish, for whole body parameters and organ indices, a BMDL could be assessed as the dose-response model was significantly better than the response model that predicts no dose response (null model) (AIC<AICnull-2), and the best fitted dose response model (lowest AIC) was better than the full response model (AICmin<AICfull+2) (Table 6). For haematology, blood plasma parameters, liver oxidative stress markers, and histopathology, a BMDL could not be established for all parameters because none of the fitted dose-response models was significantly better than the null model (AIC>AICnull-2, indicated with “none” in table 6). For selenite exposed fish, for fewer parameters than for SeMet exposed fish, a BMDL could be established. This is due to the use of only three experimental groups in the selenite dose-response assessment compared to five in the SeMet exposed fish (see mortality above). As for SeMet, a lack of BMDL was due to no significant difference of the dose-response models compared to the “null” model (AIC<AICnull-2, indicated as “none” in table 6). In addition, for several parameters (Hct, RBC, plasma glucose and creatininee, GSH, GSSG, and histological lipid intracytoplasmatic vacuolization), the best fitted dose response model was not significantly different from the full model (AICmin<AICfull+2, indicated with “*” in table 6). This was not attributed to non-random errors (e.g. data error), but rather that none of the models were appropriate for the selenite data set (EFSA 2017), which can be attributed to the use of only 3 selenite dietary exposure groups.

For the SeMet exposed fish, the parameters with the lowest BMDL were spleen somatic index, plasma creatinine, and histopathological degeneration and focal necrosis, with a BMDL05, BMDL20 and BMDL10 of 3.05, 2.29, and 2.08 mg SeMet kg^{-1} feed, respectively. However, for spleen somatic index and plasma creatinine, a large variation in the 90% lower and upper 90% confidence interval (BMDL and BMDU, respectively) was seen as a BMDL:BMDU ratio exceeding 10 (Table 6). This indicates a large variation and uncertainty BMD model assessment. For the histopathological parameter degeneration and focal necrosis, the BMDL:BMDU ratio was far less (0.4), with a more certain BMD assessment. For histological quantal data, a standard BMDL10 with extra risk factor is included in EFSA’s BMD model,

which is specific for adverse histological effect. For the SeMet exposed fish, the lowest BMDL with a high certainty (low variation BMDL and BMDU) and use of extra risk factor (EFSA 2017) was the histological focal necrosis observation at 2.1 mg SeMet kg^{-1}.

For selenite exposed fish, lowest BMDLs were for spleen somatic index and plasma AP, K, creatinine, and ureic acid, with a BMDL05 of 0.002, and a BMDL20 of 0.059, 0.066, 0.026 and 0.017 mg selenite mg kg^{-1}, respectively (table 6). However, for all these parameters, a large variation in BMDL and BMDU was observed (BMDL:BMDU ratio of 2239, 90, 127, 595 and 189 respectively), indicating an uncertain BMD assessment. Similarly for Hct and RBC, a BMDL05 of 0.43 and 0.41 mg selenite mg kg^{-1} was assessed, but both parameters had a high BMDL:BMDU ratio (64 and 467, respectively). In addition, the best fitted dose response model for Hct and RBC, was not significantly different from the full model (AICmin<AICfull+2, indicated with "*" in table 6). For the liver oxidative stress and histology parameters, GSSG and intracytoplasmatic vacuolization had the lowest BMDL20 of 0.23 and BMDL10 of 0.06 mg selenite kg^{-1}, respectively. However also for these parameters a high BMDL:BMDU and/or a best fit model that was not significantly different from the full model, indicated an uncertain BMDL assessment for these parameters. Other parameters in plasma liver parameters, oxidative stress, and histopathology (plasma ALAT, liver vitamin E, and histological focal necrosis, respectively) had a low BMDL:BMDU (ratio <10) variation and a best fit dose-response that was significantly better than the null and full fit, with lowest BMDL20 and BMDL10 of 1.4, 1.6, and 1.9 mg selenite kg^{-1}, respectively. Besides for total protein plasma content, for all parameters the BMDL was lower than the ANOVA established NOAEL (Table 6).

Table 6. Benchmark doses (BMD), lower and upper 90% confidence interval (BMDL and BMDU, respectively), ration BMDL to BMDU (BMDL/BMDU), No Observed (Adverse) Effect Level (NOAEL/NOEL), and lowest observed (adverse) effect level (LOAEL/LOEL) for relevant responses in Atlantic salmon (*Salmo salar* L.) fed to graded levels of selenite and seleno-menthionine (SeMet) for 90 days

Selenomethionine					
whole body parameters	$BMDL_{05}$	$BMUL_{05}$	BMDU/BMDU	NOAEL	LOAEL
SGR	8.48	26.8		21	39
FCR	**7,78**	22,95	2,95	21	39
FI	20.35	33.51		21	39
weight	**7,98**	27,1	3,4	21	39
lenght	26,9	42	1,56	21	39
CF	23,7	53,2	2,24	39	
organ indices					
HSI	none	none		39	
LSI	none	none		39	
MSI	**3,05**	31,7	10,39	39	
heamatology					
HCT	none	none		39	
RBC	none	none		39	
HGB	none	none		39	
MCV	none	none		21	39
MCHC	none	none		21	39
MCHC	none	none		39	
plasma parameters	$BMDL_{20}$	$BMDU_{20}$	BMDU/BMDU	NOAEL	LOAEL
AP	none	none		21	39
ALT	none	none		39	
ASAT	**19,7**	40	2,03	21	39
glucose	none	none		39	
albumine	none	none		39	
creatinine	**2,29**	31,3	13,67	21	39
tot prot	37,6	120	3,19	21	39
Ureic acid	none	none		39	
Osm	none	none		39	
Ca	none	none		21	39
Cl	none	none		39	
Na	none	none		39	
K	none	none		39	
liver oxidative stress markers					
TBARS	none	none		39	
Vitamin E	10,8	22,1	2	21	39
GSH	**4,7**	26,9	5,8	6.2	16
GSSG	6,9	15,5	2,2	6.2	16
GSH/GSSG ratio	none	none		39	

Lipid	$BMDL_{20}$	$BMDU_{20}$	BMDU/BMDU	NOAEL	LOAEL
Sum lipid	31	358	11	21	39
% FFA	none	none		11	
% TAG	none	none		5.4	11
%DAG	none	none		11	
Histology	$BMDL_{10}$	$BMDU_{10}$	BMDU/BMDU	NOAEL	LOEAL
Degeneration and focal necrosis	**2.08**	4.51	0,4	21	39
Inflammation	none	none		39	
intracytoplasmatic vacuolization	none	none		39	

Selenite					
whole body parameters	$BMDL_{05}$	$BMUL_{05}$	BMDU/BMDU	NOAEL	LOAEL
SGR	none	none		11	
FCR	4	10,8	2,7	11	
FI	10,5	15,9	1,51	11	
weight	none	none		5.4	11
lenght	none	none		5..4	11
CF	**7,53**	25,6	3,4	11	
organ indices	$BMDL_{05}$	$BMUL_{05}$	BMDU/BMDU	NOAEL	LOAEL
HSI	none	none		11	
LSI	none	none		11	
SSI	0,002	4,12	2060	5.4	11
blood parameters	$BMDL_{05}$	$BMUL_{05}$	BMDU/BMDU	NOAEL	LOAEL
HCT	0,428*	27,2*	63,55	11	-
RBC	0,407*	190*	466,83	11	-
HGB	none	none		11	-
MCV	none	none		11	-
MCHC	none	none		5.4	11
MCHC	none	none		11	-
plasma parameters	$BMDL_{20}$	$BMDU_{20}$	BMDU/BMDU	NOEL	LOEL
AP	0,059	5,32	90,17	5.4	11
ALAT	**1,36**	10,8	7,94	11	-
ASAT	none	none		11	-
glucose	10.8*	62.4*	5,77	11	-
albumine	none	none		11	-
creatinine	0.0262*	15.6*	595	11	-
tot prot	none	none		11	-
Ureic acid	0,0168	3,17	189	11	-
Osm	none	none		11	-
Ca	none	none		11	-
Cl	none	none		11	-
Na	none	none		11	-

K	0,066	8,4	127,3	11	-
liver oxidative stress	$BMDL_{20}$	$BMDU_{20}$	BMDU/BMDU	NOEL	LOEL
liver TBARS	none	none		11	-
liver vit E	1,62	9,02	5,57	5.4	11
GSH	3,67*	136*	37,06	0.45	5.4
GSSG	0,203*	7,28*	35,86	0.45	5.4
Lipid	$BMDL_{20}$	$BMDU_{20}$	BMDU/BMDU	NOAEL	LOAEL
Sum lipid	mar.97	200	50	5.4	11
% FFA	none	none		11	
% TAG	0.36	125	347	5.4	11
%DAG	none	none		11	
histopathology	$BMDL_{10}$	$BMDL_{10}$	BMDU/BMDU		
Degeneration and focal necrosis	**1,89**	7,21	3,81	0.45	5.4
Inflammation	none	none		11	-
Hepatocyte intracytoplasmatic vacuolization	0,06*	0,19*	2,86	5.4	11

AICmin<AICfull+2, indicated with "*"

Discussion

Mortality, growth, and hematology

In general, inorganic Se is considered more toxic than organic Se forms (Thiry et al., 2012). However, also for the organic SeMet a narrow window of requirement and toxicity has been observed for Nile tilapia (*Oreochromis niloticus*) (Lee et al., 2016), and juvenile rainbow trout (*Oncorhynchus mykiss*) appears to have threshold levels for chronic dietary SeMet toxicity that is in the same range as for dietary selenite (Hamilton, 2004; Vidal et al., 2005).
In the present study, selenite was more acute toxic than SeMet, as exposure to 29 mg kg^{-1} selenite (0.29 mg/kg body weight (BW)/day) caused acute toxicity while fish fed up to 39 mg kg^{-1} WW SeMet (0.39 mg/Kg BW/day) showed no mortality. In the present trial, a feed level of 11 mg k^{-1} selenite kg ww (0.11 mg/Kg BW/day) was not acute toxic to the fish, as observed in earlier trails with larger Atlantic salmon (572g) fed 15 mg kg^{-1} (0.14 mg/Kg BW/day) (Berntssen et al., 2017). A narrow range seems to exist between sublethal chronic toxicity and acute toxicity (mortality), as a 2.6 fold increase from none acute toxic dietary selenite levels gave mortality in the present study (0.11 versus 0.29 mg/kgBW/day, respectively). Atlantic salmon and rainbow trout seem to have the same sensitivity to excess dietary selenite exposure, as rainbow trout fry (1.3 g) fed with a daily dose in the same range as the present study (0.38 versus 0.29 mg/kg BW/day, present study) showed mortality (Hilton et al., 1980). In contrast, rainbow trout start-feeders (0.6 g) fed up to 11.4 mg kg^{-1} selenite (estimated 0.52 mg/Kg BW/day) showed a marginal but none significant mortality (Hilton and Hodson, 1983). Similar as for Atlantic salmon in the present study, SeMet fed rainbow trout (0.28-26 g) or cutthroat trout (9.7 g) did not show mortality at any of the dietary exposure studies with feed levels ranging from >7.4-18 mg SeMet kg^{-1} DW (0.14- 0.31 mg/kgBW/day) (Vidal et al., 2005; Rider et al., 2009; Hardy et al., 2010; Knight et al., 2016). In contrast, for chinook salmon (~ 1 g) fed 9.6 mg kg-1 SeMet, survival was reduced (Hamilton et al., 1990) (Table 7).

For overall none lethal adverse effects that are not specifically related to Se toxic pathways, growth was significantly reduced at 11 mg selenite kg^{-1} ww (0.11 mg/kg BW/day). Similarly, earlier Atlantic salmon trials fed 15 mg selenite kg^{-1} ww with a slightly higher daily dose exposure (0.14 mg/kgBW/day) showed reduced growth (Berntssen et al., 2017). In contrast, rainbow trout (26 g) fed a similar daily selenite dose (0.14 mg/KgBW/day) showed no reduced growth (Rider et al., 2009), and only daily selenite dose as high as 0.38-0.52 mg/kgBW/day caused growth impairment in juvenile (0.6-1.3g) rainbow trout (Hilton et al., 1980; Hilton and Hodson, 1983). Atlantic salmon fed 21 mg kg^{-1} (0.21 mg/ kg BW/day) SeMet, had no significantly reduced growth in the present trial. For other SeMet exposed salmonids, however, initial-feeding (0.3-0.4 g) rainbow trout had reduced growth at lower estimated SeMet daily doses (estimated 0.04 mg/kg BW/day or 0.12 mg/Kg BW/day, respectively)(Vidal et al., 2005; Knight et al., 2016). In contrast, adult rainbow trout (26-100g) fed 7.4-8.9 mg SeMet kg-1 (0.15-0.18 mg/kg BW/day) had no reduced growth (Rider et al., 2009; Pacitti et al., 2016a).

In the present study, no significant differences were observed in hematocrit, hemoglobin, or red blood cells, despite apparent adverse effects such as liver histopathology (see below) and mortality (above) in selenite fed fish. Thus indicating that hematology as a general adverse effect parameter is not applicable for dietary Se toxicity assessment. Similarly in other studies on rainbow trout, the apparent dietary selenite induced toxicity was not expressed as alterations in hematology (Hilton et al., 1980; Hilton and Hodson, 1983). However, organic selenium fed bluegills (*Lepomis macrochirus*) showed abnormally shaped erythrocytes (Finley, 1985), and combined waterborne and dietary selenium exposed green bluegill showed significant reduced hematocrit, causing a lowered mean corpuscular hemoglobin concentration (MCHC), which is a measure of the concentration of hemoglobin in a given volume of packed red blood cells, and reduced MCHC causes impaired respiratory capacity (Lemly, 1993b). In the present study, fish fed 11 mg kg^{-1} selenite or 16 mg kg^{-1} SeMet had

reduced MCHC, although the individual concentration of hemoglobin and red blood cells did not alter significantly, which reflects a reduced hemoglobin per red blood cell possibly affecting oxygen carrying capacity.

Organ toxicity

In the present study, liver Se levels were higher in fish fed dietary selenite compared to fish fed SeMet, as was observed in earlier trials (Berntssen et al., 2017). Dietary selenite and SeMet seem to distribute differently, whereas selenite accumulated relatively more in liver, SeMet accumulated relatively more in whole fish and muscle (Berntssen et al., 2017). This preferential accumulation of SeMet in muscle and whole body compared to selenite, is attributed to the difference in metabolism of SeMet compared to selenite (Rider et al., 2009; Fontagne-Dicharry et al., 2015; Godin et al., 2015). Selenomethionine can be directly and nonspecifically be incorporated in any protein containing methionine, while selenite needs to form hydrogen selenide (H_2Se) before being incorporated in specific selenoproteins (Suzuki, 2005; Godin et al., 2015). In the present study, the higher liver loads were associated with more prevailed liver toxicity for selenite fed Atlantic salmon compared to SeMet fed Atlantic salmon. The most clear liver histopathology was hepatocyte degeneration and focal necrosis, which occurred significantly in fish fed 5.4 mg selenite kg^{-1} or fish fed 21 mg SeMet kg^{-1} ww. The hepatic somatic index, however, did not alter significantly in any of the selenite and SeMet fed fish. In addition, increased inflammation was observed in both selenite and SeMet fed fish, however, none of these changes were significant. Earlier studies with other fish species also show liver histopathology such as hepatocellularvacuolar degeneration and necrosis in excess dietary organic Se exposed white sturgeon (Acipenser transmontanus) (Tashjian et al., 2006) or bluegill (Finley, 1985). In contrast, other studies with white sturgeon fed graded levels of SeMet that showed a dose- dependent increase in frequency and size of melanomacrophage aggregates, had no liver necrosis or general cell health (De Riu et al., 2014; Zee et al., 2016a), and in contrast to the present study, rainbow trout fed selenite had no histopathological lesions (Hilton et al., 1980). In the present study, the observed liver histopathology was associated with elevated plasma AP and ALAT (fish fed 5.4 and 11 mg kg^{-1} selenite, respectively), which are markers of liver toxicity due loss of liver specific enzymes to the blood stream. Only fish fed 39 mg SeMet kg^{-1} showed altered AP and ASAT levels, coinciding with a lower potential in liver pathology/toxicity of dietary SeMet compared to dietary selenite. In addition to liver toxicity, both dietary selenite and SeMet are also known to induce renal histopathological toxicity (Hilton et al., 1982; Finley, 1985; Tashjian et al., 2006; De Riu et al., 2014; Zee et al., 2016a). The present study did not include renal histopathology evaluation, however, plasma markers of renal function such as altered plasma creatinine and total protein were significantly altered in Atlantic salmon fed 39 mg/kg^{-1} SeMet, while fish fed selenite had no altered plasma creatinine and total protein. In addition, plasma ureic acid decreased in both SeMet and selenite exposed Atlantic salmon (see benchmark dose assessment under), however, with no significant ANOVA difference among the exposure groups. Interesting, SeMet and selenite fed fish had no significant effect on osmoregulation as seen from unaltered plasma osmolality, K, Na, and Cl, however, plasma Ca was significantly reduced in dietary SeMet fed Atlantic salmon. Fish exposed to dietary or waterborne selenium showed renal calcinosis and deposits in earlier rainbow trout studies (Hilton and Hodson, 1983)

Markers of lipid and oxidative stress

In the present study, for Atlantic salmon fed 11 mg kg^{-1} selenite a significant reduction in histopathological lipid intracytoplasmatic vacuolization was observed, concurrent total liver lipid was reduced in fish fed 11 mg kg^{-1} selenite or 39 mg kg^{-1} SeMet exposed fish, with significantly reduced liver triglyceride (TAG) in 11 mg kg^{-1} selenite fed fish. Earlier dietary

SeMet fish studies showed an altered energy storage (De Riu et al., 2014) as seen from liver histological changes such as cytoplasmic glycogen depletion (Teh et al., 2004; De Riu et al., 2014) and fatty vacuolar degeneration (Teh et al., 2004; Zee et al., 2016a). Furthermore, as in the present study, juvenile rainbow trout exposed to selenized-yeast worms had reduced liver triglyceride (TAG) and disturbed lipid synthesis and metabolism (Knight et al., 2016; Pacitti et al., 2016b; Berntssen et al., 2017). Wide-scope transcriptomic assessment showed that organic Se increases the expression of networks for growth related signaling cascades in addition to those related to fatty acid synthesis and metabolism (Knight et al., 2016). The disruption of metabolites related to triglyceride processing and storage, as well as gene networks for epidermal growth factor and Notch signaling in the liver, were suggested to represent key molecular initiating events for adverse outcomes related to growth and Se toxicity in fish (Knight et al., 2016). As reported in earlier Atlantic salmon trials (Berntssen et al., 2017), markers of oxidative stress such as GSH and vitamin E, were some of the most sensitives responses to both selenite and SeMet exposures. Oxidative stress has been suggested as a central mechanism in selenite and SeMet mediated toxicity in fish (Miller et al., 2007; Misra and Niyogi, 2009; Misra et al., 2012; Hursky and Pietrock, 2015; Lee et al., 2015), although recent studies report dietary SeMet induced toxicity without clear oxidative stress (Knight et al., 2016; Zee et al., 2016b). Two mechanisms of Se mediated redox cycling and GSH consumption have been identified; one is production of superoxide anion by Se compounds (Spallholz et al., 2004), where selenite is much more potent than SeMet (Goswami et al., 2012), and the other is oxidation by flavin containing monooxygenase to selenoxides, which are potent oxidizers of GSH and protein thiols (Lavado et al., 2012). Both the selenite and SeMet groups had reduced GSH and cysteine, with less reduction in the SeMet-yeast group compared to the selenite group. As cysteine is part of the GSH tripeptide, depletion of both cysteine and GSH indicate use of GSH during high selenite and SeMet-yeast exposures. Lowered GSH and a shift from reduced to oxidized glutathione (GSH:GSSG) together with increased anti-oxidant enzymes was observed in selenite and SeMet exposed rainbow trout hepatocytes (Misra and Niyogi, 2009). The lowered GSH at excess selenite and SeMet intake could be due to GSH's direct role as antioxidant or substrate to antioxidant enzymes such as glutathione peroxidase (GPx), but also due to GSHs involvement in selenite and SeMet cellular metabolism. Selenite reacts with GSH and produces hydrogen selenide, which can form ROS (Lin and Spallholz, 1993) that further increases the demand for antioxidants such as GSH. SeMet is methylated to methylselenol forms for further excretion, if not directly incorporated in general body proteins (Suzuki, 2005). The redox cycling of these methylselenols in the presence of GSH has been suggested to produce oxidative stress (Palace et al., 2004). In the present trial, however, both GSH and GSSG were lowered and the ratio GSH:GSSG did not alter indicating that total GSH was used as part of Se and Semet cellular metabolism rather than anti-oxidant role. The latter could also explain the sensitive (at lowest dose) GSG/GSSG response. The depletion of vitamin E indicates oxidative stress, which was more pronounced in selenite exposed fish compared to SeMet exposed fish (significant decrease 11 mg kg^{-1} selenite versus 39 mg kg^{-1} SeMet). The oxidative stress did not lead to a significant formation of lipid peroxidation, as seen from increased TBARS, as was reported in an earlier trial (Berntssen et al., 2017)

Comparison literature NOAEL and LOEAL and bench mark dose

Table 2 in the supplementary material gives an overview of the reported NOAEL and LOAEL in previous studies on graded (more than two exposure groups) levels of organic and inorganic selenium in several fish species. The present paper will focus on comparing the studies on rainbow trout and Atlantic salmon (summarized in Table 7). As none of the previous studies have used bench mark dosing to assess safe levels of dietary exposure, the NOAEL and

LOAEL of this study are given as well. The earlier established NOAEL will be compared with the newly assessed BMDL from this study as a safe limit for Se supplementation with regards to fish welfare.

The lowest LOAEL for SeMet in rainbow trout variated from 0.071-0.12 mg kg BW-1 day-1 (Vidal et al., 2005; Knight et al., 2016) and for selenite from 0.14-0.45 mg kg BW-1 day-1 (Hilton et al., 1980; Rider et al., 2009). This variation depends on life stage, relative feed intake, form of Se inclusion (as Se-yeast, L-SeMet, or part of natural feed), duration of exposure, and selected end-points of toxicity. Especially converting feed concentrations to daily dose per Kg body weight is of importance for comparison of studies using different life stages, as relative daily feed intake (and hence daily Se dose) decreases with increased fish size (Austreng et al., 1987). The wide range of LOAEL and higher LOAEL for selenite than SeMet, indicates the difficulty establishing threshold levels of toxicity when comparing literature, even from one species and with correction for assumed feed intake.

The assessment of safe limits is even more variable as the lowest NOAEL is often expressed as the control group, with NOAEL for SeMet in rainbow trout variating from 0.006-0.013 mg kg DW-1 day-1 (Vidal et al., 2005; Knight et al., 2016) and 0.07- 0.13 mg kg BW^{-1} dau^{-1} for selenite (Hilton et al., 1980; Rider et al., 2009). In addition to variation between studies, the use of analysis of variance (ANOVA) type of analysis within a trial leaves uncertainty as to where the toxicity threshold lies between the NOAEL and LOAEL, or even lower than the NOAEL. For example, an earlier dietary SeMet (as Se-yeast) dose-response study with juvenile splittail (*Pogonichthys macrolepidotus*) that established a NOAEL-LOAEL of respectively 2.7-6.6 mg kg-1 DW (Teh et al., 2004), was re-analysed by using a logistic regression to derive effective concentration 10 (EC10) re-establishing threshold values of 0.9 mg kg-1 dw feed (Rigby et al., 2010).

In establishing safe dietary limits, not only the threshold levels of toxicity is of importance but also the dietary levels that give no toxic effect. The introduction of the lower bound bench mark dose (BMDL) allows establishing of safe limits based on a common assessment of different dose response curve models fits that are associated with a specific change in response (the bench mark response; BMR)(EFSA, 2017b). The lower bound bench mark dose (BMDL) gives the lower 90% variant of dose response model fit, which is defined as the dose that is not giving an adverse effect and is hence an alternative to NOAEL(EFSA, 2017b). For direct continuous adverse effects data (reduced growth, feed conversion, organ somatic indices) a BMR change of 5% is used ($BMDL_{05}$) while for quantal histopathological data a BMR of 10% with extra risk is used ($BMDL_{10}$ extra risk) to set safe levels(EFSA, 2017b), The acceptable BMR changes for biomarker of adverse effect or exposure (EFSA, 2017a) is less well defined, but for plasma responses (e.g. ALT ASAT) a BMR of 20% was suggested (EFSA, 2017b). As the biomarkers of adverse effect or exposure are early warning for toxic mode of actions that lead to later adverse effects, it could be argued that a larger change in response may be chosen to set safe limits. As the threshold levels of toxicity strongly depend on exposure duration (Teh et al., 2004) and most chronic trial only cover 10% of a life cycle (3 months), the use of early biological markers of selenite or SeMet toxicity is useful.

In the present study, for dietary selenite the best dose-response model fits (lowest BMDL and BMDU variation and AIC) with the lowest BMDL were assessed for plasma ASAT, liver vitamin E and histopathology, giving a safe dietary dose of 1-2 mg/kg WW (dose 0.01-0.02 mg/kg BW/day) of total Se for selenite supplementation. However, the loss of two dose group due to mortality and the significant reduction of GSH and GSSG at the lowest exposure level warrants dose-response studies in Atlantic salmon in the range of 0.5-5 mg kg-1 ww to assess more accurately the safe limit in Atlantic salmon with regards to animal welfare. For dietary SeMet a

more accurate BMDL dose-response assessment could be made giving lowest BMDL for safe animal welfare limits of 2.8 mg /kg ww (dose 0.03 mg kgBW-1 day-1) based on liver histopathology and plasma creatinine model fits. The present study confirmed a higher relative toxicity for selenite than for SeMet and the safe limits are in the same range as the earlier reviewed general non effect levels for total selenium of <2-3 mg/kg DW in fish (Lemly, 1993a; Hamilton, 2004).

In conclusion, feed levels of 1-2 mg kg-1 (0.11-0.21 mg /Kg BW/day) and 2.8 mg kg-1 (0.29 mg/Kg BW/day) total Se for respectively selenite and SeMet supplementation to a low fish meal (10%) based diets with background levels of 0.45 mg kg-1, were safe with regards to fish welfare. SeMet is known to accumulate in the muscle (Berntssen et al., 2017), and as food safety is part of the dietary Se limit assessment, further studies are warranted on the feed-to-fillet transfer of dietary Se supplementation to assess food safety.

Table 7, Literature overview of none observed adverse effect level (NOAEL) and lowest observed adverse effect levels (LOAEL) in rainbow trout (*Oncorhynchus mykiss)* and Atlantic salmon (*Salmon salar*) fed graded levels of inorganic or organic selenium.

	NOAEL	LOAEL	Feed intake	Se form	length	endpoint	reference
rainbow trout	mg/kg	mg/kg	% BW/Day		days		exposure range
79 g	?	9 mg/kg	?	selenite	294 days	mortality	Goettl and Davies, 1978 from Hamilton et
1,3 g	3.67 mg/kg DW	13 mg/kg DW	2.9*	selenite	140 days	mortality/growth/FCR	Hilton et al. 1980
	13.06 mg/kg DW	*		selenite		no hematology, no histopathology	0.07, 0.15, 0.28, 1,25, 3.67, 13.06 mg/kg
0.6 g	11.4 mg/kg DW	*			112 days	No significant mortality	Hilton and Hodson 1983
	6.6 mg/kg DW	11.4 mg/kg DW	4.6*			Reduced growth	0.6, 6.6, 11.4 mg/kg DW
	11.4 mg/kg DW	*				No hematology or blood plasma parameters	
26 g	7.1 mg /kg ww	*	2	seleinte	70 days	no reduced growth FCR/no oxidative stress/Hct	Rider et al. 2009
	3.9 mg/kg ww	7.1 mg/kg ww				increased oxidative stress after physical stress	0.73, 2.3, 3.9, 7.1 mg/kg ww
26 g	7,4 mg/kg ww		2	Se-yeast	70 days	No mortaility no redued growth	Rider et al. 2009 organic 2 (2.4), 4 (4.1), 8
0.37 g	2,4 mg/kg DW	4.6 mg/kg DW	0.8 *	Selenomethionine	90 days	Reduced growth,	Vidal et al. 2005
	18 mg/kg DW					no mortaolity or oxidative stress	0.23, 4.6, 12, 18 mg kg DW
33.47 g	3 mg/kg WW	*	?	Se yeast Sel-Pex		No growth reduction or oxidative stress	Hunt ey al. 2011
							control, 2, 3, 4 mg/kg
0.28 g	1.3 mg/kg DW	7.1-19.5 mg/kg DV	1.6**	Semet worm	60 days	Reduced final weight	Knight et al. 2016
	7.1 mg/kg DW	19.5 mg/kg DW				Reduced liver TAG	1.3, 7.1, 10.7, 19.5, 31.8 mg/kg DW
	1.3 mg/kg DW	7.1 mg/kg DW				microarray lipid metabolism	
100 g	8.94 mg/kg ww	*	2	Se-Plex	70 days	No reduced growth	Pacitti et al. 2015
							0.87, 1.46, 4.81, 8.94 mg/kg ww
Atlantic salmon							
147 g	5.4 mg/kg	11 mg/kg		Selenite	90 days	Mortality	this study
	0.45 mg/kg	5.4 mg/kg	1.1			Histopathology plasa enzymnes	0.45, 5.,4, 11, 29, 60
	0.45-5.4 mg/kg w	5.4-11 mg/kg				oxidatve stress (GSSG and vit E,)	
572 g	15 mg/kg WW	*		selenite	90 days	No mortality	Berntssen et al. 2017
	1 mg/kg ww	15 mg/kg ww				Reduced growth	0.35, 1, 15 mg/kg WW
	1 mg/kg ww	15 mg/kg ww	0.9			Oxidatev stress (GSSG and vit E)	
	1 mg/kg ww	15 mg/kg ww				Metablomics lipid pathway distrubance	
147 g	39 mg/kg ww	*		selenomentione	90 days	No mortality	this study
	21 mg/kg ww	39 mg/kg ww	1.1			Histopathology plasma enzymens	0.45, 6.2 , 16, 21, 39 mg/kg ww
	6.2-21 mg/kg ww	16-39 mg/kg ww				oxidatives tress (GSH+GSSG and Vit E)	
572 g	15 mg/kg WW	*	0.9	selenomentione	90 days	No mortality, reduced growth, oxidative stress	Berntssen et al. 2017
	1 mg/kg ww	15 mg/kg ww				Metablomics lipid pathway distrubance	0.35, 2, 15 mg/kg WW

*Estimated daily feed intake based on given specific growth rate (SGR) and feed conversion factor (FCR)
**Estimated feed intake based on conversion of wet weight daily feed intake of worms to feed concentration expressed as dry weight on the assumption of 30% dry material

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Supplementary data

Table 1. Description of the semi-quantitative scoring system using different parameters to assess the liver of Atlantic salmon fed the seven experimental feeds containing different levels of selenomethionine and selenite

Score	Parameter
Hepatocyte intracytoplasmatic vacuolization	
0	Not observed
1	Few vacuoles
2	Medium vacuolization of hepatocytes
3	Severe presence of vacuoles
Degeneration and focal necrosis	
0	None
1	Mild (acidophilic bodies, hepatocyte degeneration and/or scattered foci of hepatocellular necrosis in less than 1/3
2	Marked (changes observed in more than 1/3 of the section)
Inflammation	
0	No inflammation
1	Mild (sprinkling of inflammatory cells)
2	Marked

Table 2, Literature over view of none observed adverse effect level (NOAEL) and lowest observed adverse effect levels (LOAEL) in different fish species fed graded levels of inorganic or organic selenium.

Bluegill								
19.1 g end	14 mg/kg ww	31 mg/kg ww	Selenite	259 days	reduced weight	Woock et al. 1987 from Hamilton et al. 20		
	14 mg/kg ww	31 mg/kg ww			Larval survival	0.79, 14, 31 mg/kgb ww		
19.1 g end	13 mg/kg ww	30 mg/kgww	Selenomethionine	260 days	Mortality and reduced weight	Woock et al. 1987 from Hamilton et al. 20		
	13 mg/kg ww	30 mg/kgww			Larval survival	0.79, 3.6, 13, 30 mg/kg ww		
adulr	16.8 mg/kg DW	33 mg/kg (DW)	Selenomethionine	140 days	reproduction	Coyle et al. 1993 from Hamilton et al. 2004		
						0.8, 4.6, 16.8, 33.3 mg/kg DW		
0,2 g	2.3 mg/kg ww	6,5 mg/kg ww	Selenomethionine	59 days	mortality	Cleveland et al. 1993 from Hamilton et al.		
white sturgeon				72 days				
124 g	5.6 mg/kg DW	22.4 mg/kg DW	Selenomethionine		mortality/euthanization	Zee et al. 2016		
	1,4 mg kg/DW	5.6 mg/ kgDW			edema	(1.4, 5.6, 22.4, 104 mg/kg DW)		
	22.4 mg/kg DW	104 mg/kg DW			weigth and length			
	22.4 mg/kg DW	104 mg/kg DW			HSI and histology lipid stores cell surface			
29 g	192 mg/kg		Selenomethionine		no mortality	Tashjian et la. 2006		
	20.5 mg/kg DW	42 mg Se/kg			reduced Swimming activity and growth	0.4, 9.6, 20.5, 41,7, 89.9, 191 mg/kg DW		
	9.6 mg/kg DW	20.5 mg/kg			Histopathology inthe liver and kidney			
30 g	77 mg/kg DW		Selenomethionine		No mortality	DeRiu et al.		
	2.2 mg/kg DW	19.7 mg/kg DW			HSI increase	2.2, 19.7, 40.1, 77.7 mg/kg dw		
	19.7 mg/kg DW	40.1 mg/kg DW			growth reduction			
	19.7 mg/kg DW	40.1 mg/kg DW			whole bbody energy			
green sturgeon								
30 g	40 mg/kg DW	77.7 mg/kg DW	Selenomethionine		Mortality	DeRiu et al.		
	2.2 mg/kg DW	19.7 mg/kg DW			reduced growth	2.2, 19.7, 40.1, 77.7 mg/kg dw		
	19.7 mg/kg DW	40.1 mg/kg DW			HSI reduced			
	2.2 mg/kg DW	19.7 mg/kg DW			whole body energy			
beluga Sturgeon (huso huso)								
3.5 g	20,3 mg/kg DW		Selenomethionine		no differences heamatology	Arshed et al. 2011		
					no histopathology	1.26, 2.44, 3.01, 5.37, 11.56, 20.26 mg /kg		
					no adverse growth			
splittail								
6.8 g	2.7 mg/kg DW	6.6 mg/kg DW	Seleno-yeast	252 days	deleterious health, deformaties	Teh et al. 2004		
	12.6 mg/kg DW	26 mg/kg DW			mortalities	0.4, 0.7, 1.4, 2.7, 6.6, 12.6, 26.0, and 57.6 n		
	12.6 mg/kg DW	26 mg/kg DW			growth			
	2.7 mg/kg DW	6.6 mg/kg DW			liveer pathology			

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6 Food safety

Modelling the feed-to-fillet transfer of selenite and selenomethionine supplements in plant based feeds to farmed Atlantic salmon (*Salmo salar*).

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Abstract

The transfer of dietary selenite and selenomethionine (SeMet) to the fillet of farmed Atlantic salmon was studied. The uptake and elimination rate constants of the two selenium (Se) forms were determined in seawater adapted Atlantic salmon (initial weight 174 g) fed on either selenite or SeMet enriched diets (5.4 and 6.2 mg Se kg^{-1} for selenite and SeMet, respectively) for 90 days, followed by a 90 days depuration period with feeding on high plant-based control diets (0.4 mg kg^{-1} Se). Dietary SeMet had a high biomagnification factor than selenite (1.62±0.23 versus 0.096±0.15 for SeMet and selenite, respectively) due to a significantly ($p<0.05$) higher uptake rate (0.148±0.016 versus 0.012±0.001, respectively) and slower elimination rate (0.98±0.39 versus1.8±0.45, respectively). The fillet half-lives of selenite and SeMet were 779±188 and 339±103 days, respectively. The elimination and uptake rates were used in simple one-compartmental kinetic model to predict levels in fillet based on long term (whole production cycle) feeding with given dietary Se levels and aquaculture production parameter such as feed intake and growth rate. Model predictions were validated with experimental uptake and elimination data as well as comparing model predicted values with surveyed levels in commercial feed and farmed Atlantic salmon, which gave a good correlation between predicted and observed values. Model predictions for salmon fed plant based feeds low in natural Se and with supplemented with either 0.2 mg Se kg^{-1} of selenite or SeMet gave predicted of 0.042 and 0.058 mg Se kg-1 ww. Based on these predictions and earlier EFSA risk assessment of Se supplementation on food producing land animals, the supplementation with 0.2 mg Se kg^{-1} selenite would likely protect food safety for the most sensitive consumer group (toddlers). However, supplementing farmed animals, including salmon, with 0.2 mg Se kg^{-1} SeMet would give a slightly (113%) higher Se intake than the set upper limit for toddlers (60 µg day^{-1} for total Se intake from all food products).

Introduction

Due to a rapid growth in aquaculture and limited access to marine resources, fish oil and fish meal in feeds for carnivorous marine species such as Atlantic salmon (*Salmo salar*) have been replaced with plant ingredients the last decade (Ytrestoyl et al., 2015). The change from marine to plant feed ingredients will alter the nutritional composition of salmon feeds, reducing essential micro-nutrients that are naturally high in fish meal and oil such as vitamins and minerals (Sissener et al., 2013). Selenium (Se) is one of the essential minerals that is known to be higher in fish meal than plant ingredients (Betancor et al., 2016), although plant products can differ largely in Se content according to Se concentration in soil (Alfthan et al., 2015). A decline of Se in Norwegian produced commercial salmon feed during the last decade has been attributed to the decreased use of fish meal (Sissener et al., 2013). Selenium concentration in Atlantic salmon flesh were lower when fed on plant protein replacement feeds compared to marine protein feeds (Betancor et al., 2016). Selenium is a well-known essential trace element (Ullah et al., 2018) active as part of functional selenoproteins (Kryukov and Gladyshev, 2000) involved in physiological processes such as antioxidant defense (glutathioneperoxidases) (Toppo et al., 2008) and thyroid homeostasis (deiodinases) (Schweizer and Steegborn, 2015). Of all food products, seafood has some of the highest natural background Se levels (EFSA, 2010; Ullah et al., 2018). Several studies on fish nutrition have suggested Se supplementation to plant based feeds to restore or maintain Se levels in farmed fish as a Se source for consumers (Betancor et al., 2016; Pacitti et al., 2016). In addition, Se supplementation to plant based feed to marine carnivorous fish has been suggested to be needed to cover animal nutrient requirement or ensure health robustness of the farmed fish (Fontagne-Dicharry et al., 2015; Godin et al., 2015; Pacitti et al., 2015; Ilham et al., 2016a; Ilham et al., 2016b). In the European Union (EU), feeds can be supplemented with organic (e.g. selenized yeasts) Se at a maximum authorised level of 0·2 mg/kg (Regulations (EU) No 427/2013; 445/2013; 121/2014; 847/2014 and 2015/489). The maximum limit for total Se in animal feeds including fish feed has been set at 0.5 mg/kg feed (EC 1831/2003 and amendments).

In addition to being an essential element, excess Se is known to be toxic at low concentrations (Jablonska and Vinceti, 2015) and a narrow window exists between requirement and toxicity for most vertebrates, including humans (Rayman, 2012). The EU recommended daily Se intake for adults in the is 70 µg day (EFSA, 2014), while a 4-fold higher intake of 300 µg day should not be exceeded to guarantee food safety and consumer health (EC, 2000). Concern has been expressed for excess Se intake for consumers of animal food products (egg, milk, and meat) from farmed land animals that are raised on Se supplemented feeds (EFSA, 2011b). This accounts in particular for the safety of children of 1-3 years of age (toddlers), which have a recommended upper limit (UL) of intake of 60 ug /day (EC, 2000). The supplementation of SeMet to feed is more limiting compared to selenite, as edible tissues and animal products—particularly meat, eggs and milk—from animals fed diets supplemented with organic Se contain significantly more Se than those from animals given inorganic sources of Se (EFSA, 2013). Similarly for fish, SeMet has a relative higher accumlation in muscle compared to selenite (Kleinow and Brooks, 1986a; Rider et al., 2009; Fontagne-Dicharry et al., 2015; Godin et al., 2015; Berntssen et al., 2017). Based on the high transfer of supplemented dietary SeMet to edible part of food producing land animals (milk, egg and pig), the current upper limit of SeMet supplementation has been set to ensure food safety. However, such feed-to food transfer assessment is lacking for farmed fish species (EFSA, 2013).

Several toxico-kinetic transfer models have been used to describe the fate of dietary contaminants in food-producing animals, including Atlantic salmon, which are valuable tools in predicting food contamination at different feed compositions and different feed contamination

scenarios (Hoogenboom et al., 2010; Berntssen et al., 2011c). Fish transfer models are based on uptake and elimination kinetics and vary from simple one-compartmental transfer models (Berntssen et al., 2007; Brambilla et al., 2007; Berntssen et al., 2011a) to multi-compartmental physiological-kinetic based (PKB) models (Nichols et al., 2004; Berntssen et al., 2011c; Berntssen et al., 2013), which allow a simple one-compartmental kinetic modeling of feed-to-fillet transfer (Berntssen et al. 20016). Kinetic studies on dietary Se in fish have shown a high intestinal absorption with a higher uptake rate for SeMet than inorganic Se (Kleinow and Brooks, 1986a, b), and Se elimination is dominated by gill and urine with lesser routes by bile and mucus (Kleinow and Brooks, 1986b). The biological half-life of Se from tissues decreased with increased Se loading except in the liver, suggesting a rate-limiting metabolic transformation of Se for excretion in this organ (Hilton et al., 1982). Hilton and Hodson (1983), reported liver and kidney elimination half-life of 19-45 and 26-28 days, depending on Se feed concentrations. Similarly, in long-term graded fed SeMet cutthroat trout (*Oncorhynchus clarkii*), the whole body elimination increased with increased initial body burden (Hardy et al., 2010), with whole-body half-lives variating from 518 to 84 days (Hardy et al., 2010).

Although routes of elimination and organ and whole body elimination rates have been assessed, the muscle uptake and elimination kinetics are lacking that can form the basis for feed—to-food transfer assessment. The present study aims to assess the uptake and elimination of selenite and SeMet from the muscle of Atlantic salmon fed elevated levels for three months followed by an extended (~ 3 months) depuration period. The present report aims to provide basic kinetic data for dietary selenite and SeMet. Future feed-to-fillet transfer assessment will be based on physiological based toxico-kinetic (PBK) multi- compartmental models based on earlier published Atlantic salmon models (Berntssen et al., 2011d; Berntssen et al., 2013).

Material and methods

Ethic statement

The experiment was approved by the Norwegian Food Safety Authority (Mattilsynet; FOTS ID: 9003) and performed in compliance with national and international ethical standards.

Experimental conditions

The feeding trial was carried out at NOFIMA (Sunndalsøra, Norway) between November, 15 2016-March 3 2017. Details in the experimental set-up will be published elsewhere (Berntssen et al., 2018). Briefly, a total of 630 Atlantic salmon smolt (*Salmo salar*, L., Salmobreed, 6 months, both genders) with an average initial weight of were randomly distributed into 9 tanks with 70 fish in each tank with an initial weight of 147 ± 4 g (mean ± SD, n=30). Prior to the experiment, all fish were fed the control diet (background level of 0.45 mg kg-1 total selenium) during a 2-week acclimation to holding facilities.
The fish were acclimated to the holding facilities for 2 weeks while being fed a low selenium plant based control diet with background total selenium levels of 0.45 mg kg-1. After the acclimatization period, fish in randomly selected triplicate tanks were fed selenite or SeMet supplemented diets at a nominal levels of 5 mg kg^{-1} (analysed levels 5.4 ± 0.09 and 6.2 ± 0.2 mg kg-1, for selenite and SeMet, respectively, mean±SD, n=3), for 90 days. Following the dietary selenite and SeMet exposure period, the fish were given control diets during an depuration period of 90 days. The feeding regime was based on automatic feeders under a photoperiod regime with 24h light. Six daily meals were provided with 4 hours between the meals, with to a level approximating 1.1 % of body weight per day. The feeding rate was

adjusted for growth biomass increase, which was assessed by measured average weight gain of the sampled fish per sampling time point. Fish were routinely monitored for nutritional performance and appetite throughout the experiment. Unconsumed feed pellets were collected and weighed once per day, for calculation of feed intake, feed conversion and Se exposure. To avoid possible leakage from feces or pellets to the water, a relative high water flow-through was maintained of 12 L min−1 per tank. During the accumulation period three fish from each tank were sampled at day 0, 4, 8, 20, 45, 75 and 90. During the depuration period three fish per tank were sampled at day 0, 2, 6, 12, 24, 72 and 90. Fish were randomly collected from the tanks, anesthetized in a bath of tricaine methanesulfonate (FINQUEL MS-222; ~ 60mg L^{-1}), and sacrificed by a blow on the head. Fish were stored at -28 °C and at the end of the experiment all fish were thawed, weighed and filleted (whole fillet muscle with skin on the left side of the salmon, fillet weight per fish was registered), three fish per tank were pooled (n=3 per sampling point), and analysed for selenium.

Se analyses

Diets and tissues of fish were digested using the microwave-acid decomposition method based on the method described by Berntssen et al. (2017), modified after Julshamn et al. (2007). Briefly, samples were weighted (0.20 – 0.25 g) into 15 mL quartz digestion vessel (Milestone Srl, Sorisole, BG, Italy), containing deionized water (0.5 mL; Milli-Q, Merck Millipore, Oslo, Norway). Concentrated nitric acid (HNO_3, 2 mL, Fluka, Sigma-Aldrich, Oslo, Norway) were added to all samples. The vessels were capped and placed in the Ultrawave (UW, SRC, Milestone, Shelton, CT, USA), with a container of 30 mL Milli-Q water and 5 mL hydrogen peroxide (H_2O_2, Emsure ACS, ISO, 32% w/w; VWR, Oslo, Norway). The gas pressure in the UW was set to 40 bar and the temperature increased incrementally to 260 °C. Freeze-dried muscle samples were diluted to 25 mL and 10 mL, respectively, with Milli-Q water. Total Se concentration were determined in the digests using ICP-MS (iCAP-Q and FAST SC-4Q DX auto sampler, both Thermo Fisher Scientific Inc, Waltham, Massachusetts, USA). An external calibration curve was made from freshly prepared multi element standard diluted to appropriate concentrations by 5% (v/v) HNO_3. The instrument was used with a collision cell gas, using He KED (kinetic energy discrimination) mode for interference removal. A solution of internal standard (Ge, Rh and Tm, Thermo Fisher Scientific Inc) was added on-line for correction of instrumental drift during the analysis. The instrument was optimized using a tuning solution (1 ppb tuning solution B, Thermo Fisher Inc, in 2% HNO_3 and 0.5% HCl (puriss. p.a, Sigma-Aldrich) prior to analysis. Plasma power was set to 1550 W, carrier/nebulizer gas flow to 1.05 L/min, the plasma/auxilliary gas flow to 0.8 L/min, and He gas (CCT1) flow was 4.6 mL/min. Isotope ^{78}Se was monitored, and the integration time was 0.1 sec. Oyster Tissue (OT, CRM 1566 b, National Institute of Standards and Technology, Gaithersburg, MD, USA) and Lobster Hepatopancreas (TORT-3, National Research Council Canada, NRC, Ontario, Canada) were used as reference materials for the analysis and analysed values were in agreement with certified values. The LOQ for total Se is 0.01 mg kg^{-1}.

Model description

A simple one-compartment fish model derived from Sijm et al. (1992) was employed to predict total Se fillet concentrations in fish fed with different feed selenite and SeMet supplementation levels. The model describes the feed-to-fish transfer as the product of feed concentration ($C_{feed,}$ mg Se kg^{-1}), feeding rate (F, % body weight day^{-1}), uptake rate (α, mg Se day^{-1}), initial concentration in the fish fillet ($C_{fish0,}$ mg Se kg^{-1}), growth dilution (γ, % body weight day^{-1}), and

physiological elimination rate (K, day^{-1}) of physiological elimination (Figure 1)(Berntssen et al., 2007; Berntssen et al., 2016).

With a specific uptake and elimination rate constant for organic and inorganic Se at a certain feeding rate and dietary concentration (C_{feed}), the concentration of a chemical in a fish (C_{fish}) at a given time can be described as

(1) $$C_{\text{fish}}(t) = \frac{\alpha F t}{K+\gamma} C_{feed}\,(1-e^{-(K+\gamma)t}) + C_{filsh0}^{\;\;-(K+\gamma)t}$$

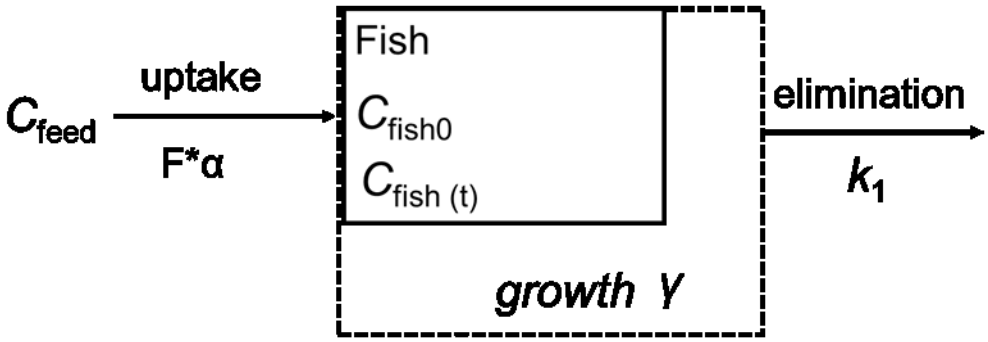

Figure 1. Schematic representation of the feed-to-fillet transfer kinetics in Atlantic salmon: C_{feed} concentration in feed; F is feeding rate; α, uptake rate ; C_{fish0} initial concentration in fish; γ, growth; K_1, elimination.

Statistics

The dietary selenite and SeMet uptake and elimination kinetics in Atlantic salmon were assessed by a simple one-compartmental kinetic model, as described earlier for Atlantic salmon exposed to dietary endosulfan (Berntssen et al., 2008), toxaphene (Berntssen et al., 2011b) and dioxins (Berntssen et al., 2007; Berntssen et al., 2016). As detectable selenium levels were present in the acclimatized fish and the low selenium control diet, data from the selenite and SeMet dietary groups were corrected for fish from the control group to compensate for background selenium levels. Growth rates were calculated by fitting fish weight to the equation; ln fish weight= *a*+*b***t*, where *a* is a constant, *b* the growth rate (g day^{-1}), and *t* the time of experiment. The elimination constant (K_{el}) was determined by fitting concentration data to a first-order decay curve; ln $\underline{C}_{fillet}$ = $\underline{a}$ + $\underline{k}_{el}$. Elimination half-lives ($t_{1/2}$) are ln2/ $\underline{k}_{el}$. The weight of the fish increased from 147g to 468g during the accumulation period and from 468g to 953g during the elimination period. This growth itself would give a decrease in fillet Se concentration of ~220% during the accumulation period and ~100% during the elimination period. To compensate for the growth dilution, the uptake and elimination rates were assessed at total fillet Se amount.

The uptake rates were calculated by fitting (Statistica, Statsoft Inc., Tulsa USA, 1993) the concentration data to the integrated form of the kinetic rate equation (1) for constant dietary exposure (Bruggeman et al., 1981)

(2) $$\alpha = \frac{C_{\text{fish}}(t) \cdot k_{\text{el}}}{F \cdot \text{C}_{\text{feed}} [1 - \exp(-k_{el} \cdot t)]}$$

where C_{feed} is the Se concentration (mg g^{-1} wet weight) in feed; α is the uptake rate constant; and F is feeding rate (g feed g^{-1} fish d^{-1}).

The biomagnification factor was determined with the following formula (2) (Muir and Yarechewski, 1988).

(3) $$BMF = \frac{\alpha F}{k_{el}}$$

All statistics were performed using the program Statistica (Statsoft Inc., Tulsa USA). Statistical differences in Se concentrations and amount between sampling points were assessed one-way ANOVA, followed by Tukey's HSD post hoc test at a significance level of 0.05 (Zar, 1984).

Selenium input data and model scenarios

The following feeding scenarios were used to estimate fillet Se levels, Scenario 1: average selenium levels from Norwegian feed surveillance in 2016 (1.1 mg kg^{-1}), Scenario 2+3: average selenium levels from Norwegian feed surveillance supplemented with SeMet or selenite to the current upper limit for SeMet supplementation (1.1+0.2=1.3 mg kg^{-1}). Scenario 4: Selenium background levels for fish feed with high plant and low (10%) marine protein (0.4 mg kg^{-1}). Scenario 5+6: Selenium levels for fish feed with high plant and low (10%) marine protein (0.4 mg kg^{-1}) supplemented with SeMet or selenite at the current upper limit for SeMet supplementation level (0.4+0.2=0.6 mg kg^{-1}). For long term model prediction in the different feed scenarios, an average feeding rate of 0.78 % BW day^{-1} and growth rate of 0.64 % day^{-1} was used with a duration of 13 months as could be expected in a commercial seawater food production cycle. The final growth phase started with a 100 g post-smolt and ended with an expected consumer sized weight of 4.5 kg (industrial model of Cargill EWOS innovation, Dr. K. Ruohonen pers.Com)(Berntssen et al., 2016). As initial fillet concentrations average Atlantic salmon levels of 0.10 mg kg^{-1} ww for a 100g pre-smolt was used.

Farmed salmon food safety assessment

EFSA made and earlier food safety risk assessment for the feed organic and inorganic Se supplementation to food producing land animals (EFSA, 2011). This risk assessment did not include the consumption of farmed seafood which was reared on Se supplemented feeds. In the present study, the EFSA (EFSA, 2011) opinion is used and extended with farmed Atlantic salmon fed on high plant-based diets that is supplemented with L-SeMet or selenite to the level of SeMet which is currently legal to add to all food producing animals (0.2 mg kg-1).

The following assumptions and input data were used as described in the EFSA 2011b opinion.

1) Toddlers are the most likely exposed consumer group with an upper limit for total Se intake of 60 µg day^{-1}(EC, 2000).

2) Toddlers have a background intake of 10 µg Se day^{-1} for vegetables, fruits, and cereals (EFSA, 2011).
3) Toddlers Se intake by food is based on the combined estimated levels of all farmed food producing animals when fed on Se supplemented feeds
4) As food intake assessment he 95 percentile toddlers consumption data for meat, milk, diary, and eggs are used (EFSA, 2011, 2015), which is extended with the median of the 95 percentile salmon intake of 29 g day^{-1} based on the EFSA Comprehensive European Food Consumption Database for consumers only (EFSA, 2015).
5) Background Se levels in control (low or unsupplemented) food classes are based on the EFSA 2011b opinion, and the predicted levels in Atlantic salmon when fed low Se unsupplemented high plant feeds (this study)
6) Background levels are multiplied with a factor that expresses the relative increase at a given supplementation level compared to the background Se level per food class (supplementation increase factor) (EFSA, 2011).

Results and discussion

Details on fish weight, length, condition factor, and liver somatic index will be published elsewhere (Berntssen et al., 2018). No significant differences were observed in any of the mentioned parameters observed in fish fed control or SeMet or selenite-enriched diets during the 90-day feeding period. No mortality was observed in control or the 6.4 and 5.2 mg kg^{-1} SeMet and selenite exposure groups. Further details on adverse effects on fish welfare after graded selenite and SeMet dietary exposure will be published elsewhere (Berntssen et al., 2018).

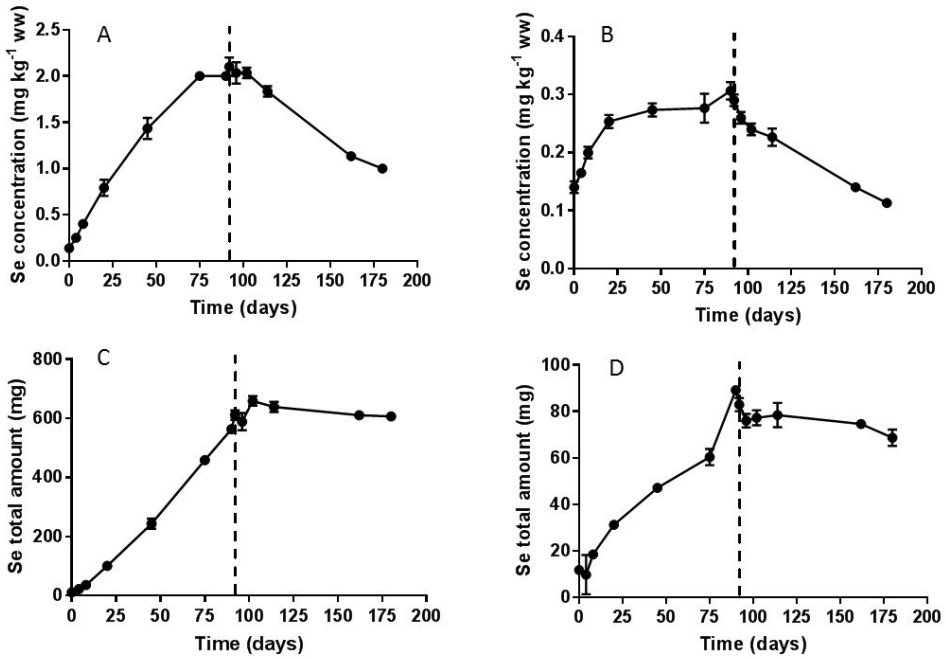

Figure 2. Concentration (mg kg^{-1} ww, A-B) and total amount (mg, C-D) of in Atlantic salmon fillet fed selenomethionine (A+C) and selenite (B+D) supplemented feeds (6.2 and 5.4 mg kg^{-}

[1], respectively) for 90 days followed by a 90 days elimination period (mean ± SD, n=3 of 3 pooled fish, per time point). Dashed vertical line signifies the end of the exposure period.

Figure 2 gives the fillet Se concentration and total fillet Se amount in Atlantic salmon fed SeMet or selenite supplemented plant based diets followed by control feed. Fish fed SeMet showed a significant increase in fillet concentrations between all subsequent sampling points besides the last two sampling points of the accumulation period (between 75 and 90 days). Fish fed selenite had a significant increase between sampling point until day 20, after which an apparent steady state condition was reached as fillet Se concentrations did not significantly increase between sampling days from day 20 to 90. In contrast, when expressed as total amount Se per fillet, thus compensating for growth dilution, both SeMet and selenite exposed fish had a continuously significantly increase at all sampling points of the accumulation period. After ending the exposure to SeMet and selenite supplemented feed, SeMet fillet amounts continued to increase for 6 days, whereas selenite levels decreased immediately after changing to control diets. The total fillet Se amount decreased significantly during the depuration period for both SeMet and selenite fed fish. However, for both exposure groups elimination was slow and baseline Se levels were not met during the depuration time used in the present trial. Based on the estimated half-lives (~ 790 and 340 days for SeMet or selenite fed fish, respectively), no steady state in both SeMet and selenite accumulation is expected to be reached during a normal seawater production cycle of > 12 months. The use of the presented one-compartmental transfer model allows predictions of Se levels during an average production cycle. In addition, final fillet Se levels are not only determined by the level of dietary Se supplementation but also aquaculture production parameters such as relative feed intake and growth rates (Berntssen et al., 2005) affect fillet levels. The present model allows the use of commercial relevant feed intake and growth rate to provide a transfer assessment at different whole cycle feeding scenarios (see under). The use of a short (sub-chronic 10% of life-cycle, ~3 months) experimental exposure feeding trial, without uptake and elimination kinetics assessment, would not appropriate assess Se fillet levels reflecting a commercial seafood production cycle.

Table 1 gives the uptake and depuration rates as well as biomagnification factor. The uptake rate of SeMet was significantly higher, and elimination significantly ($P<0.05$) lower than that of selenite. The BMF was consequently significantly higher for SeMet compared to selenite. Earlier trials showed a higher whole body ^{75}Se-isotope uptake and lower elimination, resulting in a higher accumulation of SeMet compared to selenite (Kleinow and Brooks, 1986a). This difference in SeMet and selenite uptake and relative accumulation can be explained by differences in protein incorporation. Some of the absorbed selenium from SeMet is metabolised to dihydrogen selenide to be utilised in selenium pathways and specific seleno-proteins, whereas another portion is non-specifically incorporated into the general body proteins as substitute for the common amino acid methionine (Met) (EFSA, 2014). In contrast, selenite can only become part of the specific selenoprotein pool after reacting with GSH to form hydrogen selenide before being incorporated in specific seleno-proteins (Suzuki, 2005). Liver is the main organ for Se metabolisation, and at excess intake both SeMet and hydrogen selenite can be excreted by metylation using s-Adenosylmethionie (SAM) as metyldonor (Suzuki, 2005). The none-specific incorporation of SeMet in the general protein pool as opposed to the specific incorporation of selenite in selenoproteins as seleno-cysteine (SeCys), is reflected by the higher muscle Se levels in fish fed organic Se compared to inorganic Se in the present and earlier studies (Rider et al., 2009; Fontagne-Dicharry et al., 2015; Godin et al., 2015; Berntssen et al., 2017). The different protein incorporation of dietary selenite and SeMet,

was reflected by the increase in whole body SeMet when rainbow trout fry is fed with Se-yeast, while when fed with selenite SeCyst is the main seleno-amino acids (Godin et al., 2015).

Earlier studies on oral Se kinetics in fish include assessment on absorption, based on blood kinetics (Kleinow and Brooks, 1986b), elimination pathways (urine, bile, gill and mucus)(Kleinow and Brooks, 1986a), and liver and kidney elimination half-lives (Hilton et al., 1982; Kleinow and Brooks, 1986b). Few studies have included the long term muscle kinetics that reflect the selenite and SeMet specific kinetics that include their difference in protein incooperation. Cutthroat trout fed either a basal diet (1.2 mg Se kg^{-1} diet) or a basal diet supplemented with SeMet to a final level of 5.2, 7.2, 9.2, 11.2 mg Se kg^{-1} diet for 2.5 years, showed a dose dependent whole body half-lives of respectively 515, 131, 103, and 81 days (Hardy et al., 2010). In the present study, fillet SeMet elimination levels in 6.2 mg Sekg^{-1} as SeMet pre-fed fish were in the same order with a t½ of 779 days, while fish pre-fed 5.2 mg kg^{-1} selenite had a t½ of 339 days. The low elimination rate for both SeMet and selenite (t½ >12 months) reflects the low release of Se from proteins in the fillet. The lower elimination of SeMet compared to selenite is likely the results of the larger a-specific protein pool in which SeMet is incooperated compared to the more limited seleno-protein pool for selenite. The lower elimination in the present study compared to that of Hardy et al. (2010) can be attributed to difference between the two studies such as assessment of whole body versus fillet elimination, differences on growth correction, and differences in a multiple time-course elimination assessment in the present study versus a half-live assessment based on start and end-point of elimination as in the Hardy et al. (2010) study.

Table 1. Dietary selenomethionine (SeMet) and selenite, uptake rate (α), half-life (t ½), and elimination (k_2) constants, and biomagnification factor (BMF) in Atlantic salmon (*Salmo salar* L) fed selenomethionine and selenite supplemented feeds (6.2 and 5.4 mg kg^{-1}, respectively) for 90 days followed by a 90 days elimination period (mean ± SD, n=3 of 3 pooled fish, per time point). Values with the same superscripts are not significantly different ($p<0.05$).

	Feed concentration (mg kg^{-1})	□	t ½ (days)	k_2 (10^{-3}days^{-1})	BMF
SeMet	5.4	0.148±0.016^{a}	779±188^{a}	0.98±0.39^{a}	1.62±0.23
Selenite	6.2	0.012±0.001^{b}	339±103^{b}	1.8±0.45^{b}	0.096±0.15

Based on the growth corrected uptake and elimination rates a simple one compartmental transfer model was established and validated with the analysed values of the present trial. Figure 3 gives the observed and predicted concentrations for SeMet and selenite during the accumulation and elimination as calculated by equation (3). A significant ($p<0.001$) linear correlation was discerned between observed and predicted fillet concentrations (r^2=0.88 and 0.65, for observed-predicted SeMet and selenite, respectively). Further model validation was made with the data from an earlier long-term (whole seawater production cycle) Se feeding trial in which Atlantic salmon was fed a high fishmeal diet with high Se levels (1.1-1.0 mg kg^{-1}) or a low fishmeal diet with lower Se levels (0.7-0.6 mg kg^{-1})(Betancor et al., 2016). Based on the reported feed concentrations, feeding rate, and growth rate, and feeding duration, model predicted Se levels were respectively 0.098 and 0.053 mg kg^{-1} WW, while observed values were 0.0.96 and 0.050 mg kg^{-1} WW, respectively.

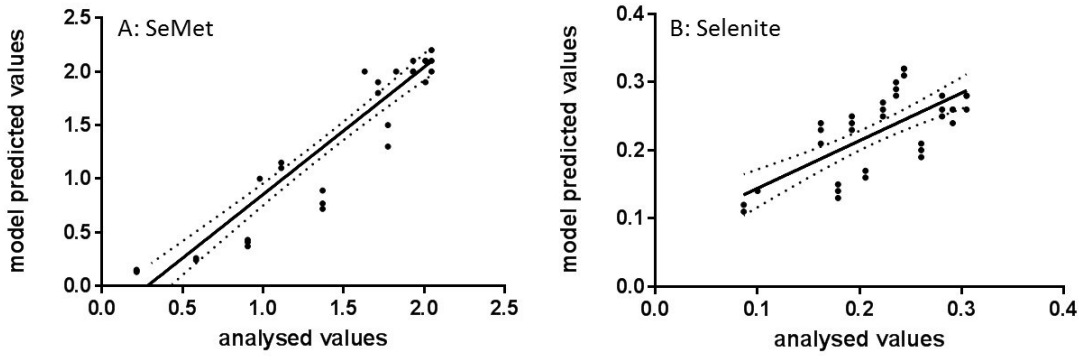

Figure 3 (A-B). Observed versus model predicted fillet concentrations (mg kg^{-1} wet weight) of selenomethionine (SeMet) and selenite in Atlantic salmon fed on SeMet or selenite enriched diets, followed by a depuration period where fish were fed control diet (*n* = 39 for both selenite and SeMet).

Model input data and model scenarios

The simple one compartmental model, based on uptake and elimination kinetics and aquaculture performance parameters (growth rate and feed intake), was use to estimate the Se fillet levels in consumer sized Atlantic salmon (4.5 kg) when fed with different SeMet and selenite supplementation levels to basal feeds with current (surveillance) Se levels (scenarios 1-3) or low background levels of Se (low fish meal and high plant meal inclusion levels) (scenarios 4-6). Surveillance data on both commercially available Norwegian produced Atlantic salmon feeds and Atlantic salmon fillet from consumer sized Atlantic salmon sampled from fish farms were used as input data to the feed-to-fillet transfer models.

Table 2. overview of model predicted Atlantic fillet levels based on a simple one compartmental kinetic model.

Feed scenarios (number)	Feed concentration (mg Se kg^{-1})	Predicted fillet concentration (mg Se kg^{-1} ww)
surveillance average (1)	1.10	0.12
surveillance average+ 0.2 SeMet (2)	1.30	0.14
surveillance average+ 0.2 Selenite (3)	1.30	0.11
high plant background (4)	0.40	0.039
high plant + 0.2 SeMet (5)	0.60	0.058
high plant + 0.2 Selenite (6)	0.60	0.042

Based on average surveillance Norwegian produced commercial salmon feed data of 1.1 mg kg^{-1} (Sanden et al., 2017), model estimations gave fillet Se levels of 0.12 mg kg^{-1} ww. Average randomly sampled fillet levels in 2016 of Norwegian produced Atlantic salmon were 0.13 mg kg^{-1} ww with min. and max of 0.065- and 0.27 mg kg^{-1} ww (n=190), respectively(IMR, 2017). Surveillance on commercial feed showed further a large variation in total Se levels with a min.-max. level of 0.3 -17 mg kg^{-1}. Surveillance of mineral mixes used in salmon feeds showed average levels of 24 mg kg^{-1} (min—max. 1-91 mg kg^{-1}, n=8), thus indicated a general supplementation of Se to salmon feed (Sanden et al., 2017). The current total upper limit for total Se in animal feeds when supplemented with Se is 0.5 mg kg^{-1}, indicating that on a general basis commercial salmon feed exceed the upper limit of supplementation. For organic Se a specific supplementation levels of 0.2 mg kg^{-1} is allowed. When adding the legal allowed supplementation of 0.2 mg kg^{-1} SeMet to average commercial feed levels (1.1 mg kg^{-1}), fillet predicted values was increased with 16% to 0.14 mg kg^{-1} ww, while when supplemented with 0.2 mg kg^{-1} selenite, the predicted fillet levels increased with 2,7% to 0.11 mg kg^{-1} ww. When supplementing 0.2 mg kg^{-1} SeMet or selenite to a basal high plant diet with a low background Se level of 0.4 mg kg^{-1}, the Se fillet increase in salmon fed unsupplemented high plant feed to supplemented high plant feed was 33% and 13% to a level of 0.058 and 0.042 mg kg^{-1}, respectively. Feeding future high plant based feeds with a low background Se level with or without 0.2 mg kg^{-1} selenite gave muscle Se levels that are lower than surveyed in commercial produced salmon in 2016 (<0.065 mg kg^{-1}). When 0.2 mg kg^{-1}SeMet was supplemented, the predicted fillet levels were higher (0.058 mg kg^{-1}), but still in the lower range of surveyed fish (<0.065 mg kg^{-1}). The lower model estimate fillet levels compared to levels found in commercially reared farmed salmon is most likely due to the higher Se background levels used in commercial salmon feeds due to higher inclusion of fishmeal compared to this study. In 2013 it was estimated that salmon feed contained 18% fish meal (Ytrestoyl et al., 2015) versus 10% used in the present study. In addition, analyses of mineral mixes show that Se is likely supplemented to commercial salmon feeds at variable levels (Sanden et al., 2017). Using feed Se levels as surveyed in commercial salmon feeds (1.1 mg kg^{-1}), gave model predicted fillet values that are similar found in surveyed commercial Atlantic salmon fillet (~ 0.12-0.13 mg kg^{-1}). In the food safety assessment predictions of fillet Se levels from Atlantic salmon reared on high plant based diets with low Se background levels are used as an example of future Atlantic salmon farming, that aims for a maximal fish meal replacement. In addition, currently (2016), Se levels in commercial reared Atlantic salmon are used, as an example of a farmed seafood reared on feeds supplemented with Se.

Food safety

A narrow window exists between requirement and Se toxicity (Rayman, 2012), and food selenium supplementation is cautioned as this could generate an increased risk of Se toxicity (Navarro-Alarcon and Cabrera-Vique, 2008). At concentrations higher than those necessary for nutrition, selenium can cause selenosis (Goldhaber, 2003), genotoxicity and carcinogenic (Valdiglesias et al., 2010; Jablonska and Vinceti, 2015), type 2 diabetes (Jablonska and Vinceti, 2015), or cause endocrine disruption by impairing synthesis of thyroid hormones (Navarro-Alarcon and Cabrera-Vique, 2008). On a biochemical level, Se can give self-induced oxidative stress (by upregulation of antioxidant proteins) and redox cycling of auto-oxidisable selenium metabolites, cause glutathione depletion, protein synthesis inhibition, depletion of S-adenosyl-methionine (cofactor for selenide methylation), general replacement of sulphur, and reactions with critical sulphydryl groups of proteins and cofactors (reviewed by ((EC, 2000) (Jablonska and Vinceti, 2015).

Earlier risk assessments have established an upper limit (UL) for Se intake of 300 µg day^{-1} for adults (EC, 2000) and 60 µg day^{-1} for toddlers (EC, 2000). Young children (toddlers) are the

most exposed groups with regards to possible excess Se intake of animals fed Se supplemented feeds (EFSA, 2011) and selenium intake which may be near or above the UL (EFSA, 2010). Seafood has some of the highest natural background on Se levels (Navarro-Alarcon and Cabrera-Vique, 2008; EFSA, 2010; Ullah et al., 2018). For example, sardines have average levels of 0.57 mg kg^{-1}, while apples 0.0045 mg kg^{-1} ww (Navarro-Alarcon and Cabrera-Vique, 2008). However, in an European food intake study on children, seafood was not the main food groups for selenium. In contrast, the food groups „cereals", „vegetables", „fresh meat" and „milk and dairy drinks", were the dominant sources for Se intake for most EU countries. This was not due to high levels of selenium present in these food groups, but due to high levels of consumption of these food groups (EFSA, 2010). However for some country studies (e.g. Italy and Spain), fish was a dominant source of Se intake for 1-2 year old children (EFSA, 2010). As toddlers have a Se intake near or above the UL, a re-assessment of the consequences for the consumer from feeding organic Se (Sel-Plex®) to food producing land animals was performed. EFSA estimated that supplementation of 0.2 Sel-Plex® mg kg^{-1} to farmed animal feed was safe with regards to the elevated levels reported in meat, egg, and milk (EFSA, 2011). In the present trial, feeds were supplemented with L-SeMet instead of Sel-Plex®, however, a later L-SeMet EFSA risk assessment assumed a similar efficacy and toxicity for L- SeMet compared to Se-yeast products such as Sel-Plex® (EFSA, 2013). The EFSA 2011 assessment for Se supplementation to food producing land animals, was made based on a background intake of 10 µg Se/day for vegetables and cereals for toddlers, the 95 percentile consumption data of food classes (meat, milk & diary, and eggs) for toddles (EFSA, 2011), background Se levels in control (low or unsupplemented) food classes, and a factor that expresses the relative increase (supplementation increase factor) per food class Se levels at a given feed Se supplementation level compared to the background Se levels.

The EFSA (2011) assessment on Sel-Plex® feed supplementation for food producing animals did not include the consumption of farmed seafood which was reared on Se supplemented feeds. The present study uses the EFSA 2011 risk assessment for Se supplementation to feed for food producing land animals and adds farmed seafood (Atlantic salmon) reared on selenite or SeMet supplemented feed as part of the same assessment protocol. For assessment of seafood intake for toddlers, and in particular Atlantic salmon, the Comprehensive European Food Consumption Database for consumers only (EFSA, 2015) is used alike the EFSA 2011 opinion for food products for land animals. The median value for the 95 percentile intake (from 10 studies) for consumption of seafood and fish for toddlers is 47 g day^{-1}, the median 95 percentile for Atlantic salmon only is 29 g day^{-1} (EFSA, 2015)(n=10 from 7 EU countries). Other fish species for toddlers include mostly feral fish species (mackerel, cod, whiting, sprat, sole) that are mainly not farmed and do not receive Se supplemented feed. As background Se levels in control (unsupplemented) Atlantic salmon fillets, the predicted fillet levels when fed on high plant based diets are used. The model predicted background Se values for Atlantic salmon fed on high plant based are 0.039 mg kg^{-1} ww, which is lower than the lower range of the surveillance data for Norwegian commercially farmed Atlantic salmon (min.-max. 0.065-0.29 mg kg^{-1}). The lower estimate is most likely due to the higher background levels used in commercial salmon feeds due to higher inclusion of fishmeal (see above). For supplementation increase factor at a Se feed supplementation of 0.2 mg kg^{-1} (the current legal limit for organic Se supplementation), the model prediction on high plant diets with and without selenite and SeMet were used. Model predictions show that 0.2 mg kg^{-1} SeMet supplementation to high plant based feeds increase Atlantic salmon Se fillet levels with a factor of 1.61, compared to fish reared on the same feed without SeMet supplementation. This estimated 0.2 mg kg^{-1} SeMet increase factor of 1.61 for salmon fillet is less than for meat products (2.2) used in the EFSA opinion on upper limits for Sel-Plex® supplementation to feed for food producing land animals (EFSA, 2011). The relative higher increase factor can partly be explained the higher

relative feed utilization by fish compared to land animals as seen from the lower feed conversion factor (amount of feed needed for growth of 1 kg of farmed animal) for fish compared to for example cows (1.1 versus 3.2, respectively).

The earlier EFSA 2011 risk assessment estimated that based on the legal supplementation of SeMet (0.2 mg kg^{-1}) to feed for food producing land animals (producing meat, milk and egg), the total intake was 56 µg day^{-1}. Including the background in take for cereals, vegetables and fruits of 10 µg day^{-1}, the UL for toddlers (60 µg day^{-1}) would be slightly exceeded by 10% (EFSA, 2011) (Table 3A). Adding farmed Atlantic salmon fed on a high plant meal based diet supplemented with 0.2 mg kg^{-1} SeMet to the EFSA 2011b toddler Se supplementation assessment (EFSA, 2011)(Table 3B), the total Se intake for toddlers would be 68 µg day, exceeding the UL with 13%. The estimated fillet levels for salmon reared on high plant meal diets supplemented with 0.2 mg SeMet kg^{-1} was is lower than the background levels of Se in Norwegian commercially farmed Atlantic salmon (0.058 versus 0.13 mg kg^{-1}, respectively, see above). Assuming that farmed salmon is reared on Se supplemented feeds, as derived from the Se levels found in mineral mixes used in commercial salmon feeds (see above), the currently (2016) commercially reared Atlantic salmon average Se level was added to the EFSA 2011 risk assessment. Adding currently commercial farmed Atlantic to the EFSA 2011 risk assessment the estimated total Se intake for toddlers was 70 µg day^{-1}, exceeding the UL with 17% (Table 3C). Adding farmed Atlantic salmon to the earlier estimated total intake of Se from food producing animals reared on Se supplemented feed increased the total Se intake with 3% for predicted levels in Atlantic salmon reared on high plant based feed supplemented with 0.2 mg kg^{-1} SeMet, or 7% for currently farmed Atlantic salmon. It has to be noted that none farmed fish species, such as mackerel that have no supplementation, average Se levels are higher than for farmed Atlantic salmon (0.54 versus 0.13 mg kg^{-1}, respectively) (IMR, 2017). With a median 95 percentile mackerel consumption for toddlers of 15 µg day^{-1} (EFSA, 2015), and adding consumption of mackerel to the EFSA 2011b Se intake risk assessment the total Se intake for toddlers would be 73 µg day^{-1}, increasing the total Se intake with 12%. Dietary selenite accumulates less than organic Se in salmon muscle, and when adding 0.2 mg kg^{-1} selenite supplemented farmed Atlantic salmon to selenite supplemented egg, milk and meat, the total Se intake would be 77% of the UL (table 3D).

Table 3. EFSA risk assessment for land animals reared on SeMet supplemented feed to the current legal level of 0.2 mg kg-1 (A). Addition of consumption of Atlantic salmon fillet to the EFSA risk assessment reared on high plant diets that are supplemented with SeMet (B). Addition of consumption of commercially available farmed Atlantic salmon to the EFSA risk assessment (C). Addition of consumption of Atlantic salmon fillet to the EFSA risk assessment reared on high plant diets that are supplemented with Selenite (D).

A) SeMet supplemented 0.2 mg kg-1, land animals only

Food	Amount consumed (kg)	Factor Se 0.2	Se in control (mg/kg WW)	Food content (mg/kg ww)	Se intake (mg)
Meat	0,09	2,20	0,107	0,235	0,021
Milk	1,05	2.36	0,010	0,024	0,025
Egg	0,035	3.84	0,074	0,282	0,010
		total food from farmed animals			0,056
		background intake from cereals etc.			0,010
				total intake	0,066
				%of UL	**110**

B) SeMet supplemented 0.2 mg kg-1, added salmon reared on high plant feeds

Food	Amount consumed (kg)	Factor Se 0.2	Se in control (mg/kg WW)	Food content (mg/kg ww)	Se intake (mg)
Meat	0,09	2,20	0,107	0,235	0,021
Milk	1,05	2.36	0,010	0,024	0,025
Egg	0,035	3.84	0,074	0,282	0,010
Salmon fillet	0,029	1,480	0,039	0,058	0,002
		total food from farmed animals			0,058
		background intake from cereals etc.			0,010
				total intake	0,068
				%of UL	**113**

C) SeMet supplemented 0.2 mg kg-1, added commercial farmed salmon

Food	Amount consumed (kg)	Factor Se 0.2	Se in control (mg/kg WW)	Food content (mg/kg ww)	Se intake (mg)
Meat	0,09	2,20	0,11	0,24	0,021
Milk	1,05	2.36	0,01	0,02	0,025
Egg	0,035	3.84	0,07	0,28	0,010
Salmon fillet	0,029			0,13	0,004
		total food from farmed animals			0,060
		background intake from cereals etc.			0,010
				total intake	0,070
				%of UL	**117**

D) selenite supplemented 0.2 mg kg-1, added salmon

Food	Amount consumed (kg)	Factor Se	Se in control (mg/ kgWW)	Food content (mg/kg ww)	Se intake (mg)
Meat	0,090	1,30	0,107	0,139	0,013
Milk	1,050	1,45	0,010	0,015	0,015
Egg	0,035	2,60	0,074	0,192	0,007
Salmon fillet	0,029	1,10	0,039	0,043	0,001
		total food from farmed animals			0,036
		background intake from cereals etc.			0,010
				total intake	0,046
				%of UL	**77**

In conclusion, the supplementation of 0.2 mg kg-1 selenite to farmed Atlantic salmon, raised on plant based feeds. in addition to 0.2 mg kg-1 selenite supplementation to farmed land animals, did not pose a risk (intake is 77% of upper limit) for toddlers, which are the most sensitive consumer group to excess dietary Se exposure. However, supplementing 0.2 mg kg-1 SeMet instead of selenite to food producing animals, including Atlantic salmon farmed on a high plant feed supplemented with 0.2 mg kg^{-1} SeMet, exceeds the upper limit for toddlers with 13%. Furthermore, adding currently farmed Atlantic salmon fillet levels to the existing risk assessment caused the UL for toddlers to exceed with 17%. The earlier EFSA assessment on the Se intake for toddlers for land animals reared on 0.2 mg kg^{-1} supplemented feed estimated an intake exceeding the UL with 10%. Adding current farmed Atlantic salmon fillet levels to the existing EFSA risk assessment of Se supplementation to feeds for food producing land animals increased the total Se food intake for toddlers with 7%. Including model estimates of Atlantic salmon reared on high plant based feeds supplemented with 0.2 mg kg^{-1} SeMet increased the estimated total intake for toddlers with 3%.

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