



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



Berntssen M.H.G.<sup>a,\*</sup>, Betancor M.<sup>b</sup>, Caballero M.J.<sup>c</sup>, Hillestad M.<sup>d</sup>, Rasinger J.<sup>a</sup>, Hamre K.<sup>a</sup>, Sele V.<sup>a</sup>, Amlund H.<sup>a</sup>, Ørnstrud R.<sup>a</sup>

<sup>a</sup> Institute of Marine Research (IMR), P.O 1870, Nordnes, N-5817, Bergen, Norway

<sup>b</sup> Institute of Aquaculture, Faculty of Natural Sciences, University of Stirling, Stirling FK94LA, United Kingdom

<sup>c</sup> Department of Morphology, Veterinary School, University of Las Palmas de Gran Canaria, Trasmontana s/n, 35416 Arucas, Las Palmas, Spain

<sup>d</sup> BioMar AS, Trondheim, Norway

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### ABSTRACT

The use of plant-based feeds warrants the supplementation with selenium (Se) to cover the requirement for Atlantic salmon. Depending on its chemical form, 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<sup>-1</sup>, 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<sup>-1</sup> ww) or organic selenomethionine (SeMet) (0.45, 6.2, 16.2, 21, or 39 mg kg<sup>-1</sup> 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 limits were set by model-fitting the effect data in a dose-response (lower bound) bench mark dose (BMDL) evaluation. Fish fed the two highest selenite levels showed mortality while fish fed SeMet had no mortality. Fish fed 5.4–11 mg selenite kg<sup>-1</sup> feed showed significantly (ANOVA, Tukey's *t*-test, *p* < .05) increased liver oxidative stress, as seen from altered hepatic GSH and vitamin E levels, and liver damage as seen from increased plasma ALAT 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 plasma proteins in fish fed  $\geq 21$  mg kg<sup>-1</sup>, compared to control. For selenite exposed fish, a safe feed limit (BMDL) was set at 1–2 mg kg<sup>-1</sup> ww feed (daily dose 0.01–0.02 mg kg BW<sup>-1</sup> day<sup>-1</sup>), based on plasma ALAT increase, liver vitamin E depletion, and liver histopathology. For SeMet fed fish, the safe feed limit was higher than for selenite with a BMDL of 2.8 mg kg<sup>-1</sup> ww (dose 0.03 mg kg BW<sup>-1</sup> day<sup>-1</sup>), based on liver histopathology and plasma creatinine. In conclusion, with regards to fish health, 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<sup>-1</sup> feed, respectively, in a high plant-based salmon feed with background levels of 0.45 mg Se kg<sup>-1</sup>.

### 1. Introduction

Due to a rapid growth in aquaculture and limited access to marine resources, fish oil and fish meal in feeds to carnivorous marine fish species such as Atlantic salmon (*Salmo salar*) have been replaced with plant ingredients the last decades (Ytrestoyl et al. 2015). The change from marine to plant feed ingredients will alter the nutritional composition of salmon feeds, reducing the levels of 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 the Se concentrations in soil (Alfthan et al. 2015). The observed Se decline 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 presence of phytates (Denstadli et al. 2006). Selenium concentration in Atlantic salmon flesh was lower when fed on plant protein replacement feeds compared to marine protein feeds (Betancor et al. 2016). Several studies have indicated the need for Se supplementation in plant-based feed to marine carnivorous fish (Fontagne-Dicharry et al. 2015; Godin et al.

\* Corresponding author.

E-mail address: [marc.berntssen@nifes.no](mailto:marc.berntssen@nifes.no) (M.H.G. Berntssen).

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2015; Pacitti et al. 2015; Ilham et al., 2016). Of the mineral supplements used, the organic forms, seleno-methionine (SeMet) or Se-yeast forms have a higher bioavailability than inorganic selenite forms (Rider et al. 2009; Fontagne-Dicharry et al. 2015). Studies in 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<sup>-1</sup> (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<sup>-1</sup> 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 requires toxicological assessment to set safe upper limits that protect fish health (Berntssen et al. 2017). Several studies have given an overview on 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 adverse effects of Se supplementation is important to consider. Several studies have performed graded dose-response with organic or inorganic Se 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 salmonid species.

Oxidative stress has been identified as a main toxic action (MOA) for excess dietary Se exposures (Palace et al. 2004; Miller et al. 2007; Han et al. 2010; Choi et al. 2015; Hursky and Pietrock 2015; Lee et al. 2015; Hauser-Davis et al. 2016). Also for dietary Se exposed Atlantic salmon, oxidative stress was a main driver for both high selenite and SeMet-yeast toxicity, with a higher toxicity for selenite compared to SeMet-yeast (Berntssen et al. 2017). In contrast, for white sturgeon (*Acipenser transmontanus*) (Zee et al. 2016a; Zee et al. 2016b) oxidative stress was not the main cause of dietary SeMet toxicity, and juvenile rainbow trout fed organic Se showed no oxidative stress while growth and liver lipids were reduced (Knight et al. 2016). Recent wide-scope pathway assessments, by use of metabolomics, have shown that disturbance in lipid metabolism could be an additional MOA for inorganic and organic Se toxicity (Berntssen et al. 2017). Earlier wide-scope pathway assessments by transcriptomics confirmed that disturbed liver lipid synthesis and metabolism was a central mechanism in dietary organic Se exposed rainbow trout (Knight et al. 2016; Pacitti et al. 2016a).

Assessment of biochemical markers in the central pathways of dietary selenite and SeMet toxicity can be used to assess early effects of excess selenite and SeMet exposures. Atlantic salmon fed sublethal selenite and SeMet levels (15 mg kg<sup>-1</sup>), showed reduced vitamin E, formation of peroxidative products, and reduction in glutathione as markers of oxidative stress, while altered lipid composition were used as markers of disturbed lipid metabolism (Berntssen et al. 2017). The use of biomarkers in central pathways of toxicity, are valuable in sub-chronic studies (10% of life cycle) where chronic whole-body adverse effects are expected to occur only after prolonged (life-cycle) 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), reduced energy stores (De Riu et al. 2014; Zee et al. 2016a), pathological effects on kidney and liver (Hicks et al. 1984; Teh et al. 2004;

Tashjian et al. 2006; Zee et al. 2016a), pathological effects on heart and ovaries, as well as skeleton/cranial deformation (Lemly, 2002; Hamilton, 2003; Hamilton, 2004).

Traditionally, animal health 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 establish safe levels of supplements or 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 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 health of Atlantic salmon. The present paper uses an integrated feed safety assessment on several levels of biological organization. These include the use of specific targeted biomarkers of Se toxicity mode of action (e.g. markers of oxidative stress and lipid metabolism) as well as general adverse effect parameters (plasma biochemistry, hematology, histopathology, and growth) of Se toxicity. The safe levels are assessed in a common EFSA dose-response bench mark dose regression model applied to all parameters.

## 2. Material and methods

### 2.1. 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.

### 2.2. Experimental conditions

The feeding trial was carried out at NOFIMA (Sunnalsøra, Norway) between the 15th of November 2016 and the 3th of March 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<sup>2</sup> 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 (see diet description under) 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 24 h light. Six daily meals were provided with 4 h 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 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 11 L min<sup>-1</sup> 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% HNO<sub>3</sub> (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;  $\sim 60 \text{ mg L}^{-1}$ ). The fish were sacrificed by a blow to the head and blood samples were taken from the caudal vein quickly following the initial anesthetization, using a heparinized VACUETTE® blood collection tube with  $21\text{G} \times 1'$  needle. Whole blood was divided into two aliquots, one of which was used for immediate on-site analyses of hematocrit and the other aliquot ( $\sim 200 \text{ mL}$ ) was kept on ice for erythrocyte count and hemoglobin determination, which were performed within two days after sampling. For plasma samples, the remaining whole blood was centrifuged at  $3500\text{g}$  for 10 min, and the plasma was snap-frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$  until further analysis (see below). Body weight and length of each fish was recorded, and liver, heart and spleen 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  $-80^\circ\text{C}$  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.

### 2.3. 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 (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 (10%), soya protein concentrate (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,  $\text{Na}_2\text{SeO}_3$ , DSM, Heerlen, Netherlands) or organic Se (> 98% L-selenomethionine (Excellent Se4000 Minsups, Winsford England) at a nominal concentration of 0, 5, 15, 25 and  $50 \text{ mg kg}^{-1}$ . 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 ( $1\text{--}5 \text{ mg kg}^{-1}$  (Berntssen et al. 2017)), more severe chronic sub-lethal toxicity for organic Se ( $\sim 20 \text{ mg kg}^{-1}$  dry weight (dw) organic Se as Se-yeast fed worms or  $15 \text{ mg kg}^{-1}$  SeMet ww, Knight et al. (2016) and Berntssen et al. (2017), respectively) or inorganic Se ( $\sim 9 \text{ mg kg}^{-1}$  (Hamilton 2004) and  $15 \text{ mg kg}^{-1}$  (Berntssen et al. 2017)), and severe chronic toxicity (positive control) (> 20 SeMet  $\text{mg kg}^{-1}$  (Hardy et al. 2010), >  $15 \text{ mg selenite kg}^{-1}$  (Berntssen et al. 2017)). The analysed Se level in the basal diet was  $0.45 \pm 0.04 \text{ mg kg}^{-1}$  (control),  $5.4 \pm 0.09$  or  $6.2 \pm 0.2$  (low),  $11.0 \pm 0.3$  or  $16.2 \pm 0.3$  (low-medium),  $29.4 \pm 0.9$  or  $21 \pm 0.3$  (medium),  $60.0 \pm 2$  or  $39 \pm 0.4$  (high)  $\text{mg kg}^{-1}$  for respectively the inorganic Se and organic Se supplemented diets ( $n = 3$ , mean  $\pm$  SD).

### 2.4. 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 (0.20–0.25 g) were digested by adding 0.5 mL deionized water (Milli-Q, Merck Millipore, Oslo, Norway) and 2 mL concentrated nitric acid

( $\text{HNO}_3$ , Fluka, Sigma-Aldrich, Oslo, Norway) in digestion vessels (Milestone Srl, Sorisole, BG, Italy). The capped vessels were ultrasonicated (UW, SRC, Milestone, Shelton, CT, USA, gas pressure 40 bar and the temperature increased incrementally to  $260^\circ\text{C}$ ) in a container with 30 mL Milli-Q water and 5 mL hydrogen peroxide ( $\text{H}_2\text{O}_2$ , Emsure ACS, ISO, 32% w/w; VWR, Oslo, Norway). Total Se concentration was determined by 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)  $\text{HNO}_3$ . Internal standard (Ge, Rh and Tm, Thermo Fisher Scientific Inc) was used for correction of instrumental drift during the analysis. Plasma power was set to 1550 W, carrier/nebulizer gas flow to  $1.05 \text{ L min}^{-1}$ , the plasma/auxiliary gas flow to  $0.8 \text{ L min}^{-1}$ , and He gas (CCT1) flow was  $4.6 \text{ mL/min}$ . Isotope  $^{78}\text{Se}$  was monitored, and the integration time was 0.1 s. 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.

### 2.5. 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  $4 \times$  volume of ice-cold 0.9% saline buffer ( $9 \text{ g L}^{-1}$  NaCl in  $\text{ddH}_2\text{O}$ ) for GSH analyses, or  $2 \times$  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,  $1500 \text{ g}$ ,  $4^\circ\text{C}$ ), 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 analysed spectrophotometrically for absorbance at 405 nm in a Wallac VICTOR (TM)  $\times 5$  2030 Multilabel Reader (PerkinElmer Life Sciences, MA, USA).

Vitamin E was analysed as  $\alpha$ -,  $\beta$ -,  $\gamma$ - and  $\delta$ -tocopherol isomers and  $\alpha$ -,  $\beta$ -,  $\gamma$ - and  $\delta$ -tocotrienol by high performance liquid chromatography (HPLC) according to the method described by Hamre et al. (2010). In short, the homogenized liver samples were saponified (20 min at  $100^\circ\text{C}$ ) 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. (2010). 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.

### 2.6. Lipid classes

Earlier wide-scope metabolic screening of Atlantic salmon fed inorganic and organic Se also 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  $-20^{\circ}\text{C}$ . 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  $\mu\text{L}$  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 ( $\text{BF}_3$ ) 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.

## 2.7. 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  $-80^{\circ}\text{C}$  until further analysis. Plasma concentrations of albumin and total protein, alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), 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).

## 2.8. 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).

## 2.9. 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  $\mu\text{m}$  were then stained with haematoxylin 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, Birkerød, 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 to 2 or 0 to 3 (see supplementary data Table 1 for description of scoring). All analyses were performed in a double-blinded format.

## 2.10. Statistics

In order to compare the results from this study with earlier published studies on dietary Se toxicity in fish, the 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 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 health. For continuous data (whole body, organ indices, plasma and blood parameters), two models (3 and 5) of exponential and Hill model families were fitted 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 (logistic, probit, log-logit, log probit, Weibull, gamma, and LMS (two-stage)) were assessed 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)  $-2$  ( $\text{AIC} < \text{AIC}_{\text{null}} - 2$ ), and the model with lowest AIC (AICmin) was lower than the AIC of the full model  $+2$  ( $\text{AIC}_{\text{min}} < \text{AIC}_{\text{full}} + 2$ ) (EFSA 2017b). Model averaging is recommended as the preferred method for calculating the BMD confidence interval (EFSA 2017b), and 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 where 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 BMDL is defined as the dose not expected to give an adverse effect. A default benchmark response (BMR) of 5% change was used as starting point for model fitting of apparent adverse effects (EFSA 2017b) such as reduced growth, altered organ indices or disturbance in hematology (BMDL<sub>05</sub>). For markers of liver function and osmoregulation (plasma enzymes and electrolytes) or markers of lipid peroxidative stress (vitamin E, GSSG 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<sub>20</sub>). For quantal and ordinal data (histology) the default BMR of 10% (extra risk; BMDL<sub>10</sub>) 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:



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

Specific growth rate (SGR)

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

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

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

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

### 3. Results

#### 3.1. 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<sup>-1</sup>, 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<sup>-1</sup>), 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<sup>-1</sup> selenite 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 ( $p < .05$ ) waterborne Se levels than the control group (ANOVA, tukey's  $t$ -test,  $n = 3$ ). The waterborne Se levels (mean  $\pm$  SD) in the experimental groups were 18.8  $\pm$  11.7, 18.1  $\pm$  8.1, 11.8  $\pm$  2.3, 21.4  $\pm$  1.0, 33.3  $\pm$  6.2, 12.7  $\pm$  2.7, 15.3  $\pm$  2.4, 22.4  $\pm$  11.3, 13.2  $\pm$  2.2 ( $\mu\text{g L}^{-1}$ ), for the control, 5, 11, 35, and 60 mg kg<sup>-1</sup> selenite groups and 6, 16, 21, and 39 mg kg<sup>-1</sup> SeMet groups, respectively.

#### 3.2. Liver se levels

Fish from all exposure groups had significantly elevated liver Se levels compared to the control group (Fig. 1). Besides fish fed 16 and 21 mg kg<sup>-1</sup> SeMet, fish fed graded levels of selenite or SeMet showed a significant ( $p < .05$ ) 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<sup>-1</sup> selenite had significantly ( $p < .05$ ) higher liver Se levels compared to fish fed respectively 6.2 and 16 mg kg<sup>-1</sup> SeMet. Even fish fed 11 mg kg<sup>-1</sup> selenite had significantly higher liver Se levels compared to fish fed much higher dietary SeMet levels of 39 mg kg<sup>-1</sup>.

#### 3.3. Weight, length, growth and organ index

Fish fed selenite levels of 11 mg kg<sup>-1</sup> had significantly reduced individual weights and lengths at the end of the 3 month trial. Despite the highest liver Se accumulation in this group, no significant differences ( $p < .05$ ) 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<sup>-1</sup> selenite compared to the control fish. No significant dietary differences were observed in specific growth rate

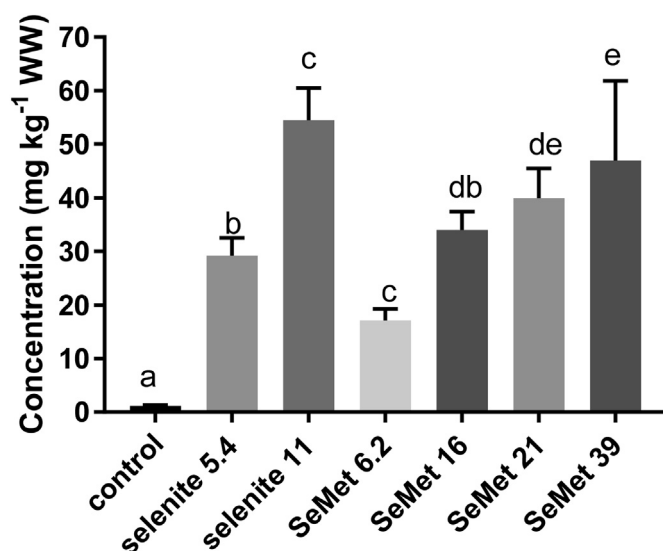


Fig. 1. Selenium (Se) concentrations (mg kg<sup>-1</sup> wet weight (ww)) in liver of Atlantic salmon (*Salmo salar*) fed control, graded levels of selenite fortified diets (5.4 and 11 mg kg<sup>-1</sup> ww, respectively), or graded levels of seleno-methionine (SeMet) fortified diets (6.2, 16, 21, and 39 mg kg<sup>-1</sup> ww, respectively) for 3 months (mean  $\pm$  SD,  $n = 15$ ). Bars with the same letters are not significantly different from each other ( $P < .05$ , one-way ANOVA, post hoc Tukey's  $t$ -test).

(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<sup>-1</sup>) had significantly reduced final individual weights and lengths, compared to fish fed the second highest SeMet level (6.2 mg kg<sup>-1</sup>), but not compared to the control fish. No significant differences were observed in fish fed dietary 6.2 mg kg<sup>-1</sup> 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<sup>-1</sup>, compared to fish fed control, 6.2 and 16 mg kg<sup>-1</sup> SeMet, respectively.

#### 3.4. 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<sup>-1</sup> selenite had significantly reduced MCHC compared to control. For SeMet exposed fish fed 39 mg kg<sup>-1</sup>, a significantly lower MCHC and MCV was observed than the 16 mg kg<sup>-1</sup> SeMet exposed group, however no significant differences were observed compared to the control group. For plasma parameters as a marker for liver injury, fish fed 11 mg kg<sup>-1</sup> selenite had only elevated alkaline phosphatase (ALP), and fish fed 5.4 mg kg<sup>-1</sup> reduced alanine aminotransferase (ALAT), compared to the control group. For fish exposed to SeMet, plasma ALP and aspartate aminotransferase (ASAT) showed a significant decrease in fish fed 39 mg kg<sup>-1</sup> SeMet, compared to control. Of the plasma ions, only Ca was significantly reduced in fish fed 39 mg kg<sup>-1</sup> 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 fish fed the highest SeMet level (39 mg kg<sup>-1</sup>), 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 ( $p = .06$ , for SeMet exposed fish) (Table 3).

**Table 1**

Final individual length (cm), weight (gr), condition factor, relative liver somatic index (LSI, %), heart somatic index (HSI, %), spleen somatic index (SSI, %) (mean  $\pm$  SD, n = 15), as well as tank specific growth rate (SGR), feed conversion ratio (FCR), and daily feed intake (FI) (mean  $\pm$  SD, N = 3), in Atlantic salmon (*Salmo salar*) fed graded levels of selenite (5.4 and 11 mg kg<sup>-1</sup>) and seleno-methionine (SeMet) (6.2, 16, 21, and 39 mg kg<sup>-1</sup>) 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 < .001, p < .01, p < .05).

	Control	Selenite 5.4	Selenite 11		p-Value
Final length	32.9 $\pm$ 1.8 <sup>ab</sup>	33.7 $\pm$ 1.7 <sup>a</sup>	31.8 $\pm$ 1.3 <sup>b</sup>		p < .001
Final weight	445 $\pm$ 102 <sup>ab</sup>	469 $\pm$ 92 <sup>a</sup>	411 $\pm$ 61 <sup>b</sup>		p < .05
Final CF	1.23 $\pm$ 0.10	1.21 $\pm$ 0.08	1.28 $\pm$ 0.09		0.33
Final LSI	1.01 $\pm$ 0.16	0.92 $\pm$ 0.12	0.99 $\pm$ 0.09		0.12
Final HSI	0.093 $\pm$ 0.013	0.11 $\pm$ 0.019	0.10 $\pm$ 0.012		0.19
Final SSI	0.099 $\pm$ 0.028 <sup>a</sup>	0.077 $\pm$ 0.014 <sup>ab</sup>	0.075 $\pm$ 0.018 <sup>b</sup>		p < .01
0–90 days					
SGR	1.24 $\pm$ 0.10	1.26 $\pm$ 0.10	1.14 $\pm$ 0.03		0.21
FCR	0.80 $\pm$ 0.07	0.77 $\pm$ 0.07	0.89 $\pm$ 0.01		0.085
FI	0.99 $\pm$ 0.02	0.97 $\pm$ 0.02	1.01 $\pm$ 0.01		0.06

	Control	SeMet 6,2	SeMet 16	SeMet 21	SeMet 39	p-Value
Final length	32.9 $\pm$ 1.8 <sup>ab</sup>	33.3 $\pm$ 2.0 <sup>a</sup>	33.1 $\pm$ 1.8 <sup>ab</sup>	32.2 $\pm$ 2.2 <sup>ab</sup>	31.0 $\pm$ 1.6 <sup>b</sup>	p < .01
Final weight	445 $\pm$ 102 <sup>a</sup>	467 $\pm$ 101 <sup>a</sup>	458 $\pm$ 91 <sup>a</sup>	405 $\pm$ 110 <sup>a</sup>	352 $\pm$ 60 <sup>b</sup>	p < .05
Final CF	1.23 $\pm$ 0.10	1.25 $\pm$ 0.10	1.25 $\pm$ 0.09	1.18 $\pm$ 0.10	1.17 $\pm$ 0.08	0.06
Final LSI	1.01 $\pm$ 0.16	1.01 $\pm$ 0.08	0.94 $\pm$ 0.14	1.06 $\pm$ 0.12	1.01 $\pm$ 0.15	0.23
Final HSI	0.093 $\pm$ 0.013	0.096 $\pm$ 0.014	0.084 $\pm$ 0.019	0.098 $\pm$ 0.014	0.092 $\pm$ 0.019	0.41
Final SSI	0.099 $\pm$ 0.028	0.084 $\pm$ 0.019	0.090 $\pm$ 0.023	0.11 $\pm$ 0.034	0.12 $\pm$ 0.067	0.07
0–90 days						
SGR	1.24 $\pm$ 0.10 <sup>a</sup>	1.28 $\pm$ 0.09 <sup>a</sup>	1.25 $\pm$ 0.03 <sup>a</sup>	1.13 $\pm$ 0.08 <sup>a</sup>	0.97 $\pm$ 0.07 <sup>b</sup>	p < .001
FCR	0.80 $\pm$ 0.07 <sup>a</sup>	0.76 $\pm$ 0.08 <sup>a</sup>	0.79 $\pm$ 0.01 <sup>a</sup>	0.90 $\pm$ 0.06 <sup>ab</sup>	1.13 $\pm$ 0.08 <sup>b</sup>	p < .001
FI	0.99 $\pm$ 0.02 <sup>a</sup>	0.97 $\pm$ 0.03 <sup>a</sup>	0.98 $\pm$ 0.02 <sup>a</sup>	1.01 $\pm$ 0.02 <sup>a</sup>	1.1 $\pm$ 0.02 <sup>b</sup>	p < .001

### 3.5. Markers of lipid metabolism

As markers of altered lipid metabolism, sum total lipid was significantly (p < .05) reduced compared to control group in fish fed 11 mg kg<sup>-1</sup> selenite and 39 mg kg<sup>-1</sup> SeMet (Fig. 2A). The relative (% of sum lipid) distribution of lipid classes was significantly affected in the 11 mg kg<sup>-1</sup> selenite group with reduced TAG compared to control groups (Fig. 2B). Although fish fed highest levels of SeMet also showed decreased TAG, this difference were not significantly different compared to control (p = .08, one-way ANOVA, Tukey's t-test).

### 3.6. 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<sup>-1</sup> selenite and 16, 21, and 39 mg kg<sup>-1</sup> SeMet. The oxidized

glutathione (GSSG) was also significantly reduced in the same exposure groups compared to the control fish, causing the ratio GSG: GSSG (data not shown) not to be significantly altered among any of the exposure groups compared to control fish. Fish fed the highest level of selenite (11 mg kg<sup>-1</sup>) and SeMet (39 mg kg<sup>-1</sup>) 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 (Fig. 3).

### 3.7. Histopathology

Hepatocyte lipid intracytoplasmic 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

**Table 2**

Blood hematocrit (Hct, %), red blood cell count (RBC, number  $\times 10^{-12}$  L<sup>-1</sup>), hemoglobin (HGB, g 100 mL<sup>-1</sup>), mean corpuscular volume (MCV, 10<sup>-5</sup> L<sup>-1</sup>), Mean corpuscular hemoglobin concentration (MCHC, g L<sup>-1</sup>), mean corpuscular hemoglobin (MCH  $\mu$ g) in Atlantic salmon (*Salmo salar*) fed graded levels of selenite (5.4 and 11 mg kg<sup>-1</sup>) and seleno-methionine (SeMet) (6.2, 16, 21, and 39 mg kg<sup>-1</sup>) for 3 months (triplicate tanks per diet, mean  $\pm$  SD, n = 15). Values with different superscripts are significantly different from each other (one-way ANOVA, Tukey's HSD test, p < .001, p < .01, p < .05).

	Control	Selenite 5.4	Selenite 11		p-Values
Hct	43.5 $\pm$ 3.52	46.0 $\pm$ 2.55	46.9 $\pm$ 2.66		0.10
RBC	1.34 $\pm$ 0.129	1.42 $\pm$ 0.106	1.43 $\pm$ 0.087		0.29
HGB	9.88 $\pm$ 0.900	10.2 $\pm$ 0.61	10.3 $\pm$ 0.54		0.71
MCV	324 $\pm$ 9.3	324 $\pm$ 17.8	328 $\pm$ 13.9		0.87
MCHC	22.7 $\pm$ 0.85 <sup>a</sup>	22.1 $\pm$ 0.62 <sup>ab</sup>	21.9 $\pm$ 0.74 <sup>b</sup>		p < .05
MCH	73.7 $\pm$ 2.54	71.6 $\pm$ 3.39	72.1 $\pm$ 3.72		0.30

	Control	SeMet 6.2	SeMet 16	SeMet 21	SeMet 39	p-Values
Hct	43.5 $\pm$ 3.52	44.9 $\pm$ 2.21	45.0 $\pm$ 3.37	45.1 $\pm$ 3.50	43.6 $\pm$ 4.14	0.098
RBC	1.34 $\pm$ 0.129	1.39 $\pm$ 0.077	1.39 $\pm$ 0.092	1.42 $\pm$ 0.118	1.38 $\pm$ 0.11	0.55
HGB	9.88 $\pm$ 0.900	10.3 $\pm$ 0.52	10.2 $\pm$ 0.70	10.4 $\pm$ 0.80	10.1 $\pm$ 0.99	0.29
MCV	324 $\pm$ 9.3 <sup>ab</sup>	323 $\pm$ 13.7 <sup>ab</sup>	325 $\pm$ 15.7 <sup>b</sup>	318 $\pm$ 13.4 <sup>ab</sup>	315 $\pm$ 18.4 <sup>a</sup>	p < .01
MCHC	22.7 $\pm$ 0.85 <sup>ab</sup>	22.8 $\pm$ 0.78 <sup>ab</sup>	22.6 $\pm$ 0.81 <sup>b</sup>	23.0 $\pm$ 0.48 <sup>ab</sup>	23.1 $\pm$ 0.59 <sup>a</sup>	p < .05
MCH	73.7 $\pm$ 2.54	73.8 $\pm$ 3.06	73.5 $\pm$ 3.33	73.3 $\pm$ 2.21	72.8 $\pm$ 4.20	0.43

**Table 3**

Plasma biochemistry alkaline phosphate (ALP, Units L<sup>-1</sup>), Alanine aminotransferase (ALAT, U L<sup>-1</sup>), aspartate aminotransferase (ASAT, U L<sup>-1</sup>), glucose (mmol), albumin (μmol), creatinine (μmol), total protein (g L<sup>-1</sup>), ureic acid (μmol), and ions Na, K, Cl (mmol) and osmolality (Osm, mOsm) in Atlantic salmon (*Salmo salar*) fed graded levels of selenite (5.4 and 11 mg kg<sup>-1</sup>) and seleno-methionine (SeMet) (6.2, 16, 21, and 39 mg kg<sup>-1</sup>) for 3 months (triplicate tanks per diet, mean ± SD, n = 15). Values with different superscripts are significantly different from each other (one-way ANOVA, Tukey's HSD test, p < .001, p < .01, p < .05).

	Control	Selenite 5.4	Selenite 11	p-Values
ALP	448 ± 168 <sup>a</sup>	624 ± 143 <sup>ab</sup>	720 ± 204 <sup>b</sup>	p < .01
ALAT	13.5 ± 3.14 <sup>a</sup>	21.2 ± 5.6 <sup>b</sup>	19.4 ± 1.42 <sup>b</sup>	p < .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
ALP	448 ± 168 <sup>a</sup>	601 ± 155 <sup>a</sup>	566 ± 153 <sup>ab</sup>	512 ± 196 <sup>ab</sup>	422 ± 17 <sup>b</sup>	p < .01
ALAT	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 <sup>a</sup>	384 ± 105 <sup>a</sup>	330 ± 81 <sup>a</sup>	333 ± 110 <sup>a</sup>	253 ± 91 <sup>b</sup>	p < .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
Creatinine	17.7 ± 6.47 <sup>a</sup>	20.2 ± 7.17 <sup>a</sup>	15.3 ± 6.33 <sup>a</sup>	13.4 ± 6.78 <sup>a</sup>	10.8 ± 4.61 <sup>b</sup>	p < .01
Tot prot	41.4 ± 7.31 <sup>a</sup>	44.9 ± 5.40 <sup>a</sup>	42.59 ± 5.56 <sup>a</sup>	40.18 ± 7.58 <sup>a</sup>	37.3 ± 6.05 <sup>b</sup>	p < .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	0.86
Ca	2.67 ± 0.18 <sup>a</sup>	2.74 ± 0.14 <sup>a</sup>	2.68 ± 0.19 <sup>a</sup>	2.68 ± 0.18 <sup>a</sup>	2.53 ± 0.12 <sup>b</sup>	p < .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

two of the evaluated parameters, liver hepatocyte vacuolization ( $p = .040$ ) and degeneration and focal necrosis ( $p < .001$ ). No significant differences were observed in the parameter inflammation among fish fed the different dietary treatment. Fish fed both 5.4 and 11 mg kg<sup>-1</sup> selenite had significantly higher hepatocyte degeneration and focal necrosis compared to the control fish. Only fish fed the two highest SeMet level (21 and 39 mg kg<sup>-1</sup>) had significantly increased focal necrosis compared to control fish. Fish fed the highest selenite levels (11 mg kg<sup>-1</sup>) had significantly reduced liver lipid intracytoplasmic 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 (Fig. 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 pancytoplasmic inclusions of refractile, eosinophilic material was occasionally observed (Fig. 4b). Degenerated

hepatocytes presented a dark nucleus with condensed chromatin, piknotic nuclei and were frequently surrounded by red blood cells and oedematous areas (Fig. 4c). No inflammatory reaction was observed surrounding the lesions. A mononuclear cell infiltrate was often observed surrounding blood vessels (Figs. 5 A & B) and bile ducts (Figs. 5 C & D), although it could also be observed in the hepatic parenchyma.

### 3.8. Bench mark dose analyses

Parameter BMDL analyses as well as NOAEL and LOAEL assessments are given in Table 5. 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 5). For hematology, blood plasma parameters, liver oxidative stress markers, and histopathology, no BMDL

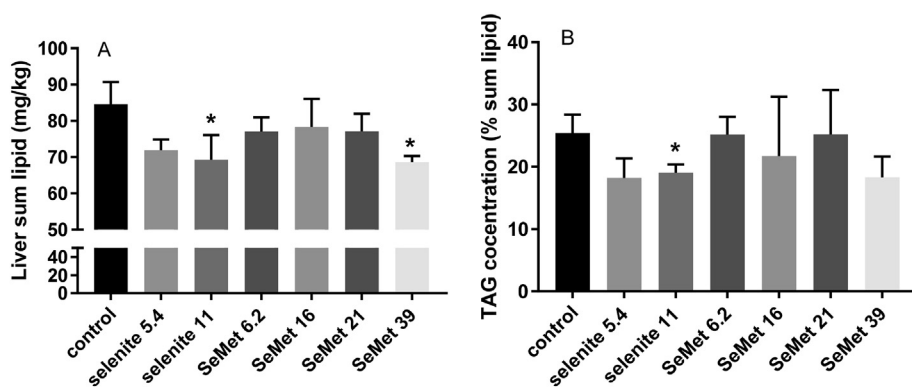


Fig. 2. Liver sum lipid (mg kg<sup>-1</sup> ww)(A), relative triacylglycerol (TAG, % sum lipid) (B), in Atlantic salmon (*Salmo salar*) fed control, graded levels of selenite fortified diets (5.4 and 11 mg kg<sup>-1</sup> WW, respectively), or graded levels of seleno-methionine (SeMet) fortified diets (6.2, 16, 21, and 39 mg kg<sup>-1</sup> WW, respectively) for 3 months (mean ± SD, n = 3). Bars with \* are significantly different from control (P < .05, one-way ANOVA, post hoc Tukey's t-test).

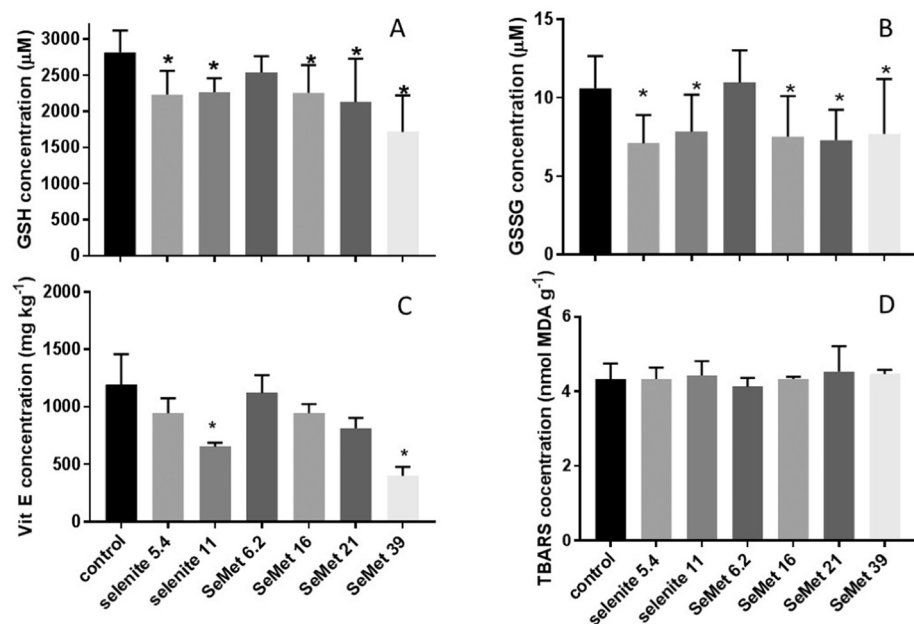


Fig. 3. Liver reduced glutathione (GSH) (A), oxidized glutathione (GSSG) (B), vitamin E (as alpha-tocopherol) (C) and thiobarbituric acid reactive substances (TBARS) (D) in Atlantic salmon (*Salmo salar*) fed control, graded levels of selenite fortified diets (5.4 and 11 mg kg<sup>-1</sup> ww, respectively), or graded levels of seleno-methionine (SeMet) fortified diets (6.2, 16, 21, and 39 mg kg<sup>-1</sup> ww, respectively) for 3 months (mean ± SD, n = 15). Bars with \* are significantly different from control (P < .05, one-way ANOVA, post hoc Tukey's t-test).

Table 4

Score for the main histopathological changes (hepatocyte intracytoplasmic 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<sup>-1</sup> WW, respectively), or graded levels of seleno-methionine (SeMet) fortified diets (6.2, 16, 21, and 39 mg kg<sup>-1</sup> WW, respectively) for 3 months (mean ± SD, n = 9). Different superscript letters denote significant differences among the dietary treatments (p < .05). Group mean scores were compared using Pearson's X<sup>2</sup> (level of confidence 95%).

	Vacuolization	Degeneration and focal necrosis	Inflammation
Control	1.4 ± 0.9 <sup>a</sup>	0.3 ± 0.2 <sup>c</sup>	0.2 ± 0.4
SeMet 6.2	0.9 ± 0.9 <sup>ab</sup>	0.5 ± 0.2 <sup>bc</sup>	0.6 ± 0.5
SeMet 16	0.8 ± 0.4 <sup>ab</sup>	0.7 ± 0.4 <sup>abc</sup>	0.6 ± 0.5
SeMet 21	0.8 ± 0.7 <sup>ab</sup>	0.8 ± 0.5 <sup>ab</sup>	0.2 ± 0.3
SeMet 39	0.9 ± 0.6 <sup>ab</sup>	1.1 ± 0.2 <sup>a</sup>	0.4 ± 0.3
Selenite 5.4	0.7 ± 0.8 <sup>ab</sup>	1.0 ± 0.5 <sup>ab</sup>	0.5 ± 0.4
Selenite 11	0.3 ± 0.5 <sup>b</sup>	1.0 ± 0.4 <sup>a</sup>	0.6 ± 0.8

could be established for all parameters because none of the fitted dose-response models were significantly better than the null model (AIC > AICnull-2, indicated with “none” in Table 5). 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 5). In addition, for several parameters (Hct, RBC, plasma glucose and creatinine, GSH, GSSG, and hepatocyte lipid intracytoplasmic vacuolization), the best fitted dose response model was not significantly different from the full model (AICmin < AICfull + 2, indicated with “\*” in Table 5). 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 2017b), which can be attributed to the use of only 3 selenite dietary exposure groups.

For the SeMet exposed fish, the parameter[s] with the lowest BMDL

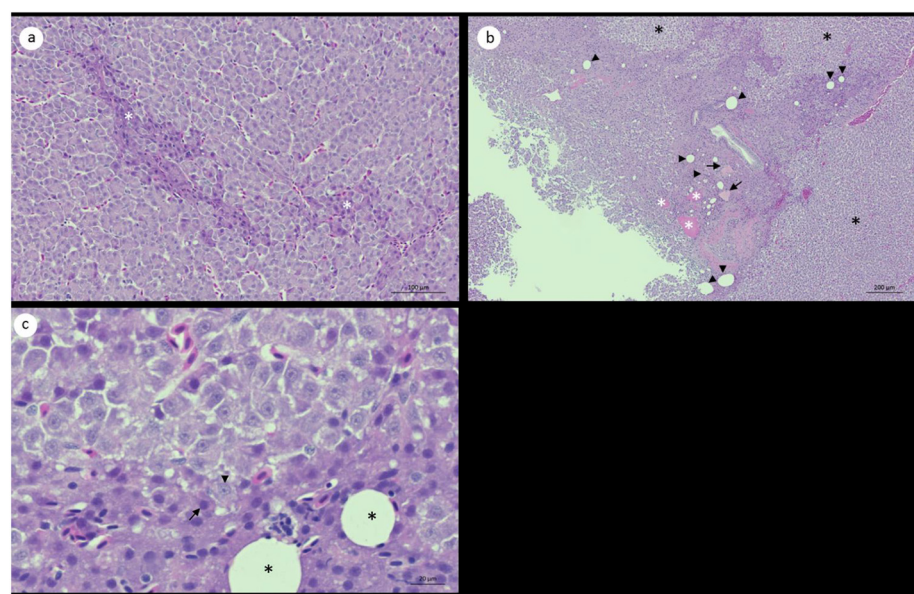


Fig. 4. (A–C) - Several degenerative and necrosis associated lesions observed in sections (5 µm) from the liver of Atlantic salmon fed selenite 11 mg kg<sup>-1</sup> (a), seleno-methionine (SeMet) 21 mg kg<sup>-1</sup> (b) and selenite 5.4 mg kg<sup>-1</sup> (c) for 3 months. (a) Basophilic focus (asterisk) 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.



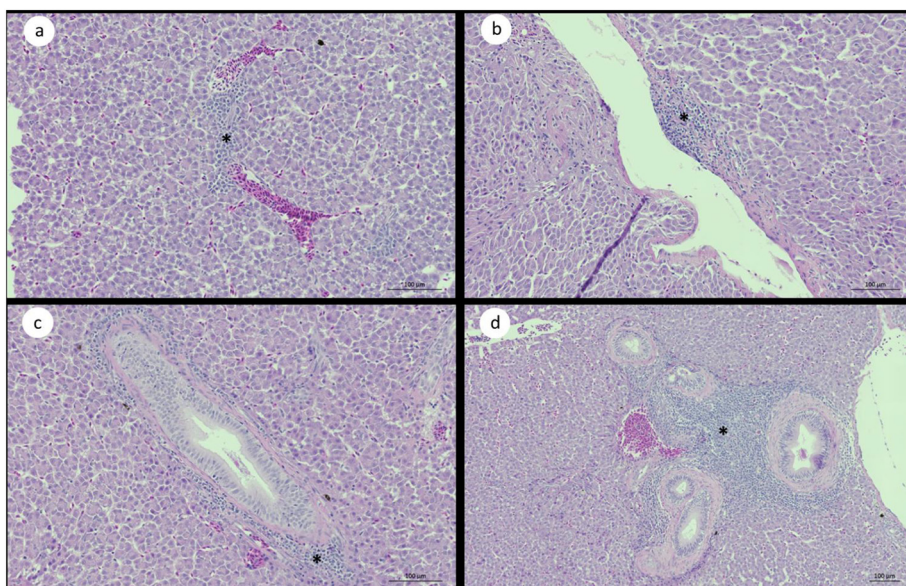


Fig. 5. (A–D)- Sections (5  $\mu\text{m}$ ) from Atlantic salmon liver fed the diets selenite  $5.4 \text{ mg kg}^{-1}$  (a), selenite  $11 \text{ mg kg}^{-1}$  (b & d) and seleno-methionine (SeMet)  $16 \text{ mg kg}^{-1}$  (c) for 3 months. A mononuclear (mainly lymphocytes) inflammatory reaction could 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.

were spleen somatic index, plasma creatinine, and histopathological degeneration and focal necrosis, with a  $\text{BMDL}_{05}$ ,  $\text{BMDL}_{20}$  and  $\text{BMDL}_{10}$  of 3.05, 2.29, and  $2.8 \text{ mg SeMet kg}^{-1}$  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 5). 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  $\text{BMDL}_{10}$  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 2017b) was the histological focal necrosis observation at  $2.1 \text{ mg SeMet kg}^{-1}$ .

For selenite exposed fish, lowest BMDLs were for spleen somatic index and plasma ALP, K, creatinine, and ureic acid, with a  $\text{BMDL}_{05}$  of 0.002, and a  $\text{BMDL}_{20}$  of 0.059, 0.066, 0.026 and  $0.017 \text{ mg selenite mg kg}^{-1}$ , respectively (Table 5). 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  $\text{BMDL}_{05}$  of 0.43 and  $0.41 \text{ mg selenite mg kg}^{-1}$  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 ( $\text{AIC}_{\text{min}} < \text{AIC}_{\text{full}} + 2$ , indicated with “\*” in Table 5). For the liver oxidative stress and histology parameters, GSSG and intracytoplasmic vacuolization had the lowest  $\text{BMDL}_{20}$  of 0.23 and  $\text{BMDL}_{10}$  of  $0.06 \text{ mg selenite kg}^{-1}$ , 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  $\text{BMDL}_{20}$  and  $\text{BMDL}_{10}$  of 1.4, 1.6, and  $1.9 \text{ mg selenite kg}^{-1}$ , respectively. Besides for total protein plasma content, for all parameters the BMDL was lower than the ANOVA established NOAEL (Table 5).

## 4. Discussion

### 4.1. 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 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  $29 \text{ mg kg}^{-1}$  selenite ( $0.29 \text{ mg kg}^{-1}$  body weight (bw)  $\text{day}^{-1}$ ) caused acute toxicity while fish fed up to  $39 \text{ mg kg}^{-1}$  ww SeMet ( $0.39 \text{ mg kg}^{-1}$  bw  $\text{day}^{-1}$ ) showed no mortality. In the present trial, a feed level of  $11 \text{ mg kg}^{-1}$  selenite kg ww ( $0.11 \text{ mg kg}^{-1}$  bw  $\text{day}^{-1}$ ) was not acute toxic to the fish, as was also observed in earlier trails with larger Atlantic salmon (572 g) fed  $15 \text{ mg kg}^{-1}$  ( $0.14 \text{ mg kg}^{-1}$  bw  $\text{day}^{-1}$ ) (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  $0.29 \text{ mg kg}^{-1}$  bw  $\text{day}^{-1}$ , 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  $0.29 \text{ mg kg}^{-1}$  bw  $\text{day}^{-1}$ , present study) showed mortality (Hilton et al. 1980). In contrast, rainbow trout start-feeders (0.6 g) fed up to  $11.4 \text{ mg kg}^{-1}$  selenite (estimated  $0.52 \text{ mg kg}^{-1}$  bw  $\text{day}^{-1}$ ) showed a marginal, but none significantly, 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  $> 7.4$  to  $18 \text{ mg SeMet kg}^{-1}$  dw ( $0.14$ – $0.31 \text{ mg kg}^{-1}$  bw  $\text{day}^{-1}$ ) (Vidal et al. 2005; Rider et al. 2009; Hardy et al. 2010; Knight et al. 2016). In contrast, for chinook salmon ( $\sim 1$  g) fed  $9.6 \text{ mg kg}^{-1}$  SeMet, survival was reduced (Hamilton et al. 1990) (Table 6), thus emphasizing the salmonid species difference in sensitivity towards dietary Se toxicity.

For overall non-lethal adverse effects that are not specifically related to Se toxic pathways, growth was significantly reduced at  $11 \text{ mg selenite kg}^{-1}$  ww ( $0.11 \text{ mg kg}^{-1}$  bw  $\text{day}^{-1}$ ). Similarly, earlier Atlantic salmon fed  $15 \text{ mg selenite kg}^{-1}$  ww with a slightly higher daily dose exposure ( $0.14 \text{ mg kg}^{-1}$  bw  $\text{day}^{-1}$ ) showed reduced growth (Berntssen et al. 2017). In contrast, rainbow trout (26 g) fed a similar daily selenite

**Table 5**

Benchmark doses (BMD), lower and upper 90% confidence interval (BMDL and BMDU, respectively), ratio 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-mentionine (SeMet) for 90 days. For parameter abbreviations see main text.

Selenomethionine					
Whole body parameters	BMDL <sub>05</sub>	BMUL <sub>05</sub>	BMDU/ BMDU	NOAEL	LOAEL
SGR	8.48	26.8		21	39
FCR	7.78	22.9	2.95	21	39
FI	20.4	33.5		21	39
Weight	7.98	27.1	3.41	21	39
Length	26.9	42.0	1.56	21	39
CF	23.7	53.2	2.24	39	
Organ indices					
HSI	None	None		39	
LSI	None	None		39	
SSI	3.05	31.7	10.39	39	
Haematology					
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 <sub>20</sub>	BMDU <sub>20</sub>	BMDU/ BMDU	NOAEL	LOAEL
ALP	None	None		21	39
ALAT	None	None		39	
ASAT	19.7	40	2.03	21	39
Glucose	None	None		39	
Albumine	None	None		39	
Creatinine	2.29	31.3	13.7	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	2.00	21	39
GSH	4.71	26.9	5.80	6.2	16
GSSG	6.91	15.5	2.20	6.2	16
GSH/GSSG ratio	None	None		39	
Lipid	BMDL <sub>20</sub>	BMDU <sub>20</sub>	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 <sub>10</sub>	BMDU <sub>10</sub>	BMDU/ BMDU	NOAEL	LOEAL
Degeneration and focal necrosis	2.8	4.51	1.6	21	39
Inflammation	None	None		39	
Intracytoplasmatic vacuolization	None	None		39	
Selenite					
Whole body parameters	BMDL <sub>05</sub>	BMUL <sub>05</sub>	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
Length	None	None		5.4	11
CF	7.53	25.6	3.4	11	

**Table 5 (continued)**

Selenite					
Organ indices	BMDL <sub>05</sub>	BMUL <sub>05</sub>	BMDU/ BMDU	NOAEL	LOAEL
HSI	None	None		11	
LSI	None	None		11	
SSI	0.002	4.12	2060	5.4	11
Blood parameters	BMDL <sub>05</sub>	BMUL <sub>05</sub>	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 <sub>20</sub>	BMDU <sub>20</sub>	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 <sub>20</sub>	BMDU <sub>20</sub>	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 <sub>20</sub>	BMDU <sub>20</sub>	BMDU/ BMDU	NOAEL	LOAEL
Sum lipid	3.97	200	50	5.4	11
% FFA	None	None		11	
% TAG	0.36	125	347	5.4	11
%DAG	None	None		11	
Histopathology	BMDL <sub>10</sub>	BMDL <sub>10</sub>	BMDU/ BMDU	NOAEL	LOAEL
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 “\*”.

dose (0.14 mg kg<sup>-1</sup> bw day<sup>-1</sup>) showed no reduced growth (Rider et al. 2009), and only daily selenite dose as high as 0.38–0.52 mg kg<sup>-1</sup> bw day<sup>-1</sup> caused growth impairment in juvenile (0.6–1.3 g) rainbow trout (Hilton et al. 1980; Hilton and Hodson 1983). Atlantic salmon fed 21 mg kg<sup>-1</sup> (0.21 mg kg<sup>-1</sup> bw day<sup>-1</sup>) 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<sup>-1</sup> bw day<sup>-1</sup> or 0.12 mg kg<sup>-1</sup> bw day<sup>-1</sup>, respectively)(Vidal et al. 2005; Knight et al. 2016). In contrast, adult rainbow trout (26–100 g) fed 7.4–8.9 mg SeMet kg<sup>-1</sup> (0.15–0.18 mg kg<sup>-1</sup> bw day<sup>-1</sup>) had no reduced growth (Rider et al. 2009; Pacitti et al. 2016a) (Table 6).

In the present study, no significant differences were observed in hematocrit, hemoglobin, or red blood cells counts, 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

**Table 6**

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
	mg kg <sup>-1</sup>	mg kg <sup>-1</sup>	% BW/Day		Days		Exposure range
<b>Rainbow trout</b>							
79 g	?	9 mg kg <sup>-1</sup>	?	Selenite	294 days	Mortality	Hamilton, 2004 From Hamilton, 2004
1.3 g	3.67 mg kg <sup>-1</sup> DW 13.06 mg kg <sup>-1</sup> DW	13 mg kg <sup>-1</sup> DW *	2.9*	Selenite	140 days	Mortality/growth/FCR No hematology, no histopathology	Hilton et al. 1980 0.07, 0.15, 0.28, 25, 3.67, 13.06 mg kg <sup>-1</sup>
0.6 g	11.4 mg kg <sup>-1</sup> DW 6.6 mg kg <sup>-1</sup> DW 11.4 mg kg <sup>-1</sup> DW	* 11.4 mg kg <sup>-1</sup> DW *	4.6*		112 days	No significant mortality Reduced growth	Hilton and Hodson 1983 0.6, 6.6, 11.4 mg kg <sup>-1</sup> DW
26 g	7.1 mg /kg ww	*	2	Selenite	70 days	No hematology or blood plasma parameters no reduced growth FCR/no oxidative stress/Hct	Rider et al. 2009
	3.9 mg kg <sup>-1</sup> ww	7.1 mg kg <sup>-1</sup> ww				Increased oxidative stress after physical stress	0.73, 2.3, 3.9, 7.1 mg kg <sup>-1</sup> ww
26 g	7.4 mg kg <sup>-1</sup> ww		2	Se-yeast	70 days	No mortality no reduced growth	Rider et al. 2009 0.73, 2.4, 4.1, 8 mg kg <sup>-1</sup> ww
0.37 g	2.4 mg kg <sup>-1</sup> DW 18 mg kg <sup>-1</sup> DW	4.6 mg kg <sup>-1</sup> DW	0.8 *	Selenomethionine	90 days	Reduced growth, No mortality or oxidative stress	Vidal et al. 2005 0.23, 4.6, 12, 18 mg kg DW
33.47 g	3 mg kg <sup>-1</sup> WW	*	?	Se yeast Sel-Pex		No growth reduction or oxidative stress	Hunt et al. 2011 Control, 2, 3, 4 mg kg <sup>-1</sup>
0.28 g	1.3 mg kg <sup>-1</sup> DW 7.1 mg kg <sup>-1</sup> DW	7.1–19.5 mg kg <sup>-1</sup> DW 19.5 mg kg <sup>-1</sup> DW	1.6**	Semet worm	60 days Reduced liver TAG	Reduced final weight 1.3, 7.1, 10.7, 19.5, 31.8 mg kg <sup>-1</sup> DW	Knight et al. 2016
100 g	1.3 mg kg <sup>-1</sup> DW 8.94 mg kg <sup>-1</sup> ww	7.1 mg kg <sup>-1</sup> DW *	2	Se-Plex	70 days	Microarray lipid metabolism No reduced growth	Pacitti et al. 2015 0.87, 1.46, 4.81, 8.94 mg kg <sup>-1</sup> ww
<b>Atlantic salmon</b>							
147 g	5.4 mg kg <sup>-1</sup> 0.45 mg kg <sup>-1</sup> 0.45–5.4 mg kg <sup>-1</sup>	11 mg kg <sup>-1</sup> 5.4 mg kg <sup>-1</sup> 5.4–11 mg kg <sup>-1</sup>	1.1	Selenite	90 days	Mortality Histopathology plasma enzymes Oxidative stress (GSSG and vit E)	This study 0.45, 5.4, 11, 29, 60 mg kg <sup>-1</sup> WW
572 g	15 mg kg <sup>-1</sup> ww 1 mg kg <sup>-1</sup> ww 1 mg kg <sup>-1</sup> ww 1 mg kg <sup>-1</sup> ww	* 15 mg kg <sup>-1</sup> ww 15 mg kg <sup>-1</sup> ww 15 mg kg <sup>-1</sup> ww	0.9	Selenite	90 days	No mortality Reduced growth Oxidative stress (GSSG and vit E) Metabolomics lipid pathway disturbance	Bertssen et al. 2017 0.35, 1, 15 mg kg <sup>-1</sup> WW
147 g	39 mg kg <sup>-1</sup> ww 21 mg kg <sup>-1</sup> ww 6.2–21 mg kg <sup>-1</sup> ww	* 39 mg kg <sup>-1</sup> ww 16–39 mg kg <sup>-1</sup> ww	1.1	Selenomethionine	90 days	No mortality Histopathology plasma enzymes oxidative stress (GSH + GSSG and Vit E)	This study 0.45, 6.2, 16, 21, 39 mg kg <sup>-1</sup> ww
572 g	15 mg kg <sup>-1</sup> WW 1 mg kg <sup>-1</sup> ww	* 15 mg kg <sup>-1</sup> ww	0.9	selenomethionine	90 days	No mortality, reduced growth, oxidative stress Metabolomics lipid pathway disturbance	Bertssen et al. 2017 0.35, 2, 15 mg kg <sup>-1</sup> 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.

apparent dietary selenite induced toxicity was not expressed as alterations in hematology (Hilton et al. 1980; Hilton and Hodson 1983). However, organic selenium fed bluegill (*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<sup>-1</sup> selenite or 16 mg kg<sup>-1</sup> 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 the oxygen carrying capacity in dietary Se exposed Atlantic salmon.

#### 4.2. 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

(Bertssen 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 (Bertssen 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 incorporated in any protein containing methionine, while selenite needs to form hydrogen selenide (H<sub>2</sub>Se) 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 histopathological finding was hepatocyte degeneration and focal necrosis, which occurred significantly in fish fed 5.4 mg selenite kg<sup>-1</sup> or fish fed 21 mg SeMet kg<sup>-1</sup> ww. The hepatic somatic index, however, was not significantly altered 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 showed liver histopathological changes in liver such as hepatocellular vacuolar 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 showed a dose-dependent ( $1.4\text{--}104\text{ mg kg}^{-1}\text{ dw}$ ) increase in frequency and size of melanomacrophage aggregates, but had no liver necrosis or general cell damage (De Riu et al. 2014; Zee et al. 2016a). Furthermore, in contrast to the present study, rainbow trout fed  $13\text{ mg kg}^{-1}$  selenite had no histopathological lesions (Hilton et al. 1980). In the present study, the observed histopathological findings in liver were associated with elevated plasma ALP and ALAT (fish fed 5.4 and  $11\text{ mg kg}^{-1}$  selenite, respectively), which are markers of liver toxicity due to loss of liver specific enzymes to the blood stream. Only fish fed  $39\text{ mg SeMet kg}^{-1}$  showed altered AP and ASAT levels, attributable to the lower potential of dietary SeMet to cause liver pathology/toxicity compared to dietary selenite. In addition to liver toxicity, both dietary selenite and SeMet are also known to induce renal toxicity observed as morphological alterations (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 histopathology evaluation of kidney, however, plasma markers of renal function such as plasma creatinine and total protein were significantly altered in Atlantic salmon fed  $39\text{ mg kg}^{-1}$  SeMet, while fish fed selenite displayed normal values. 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. Interestingly, SeMet and selenite had no significant effect on osmoregulation as seen from unaltered plasma osmolality, K, Na, and Cl, although plasma Ca was significantly reduced in 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).

#### 4.3. Markers of lipid and oxidative stress

In the present study, for Atlantic salmon fed  $11\text{ mg kg}^{-1}$  selenite a significant reduction in lipid intracytoplasmic vacuolization was observed. Concurrently, total liver lipid was reduced in fish fed  $11\text{ mg kg}^{-1}$  selenite or  $39\text{ mg kg}^{-1}$  SeMet exposed fish, with significantly reduced liver TAG in  $11\text{ mg kg}^{-1}$  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 TAG content 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 increased 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 TAG processing and storage, as well as liver gene networks for epidermal growth factor and Notch signaling, 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 sensitive responses to both selenite and SeMet exposures. Oxidative stress has been suggested as a main mode of action (MOA) in selenite and SeMet induced toxicity in fish (Miller et al. 2007; Misra and Niyogi 2009; Misra et al. 2012; Hursky and Pietrock 2015; Lee et al. 2015). It has to be noted that more recent studies reported dietary SeMet induced toxicity without clear oxidative stress in rainbow trout and white sturgeon (Knight et al. 2016; Zee et al. 2016b). In vitro trails on rainbow trout hepatocytes, showed an increase in antioxidant enzymes together with reduced levels of GSH and GSH:GSSG ratio (Misra and Niyogi 2009). The

reduction of GSH at high dietary Se exposures can be due to the role of GSH in the anti-oxidant defense as substrate to anti-oxidant enzymes (e.g. glutathione peroxidase) or as direct antioxidant. In addition, GSH is consumed in the metabolism of selenite in the formation of hydrogen selenide on its way in being incorporated in selenoproteins (Suzuki 2005; Godin et al. 2015). The metabolism of both selenite itself can cause oxidative stress in the form of formation of reactive oxidative substances in formation of hydrogen selenide (Lin and Spallholz 1993). Excess SeMet is methylated to methylselenol for further excretion (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, both GSH and GSSG were lowered and the ratio GSH:GSSG was not altered 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 at  $11\text{ mg kg}^{-1}$  selenite versus  $39\text{ mg kg}^{-1}$  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).

#### 4.4. Comparison literature NOAEL and LOEL and bench mark dose

Table 2 in the supplementary material gives an overview of the reported NOAEL and LOEL 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 6). As none of the previous studies have used bench mark dosing to assess safe levels of dietary exposure, the NOAEL and LOEL 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 feed safety.

The lowest LOEL for SeMet in rainbow trout varied from  $0.071\text{--}0.12\text{ mg kg}^{-1}\text{ bw day}^{-1}$  (Vidal et al. 2005; Knight et al. 2016) and for selenite from  $0.14\text{--}0.45\text{ mg kg BW}^{-1}\text{ day}^{-1}$  (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 LOEL and higher LOEL 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 varying from  $0.006\text{--}0.013\text{ mg kg dw}^{-1}\text{ day}^{-1}$  (Vidal et al. 2005; Knight et al. 2016) and  $0.07\text{--}0.13\text{ mg kg bw}^{-1}\text{ day}^{-1}$  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 LOEL, 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  $2.7\text{--}6.6\text{ mg kg}^{-1}\text{ dw}$  (Teh et al. 2004), was re-analysed by using a logistic regression to derive an effective concentration of 10% (EC10), giving a new threshold adverse effect level of  $0.9\text{ mg kg}^{-1}\text{ dw feed}$  (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 a safe limit based on a common assessment of different dose-response 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% variance of the 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). 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 compared to using adverse effects. 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 ALAT, liver vitamin E and histopathology, giving a safe dietary dose of 1–2 mg kg<sup>-1</sup> ww (dose 0.01–0.02 mg kg bw<sup>-1</sup> day<sup>-1</sup>) of total Se for selenite supplementation. However, the loss of two dose groups 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<sup>-1</sup> ww to assess more accurately the safe limit in Atlantic salmon with regards to animal health. For dietary SeMet a more accurate BMDL dose-response assessment could be made giving lowest BMDL for safe animal health limits of 2.8 mg kg<sup>-1</sup> ww (dose 0.03 mg kg bw<sup>-1</sup> day<sup>-1</sup>) based on liver histopathology, spleen somatic index, 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<sup>-1</sup> dw in fish (Lemly 1993a; Hamilton 2004).

In conclusion, feed levels of 1–2 mg kg<sup>-1</sup> (0.11–0.21 mg kg<sup>-1</sup> bw day<sup>-1</sup>) and 2.8 mg kg<sup>-1</sup> (0.29 mg kg<sup>-1</sup> bw day<sup>-1</sup>) total Se for respectively selenite and SeMet supplementation to a low fish meal (10%) based diets with background levels of 0.45 mg kg<sup>-1</sup>, were safe with regards to fish health. SeMet is known to accumulate in the muscle (Bertssen 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.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.aquaculture.2018.06.041>.

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