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Title:

Element content and daily intake from dietary supplements (nutraceuticals) based on algae, garlic, yeast fish and krill oils - should consumers be worried?

Article Type:

Research paper

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Highlights

- Essential and non-essential elements were determined in dietary supplements
- Most elements: daily intake is below the recommended daily intake/ legal limits
- Use of some algae based supplements can lead to excess daily intake of iron or iodine
- Levels of inorganic As above the Chinese legal limit were found in some supplements
- None of the tested dietary supplements poses are direct risk to healthy adults

Abstract

The element content of sixty seven food supplements falling into five different categories was determined with an Agilent 8800 Triple Quadrupole ICP-MS and the maximum daily intake calculated. The determined elements were: Rb, Cs, Mg, Ca, Sr, Ba, V, Cr, Mn, Fe Co, Cu, Zn, Mo, Se, I, Br, B, Al, As, Cd, Sb and Pb. The majority of supplements contained significantly less essential elements than the recommended daily intake. Exceptions were two algae based products leading to a very high iron intake. The use of 3 other algae based products would result in increased iodine intake. Of the non-essential elements determined the intake of inorganic arsenic from all supplements was below the limit set by ANSI 173, but several algae based and one garlic based supplement contained levels of inorganic arsenic above the limit set in China for food supplements. Generally garlic, fish oil and krill oil based products pose little risk of inadvertent increased intake of essential and non-essential elements. Algae based products can lead to intakes above the recommended limits for specific elements and generally contain higher amounts of all elements. None of the tested food supplements poses a direct risk to healthy adults.

Keywords

Krill oil, fish oil, essential elements, non-essential elements, garlic, food supplement, algae, food composition, food analysis

1. Introduction

Dietary supplements are used by an estimated 50 % of the population through the western world; out of these, an estimated 8 – 25 % use botanical and herbal supplements (Gershwin et al., 2010; Genuis et al, 2012; Garcia-Alvarez et al., 2014). The market for food supplements increased significantly in the last decades. In 2005 the market size in the EU was estimated to be 5 billion €; the estimate for the USA was 20 billion \$ in 2010 (Gershwin et al., 2010). Dietary supplements include vitamin and mineral supplements and plant and animal based products. Marketed as food supplements, they have to comply with laws applicable to food, but not drugs. In the US, the main applicable regulation for dietary supplements is the Dietary Supplement Health and Education Act (DSA) of 1994 and follow-on legislation like 21 CFR Part 111 (Gershwin et al., 2010). Within the EU they have to comply with Directive 2002/46/EC and follow-on legislation like 2006/37/EC. Both regulations deal mostly with production, permitted ingredients and labelling issues. The laws regulating metal contamination of food are also applicable to food supplements within the EU and the US. The American Institute for Standards has developed maximum levels for certain elements in food supplements (ANSI, 2010). The area of dietary supplements is not regulated by the laws governing the production of drugs (Pharmacopeia). In the US, supplements do not need approval by the FDA (2015), but production facilities need to be registered by the FDA, and cGMP regulation must be followed. Claims on labels in the US are not allowed to include statements concerning treatment of disease or conditions, but are allowed if supported by inconclusive evidence (FDA, 2009). Within the EU, it is required that any health claims are substantiated by scientific evidence (EC, 2002; EC, 2015b).

For this study, out of the several thousand individual products available over the counter in the USA and UK, 67 products containing the main ingredient algae, garlic, yeast, fish or krill oil were randomly chosen based on availability (market survey). Three out of the 67 studied food supplements were fortified with minerals or trace elements according to their label. All others are recommended either to increase intake of poly-unsaturated fatty acids (fish and krill oils) or to increase intake of phytochemicals thought to be beneficial to health, e.g., allicin in garlic.

During this study the aim was to determine the total element content of a variety of essential and non-essential elements in these dietary supplements using ICP-MS/MS. The determined content was used in conjunction with the maximum daily dose recommended by the manufacturers to calculate maximum daily intake resulting from use of these supplements. Calculated consumption was compared to the recommended daily allowance (RDI) or adequate intake (AI) for essential elements and minimal risk levels (MRL) for non-essential elements (where such levels have been defined). The maximum permitted levels were taken from NSF International Standard/American National Standard 173 (ANSI, 2010), United States Pharmacopeia (USP) (USP, 2015), European and Australian food / food supplement legislations (ANZ, 2015; EU, 2015a), MRL values were found in publications of the Agency for Toxic Substances and Disease Registry and the integrated Risk Information System (IRIS) of the U.S. Environmental Protection Agency (EPA) (IRIS, 2015) for the relevant elements. In addition to total arsenic levels, the level of inorganic arsenic was estimated using hydride-generation (HG) ICP-MS/MS without chromatography, since total arsenic does not reflect the toxicological potency of this element.

2. Materials and methods

2.1 Samples

In total, sixty seven dietary supplements were used (details: Table S1). Seven commercially available products based on either marine algae (kelp or fucus, n= 3), single cell algae chlorella (n=1) or spirulina (n= 3) were purchased in the UK or California. Ten garlic-based products containing dried garlic (n=7) or garlic extract (n=3) and two yeast-based products, one for use as a selenium supplement and the other a commercial food product (Marmite™), were also sourced. Additional 19 pure krill oil supplements and 30 fish oil supplements (fish oil without elemental additives n= 28) were purchased in the UK or California.

2.2 Quality controls and statistical analysis

The following certified reference materials were analysed alongside the samples: NIST1568a (Rice) Standard Reference Material 1568b Rice Flour and RM8415 (Whole Egg Powder) Reference Material 8415 Whole Egg Powder both from the National Institute of Standards and Technology (NIST, Gaithersburg, USA), ERM-BCR-211 (Rice), BCR-402 (White Clover) and BCR-062 (Olive Leaves) from the Institute for Reference Materials and Measurements (Geel, Belgium), DORM-3 (Fish Protein Dogfish Muscle Certified Reference Material for Trace Metals), DOLT-2 (Dogfish Liver Certified Reference Material for Trace MetalsDogfish Liver), TORT-2 (Lobster Hepatopancreas Reference Material for Trace MetalsLobster Hepatopancreas) and DOLT-4 (Dogfish Liver Certified Reference Material for Trace MetalsDogfish Liver) from the National Research Council of Canada, Seronorm urine blank from SERO (Norway) and IAEA-140 (Trace Elements and Methylmercury in

Seaweed) from the International Atomic Energy Agency (Vienna, Austria). Results for the different CRM's are given in Table S3 and S4.

2.3 Chemicals

Water (18.2 M Ω cm) provided from a MilliQ water purification system (Millipore, UK) was used throughout this work. Nitric acid ($\geq 69.0\%$, TraceSELECT[®]) was purchased from Fluka (Switzerland), hydrogen peroxide ($\geq 30\%$, analytical reagent grade) and hydrochloric acid (32 % laboratory grade) were from Fisher Scientific (Loughborough, UK) as was the ammonia (25 % p.a.). Triton-X100 was from BDH (UK). Calibration standards were prepared from 10 mg/kg multi element standard AccuTrace[®] (AccuStandard[®], New Haven, USA), and 1000 mg/kg B, Br, I, Sb and Mo single element standards (High-Purity Standards, USA).

2.4 Sample Preparation

Ten tablets or 10 capsules of each individual sample were weighed (whole tablet or content of capsule and additionally capsule shell of powdered samples) and pooled. The tablets were ground and homogenized with a mortar and pestle. Capsules with a powdered, paste or oil-like content were homogenized. Liquids were used directly after homogenization. All samples were stored at room temperature in 15 mL polypropylene (PP) tubes (Corning[®] Ltd, UK) prior to use.

Open or closed vessel microwave digestion (Mars5, CEM Microwave Technology Ltd, Buckingham, UK) was used for matrix digestion, all samples were digested and measured in triplicate. Open vessel digestion was used for algae and garlic based (not garlic oil) supplements. For these, a portion (100 mg weighed to 0.1 mg) of sample or CRM was placed in PP tubes. One mL HNO₃ was added and samples

were pre-digested overnight, the hydrogen peroxide (2 mL) was added to open vessel digests. Samples were heated to 95°C for 30 min. After cooling the samples were diluted with water to 50 g.

Closed vessel digestion was used for oil-based supplements using Mars5 Microwave digestion system following the recommended procedure of CEM. For this, a portion (100 mg weighed to 0.1 mg) of sample or CRM was placed in the Teflon vessels and 10 mL conc. HNO₃ was added. The vessels were closed and heated to 180°C for 30 min. After cooling the samples were diluted with water to 50 g. Multi element standards were prepared in water containing 10% HNO₃ (v/v) (closed vessel digestion) or 1 % HNO₃ (v/v) (open vessel digestion), the calibration range was from 0.1 µg/kg to 100 µg/kg. Rhodium and germanium (each 10 µg/kg in 1 % (v/v) HNO₃) were used as internal standards and added on-line in each case.

Iodine and bromine were determined in alkaline extracts. A portion (100 mg weighed to 0.1 mg) of sample was mixed with 10 g of a solution containing 1 % (v/v) ammonia and 0.1 % Triton X-100 and homogenized. Algae, garlic and yeast samples were centrifuged after a 24 h extraction period to remove solid particles. Fish and krill oils were only homogenized. Standards were made up in the same solvent. Tellurium (10 µg/kg in the same solvent) was added on-line as internal standard.

Extraction for arsenic speciation (inorganic arsenic): Samples (100 mg weighed to 0.1 mg) were mixed 10 mL of a solution containing with 2 % HNO₃ (v/v) or HCl (v/v, for fish and krill oils) and 3 % H₂O₂ (v/v) and extracted using microwave assisted extraction as described by Petursdottir et al. (2014). After cooling, the samples were weighed again and centrifuged. The supernatant was used for the determination of inorganic As by HG-ICP-MS/MS.

2.5 ICP-MS/MS

For the determination of the total element content of dietary supplements, an Agilent 8800 Triple Quadrupole ICP-MS (ICP-MS/MS, Agilent Technologies, UK) equipped with a Scott-type spray chamber and a MicroMist concentric glass nebulizer (Glass Expansion, West Melbourne, Australia) was used. The ICP-MS/MS used in this study is a quadrupole instrument consisting of one quadrupole after the focusing lenses (Q1), followed by the reaction cell and the second quadrupole (Q2) before the detector. The quadrupoles can be operated either in tandem (MS-mode, both Q at same m/z) or independently (MS/MS-mode, different m/z settings on each quadrupole for gas phase reactions). The sample and skimmer cones were made of Ni. The ICP-MS/MS was operated in no gas (MS-mode), helium (MS-mode), hydrogen (MS/MS-mode) and oxygen (MS/MS-mode) mode for different elements. Collision/ reaction cell (CRC) gas flow rates for helium, hydrogen and oxygen were 4.5 mL/min, 3.5 mL/min and 30% (~ 0.3 mL/min) respectively (further instrument parameters can be found in the electronic supplement, Table S2). The ICP-MS/MS was optimized for robustness of plasma conditions. The detailed instrument setup for HG-ICP-MS/MS is described in Musil et al. (2014).

2.6. Quantification, calculation of daily intake and statistical analysis

All concentrations of the individual digests were determined using external calibration. Mean and standard deviation for each sample were calculated from the triplicates of each sample prepared and measured. The limits of detection in solution were comparable or better than literature values (Table S5). The limit of quantification (LOQ) for each element in the different modes was calculated from the mean result of the digestion blank ($n=3$) + 10 SD (standard deviation) during each

measurement day (an average over all days ($n=12$) for the LOQ is given in Table S5). Elements present in the multi-element standard were determined using at least two different gas modes and/or isotopes. Which gas mode or isotope was used for the calculations was decided on the basis of the occurrence of interferences, LOQs and recovery rates of CRMs and also the matrix (Table S5). Al, Sb, Ba, B, Cd, Cs, Pb, Sr, I, Br and Rb were quantified in no-gas mode (MS-mode). For Pb, three isotopes were measured (206, 207 and 208) and their intensities summed up to exclude influence of different Pb-isotope signatures from samples of widespread origin on the quantified amount. As and Se are well known to suffer from molecular interferences mainly caused by chlorine and were therefore measured using helium (MS-mode), hydrogen (MS/MS mode) and oxygen (MS/MS-mode) as reaction gases, both were quantified using H₂-mode. It was found that the reaction gas mode used for the determination of arsenic has little influence on the amount of arsenic found in the matrices studied. Fe, Cu, Zn, Mn, Co, Mo, V and Cr were quantified using helium mode. Ca was quantified using H₂-mode.

Element concentrations were calculated using MassHunter (Agilent Technologies, UK). Median, mean and standard deviations were calculated using Excel (Microsoft, USA). Only the LOQ was considered (not the LOD) for reporting data below the quantification limit (reporting limit). To calculate the median values below the LOQ were substituted by LOQ/2. More extensive modelling of values below the LOQ would require significantly more samples to be measured (Croghan CW). Spearman rank order correlations were calculated using SigmaPlot 13.0 (Systat Software, Inc., USA), no further statistical tests were done. SigmaPlot 13.0 was also used for preparation of figures 1-4.

The daily elemental intake for each supplement was calculated by multiplying each determined element concentration by the maximum serving size per day as recommended on the label of each product. The size and recommended daily dose for the different tablets and capsules resulted in serving size per day from less than 1 g to nearly 10 g of product. Daily elemental intake calculations were compared with regulatory levels and recommended daily intake (RDI) values and the results are summarized in Figures 1 - 4 and Table S6-S9.

3. Results and Discussion

The majority of dietary supplements in this study are not intended as supplements for essential trace elements, but as supplements of phytochemicals or polyunsaturated fatty acids. The labels of only seven out of the 67 supplements (Table S1) indicate the intake of certain trace elements per serving; three of these seven were artificially fortified. In Tables S6a-S9a, the median daily intake (plus minimum and maximum intake) for each supplement category, the relevant RDI, UL (upper safe level of intake) and MRL (minimal risk levels) values are summarized. Tables S6b to S9b summarize the median total element content (incl. min and max values) for the different supplement categories, the average LOQ as determined in this study and some additional legislative information. Figures 1 – 4 show the span of the intake for individual elements, the RDI, AI (adequate intake) or MRL are indicated in the individual graphs.

The figures use the Rank Sum Test box plot showing 25th and 75th percentiles, with a line at the median and error bars defining the 10th and 90th percentiles, outliers are marked by dots (all data were used for preparation of box whisker plots).

Data quality for the majority of elements was checked by using several CRM's of different matrices as control for the same element, since CRM's certified for some trace elements at least are only available for algae based matrices, but not for garlic and/or fish and krill oil. The quality of the measurement was considered sufficient when the majority of concentrations from different CRM's for this element were within the certified range. Some elements in certain CRM's are known to be problematic like Al in IAEA-140 (underestimation possibly due to the presence of silica), Se in the same material is always significantly above the certified value and these values were therefore ignored. There was no trend with regard to over- or underestimation of a particular element across the different CRM's. T-test did not reveal statistically significant differences between measured and certified range, except for the elements indicated in Table S3 and S4. Recoveries for the CRM's were generally between 80 and 110 %, the calculated results were not corrected for recovery rates of the CRM's (Table S3 & S4).

3.1. Alkali and earth alkali elements

Four out of the ten alkali and earth alkali elements are known to be essential to humans with recommended daily intakes in the high mg to gram range (details see Table S6a and S6b). Of these, sodium and potassium are only mentioned here for completeness as they were not determined in this study. Rubidium, caesium, strontium and barium are not known to be essential elements for humans.

Generally the consumption of algae based supplements would result in the highest daily intake of all these elements and pure fish oils have the lowest contribution to the daily intake (Figure 1A-F). The range of intake from supplements within each

category was large and in line with data published for similar samples in the literature. The calculated intake for none of the elements was high enough to reach the UL for Ca or Mg or the MRL for strontium and barium. Statistical comparison (Spearman Rank test) showed a linear relationship between most of these elements (with $r > 0.86$, $p < 0.001$) with the exception of Cs. Daily Cs intake compared to the other elements in this group shows correlation coefficients of around $r = 0.6$ with $p < 0.001$. The most likely reason for this similarity is the ionic nature of all these elements.

Of the studied alkali elements the daily intake of Rb (up to 49 μg , median: algae 6.4 μg , garlic 2.5 μg , yeast 2.4 μg , fish oil 0.028 μg and krill oil 0.22 μg , Figure 1A) is much higher than Cs (up to 554 ng, median: algae 55 ng, garlic 14 ng, yeast 7.1 ng, fish oil 1 ng and krill oil 61 ng, Figure 1B), reflecting their prevalence in nature (Table S5a and S5b). Both elements were not determined in many studies of food supplements, but values found are comparable for supplements with similar basic ingredient (Avula et al., 2010; Genuis et al., 2012).

Among the earth alkali elements daily Ca intake (up to 61 mg) and Mg (up to 62 mg) are significantly higher than Sr (up to 0.50 mg) or Ba-intake (up to 73 μg) again reflecting at least in part their natural abundance (Figure 1C-F, Table S5a and S5b). These elements were not measured in many studies of food supplements based on botanical or animal ingredients. The concentrations found in this study are comparable with literature values (Avula et al., 2010; Genuis et al., 2012, Sivakumar et al., 2007).

Median daily intake of Mg was for algae-based samples 5.5 mg, garlic 1.2 mg, yeast 1.2 mg, fish oil 0.0076 mg and krill oil 0.51 mg (range 0 - 62 mg, Figure 1C). Mg is

an essential element for humans with a recommended overall daily intake between 310 and 420 mg. On average the studied supplements contribute 0.8 % to the average RDI of 345 mg/d. Two algae based products (A4 and A5) contribute between 15 and 18 % to the RDI respectively partly as result of the recommended high daily dose. EFSA (SCF, 2001) set 250 mg Mg as the UL for easily dissociated Mg salts as they are used for the preparation of dietary supplements (algae and garlic which contain Mg naturally are not included), also intake of Mg through normal food is not included in this calculation. Institute of Medicine (IoM, 2010) defined UL from all sources as 350 mg/d. None of the supplements contributes Mg near the UL.

Median daily intake of Ca was for algae-based samples 16 mg, garlic 0.78 mg, yeast 20 mg, fish oil 0.034 mg and krill oil 0.43 mg (range 0 - 61 mg, Figure 1D). Ca is an essential element for humans with daily requirement estimated to be between 2 and 2.5 g and a recommended daily intake between 0.7 and 1.3 g/d. None of the supplements contributes significant amounts of Ca to the RDI. EFSA (SCF, 2003d) and IoM (2010) consider 2.5 g/d as the UL for total Ca intake from all sources (food + water + supplements). The studied supplements contribute between 0 and 6 % to the RDI.

Median daily intake of Sr was for algae-based samples 212 µg, garlic 42 µg, yeast 5.4 µg, fish oil 0.072 µg and krill oil 0.77 µg (range 0 - 503 µg, Figure 1E). For non-radioactive Sr a MRL between 0.6 and 2.0 mg/kg/d is set (Agency for Toxic Substances, Disease Registry, ATSDR, 2004b). Applying the MRL all supplements are well below a 10 % cut-off limit of 42 mg/d when EPA's chronic reference dose is applied for a 70 kg adult person.

Median daily intake of Ba was for algae-based samples 9.6 μg , garlic 1.7 μg , yeast 0.30 μg , fish oil 0.14 μg and krill oil 0.42 μg (range 0 - 73 μg , Figure 1F). . All studied products contained Ba at intakes well below the limit for supplements of 1.2 mg/d set by FDA (maximum 6 % of this limit) (Genius et al., 2012) and well below the MRL of 0.2 mg/kg/d (ATSDR, 2007c).

3.2. Essential metallic elements

Among the first row transition metals, iron, zinc, copper, manganese and cobalt are known to be essential to humans in addition to molybdenum (second row transition metals). The daily intake of these required for homeostasis decreases in the order of these elements, with Co only to be known essential in form of Vitamin B12. The other five elements are required as catalytic centre in a wide range of enzymes and as part of metalloproteins. The essentiality of vanadium for humans is not yet proven and chromium is known to influence the glucose metabolism in an as yet unknown way.

Generally the use of algae based supplements would result in the highest daily intake of all these elements and pure fish oils have the lowest contribution to the daily intake (Figure 2A-H, Table S7a and S7b). The polyunsaturated fatty acids in fish and krill oil supplements can quickly be spoiled by the presence of redox-active metals e.g. Fe, which is the reason that the majority of metals are removed during processing (BIOHAZ, 2010). For example the majority of fish and krill oil supplements (46/50) did not contain detectable amounts of Fe. Fe, Cu, Zn, Co, Mn, and Mo intakes correlated strongly with each other in a linear fashion ($r > 0.7$, $p < 0.001$, Spearman Rank Test). Iron intake also correlated with Al intake ($r > 0.8$, $p < 0.001$). The daily intake varied widely between supplements of each category.

The maximum daily intake resulting from the use of one of the studied supplements for V was 48 µg, Cr 116 µg, Mn 1.3 mg, Fe 19 mg, Co 53 µg, Cu 103 µg, Zn 373 µg and Mo 4.7 µg. These maximum values do not necessarily reflect the daily requirement of these elements by humans. The concentrations/daily intakes of these elements measured in this study were similar to the majority of values found in the literature for supplements of similar basic ingredient with exceptions mentioned in the paragraph of the relevant element (Avula et al., 2012; Hight et al., 1993; Raman et al., 2004; Leblond et al., 2008; Sivakumar et al., 2007; García-Rico et al., 2007).

As is generally the case, these elements can also be toxic. Some V- and Cu-compounds, Cr in form of its hexavalent salts and Co-metal and alloys are known to be carcinogenic (International Agency for Research on Cancer – World Health Organization, IARC, 2006a & 2012). Regulated by USP (2015) are V and Cu in drugs, Cr is not a safety concern for USP, whereas ANSI (2010) states that hexavalent chromium Cr^{VI} in raw materials used for the production of dietary supplements shall not exceed 2 mg/kg and the daily intake of Cr^{VI} from the finished product shall not exceed 0.02 mg/d. None of the other elements in this group are regulated by either USP or ANSI. For the food supplement industry in the UK, guidance values for Mn, Fe, Co and Zn in vitamin and mineral supplements were developed (EVM, 2003). These levels are only applicable to 3 of the tested supplements since the majority of tested supplements do not fall within this category (Table S1).

The median daily V intake was for algae-based samples 2.2 µg, garlic 0.14 µg, yeast 0.021 µg, fish oil 0.011 µg and krill oil 0.0050 µg (range 0 to 48 µg, Figure 2A) and well below the UL of 1.8 mg/d set by IoM (2010) and the MRL of 0.01 mg/kg/d set out by ATSDR (2009), 0.009 mg/kg/d (IRIS, 2015) and below the levels set out in

USP (2015). Only algae based supplements A4 and A5 contribute between 3 and 6 % to the MRL set by IRIS for vanadium pentoxide all other products contribute less than 1 %. V concentrations in fish oil or fish-based supplements were also determined by Hight et al. (1993) and ranged from <0.1 to 5.7 mg/kg, most of the fish oils determined here contained significantly lower amounts of V, but for no obvious reason some fish and krill oil supplements contained more V than the majority.

The median daily Cr intake was for algae-based samples 1.1 µg, garlic 0.26 µg, yeast 0.15 µg, fish oil 0.55 µg and krill oil 0.031 µg (range: 0 – 116 µg, Figure 2B). Only trivalent chromium (Cr^{III}) is found in food and food supplements naturally (SCF, 2003a), therefore the carcinogenic Cr^{VI} was not considered here and no Cr-speciation was done in this study. RDI values have not yet been set, since the exact role of Cr in the human metabolism is unknown (IoM, 2010). Adequate intake (AI) is estimated to be in the range of 20–100 µg/d (IoM, 2010; SCF, 2003a). Using an average AI of 60 µg/d most supplements contribute less than 10 % to this value, but algae based products A4 and A5 are contributing significantly higher amounts (128 and 194 % of the AI). The guidance value for mineral and vitamin supplements was set to less than 10 mg/d in the UK (EVM, 2003), whereas the WHO (1996) considers intakes below 250 µg/d as safe.

Median daily intake of Mn was for algae-based samples 53 µg, garlic 5.9 µg, yeast 2.7 µg, fish oil 0.14 µg and krill oil 0.15 µg (range 0 – 13 mg, Figure 2C). No RDI for manganese is as yet established. IoM (2010) and EFSA (NDA, 2013a) estimate an AI ranging from 1.8 to 3 mg/d for adults. Estimates for the UL of Mn vary from 2 to 11 mg/d (SCF, 2000a). The majority of tested supplements contributed less than 4 % to the average AI. In the UK the Mn level in vitamin and mineral supplements should not contribute more than 4 mg to the daily Mn intake (EMV, 2003). All of the tested

supplements contributed less than 33% of this value. Use of one fish oil sample (F6) supplemented with manganese sulphate would result in intake of 0.47 mg Mn per day (labelled amount 0.3 mg/d) (~ 20 % AI, ~ 10 % of the guidance level set in the UK for mineral supplements which is in this case applicable). Algae based products A4 and A5 contributed 32 and 53 % resp., of the average AI. Intake of Mn through normal food and water is already relative high (estimate for Europe: 2 to 6 mg/d) and therefore any additional Mn intake may increase the risk of Mn toxicity (esp. neurotoxicity). Chronic intake of supplements F6, A4 and A5 may therefore pose an increased risk especially for consumers with high intake of tea infusions (esp. black tea) (Hope et al., 2006), which contribute significant amounts of Mn themselves, since the overall Mn intake from all sources for these individuals can surpass the UL from normal food intake alone. Also potentially at risk are anaemic individuals, since Mn uptake is increased when Fe stores are low.

Median daily Fe intake was for algae-based samples 486 µg, garlic 44 µg, yeast 15 µg, fish oil 7.5 µg and krill oil 3.8 µg (range 0 – 19 mg, Figure 2D). RDI values for adult men (RDI 6.7-11.4 mg) and women (RDI 15-20 mg) (IoM, 2012; NDA, 2004) are very different due to the regular loss of blood by women. Daily intake of Fe from the studied supplements exceeded in two cases (A4 14 mg and A5 19 mg) the RDI for men and was on the upper end of the RDI recommended for women of reproductive age. For both products Fe intake per serving (6 tablets 3 times daily) is indicated on the label as 3.2 and 6 mg/serving respectively. The calculated intake for A4 was with 4.6 mg 40 % higher than indicated (3.2 mg) per serving, for A5 the calculated intake of 6.3 mg was similar to the one indicated on the label. Fe intake from the majority of studied supplements is below 6 % of the RDI. EFSA decided that not enough data are available to allow determination of a UL (NDA, 2004), a

voluntary limit for supplements of 17 mg/d was set in the UK (EVM, 2003), since both algae supplements are not falling into the category of vitamin and mineral supplements this limit is not applicable to them. In the US IoM (2010) set a safe upper limit for Fe from all sources of 45 mg /d for adults, which none of the supplements alone reaches. Fe deficiency is still a risk especially for women of reproductive age (up to 14 % are estimated in Europe and North America to suffer from Fe deficiency). A small proportion of the general population (~ 1/250 Caucasian), suffering from the genetic disorder hereditary haemochromatosis (HHC) is unable to control intestinal Fe-uptake and therefore at risk from chronic Fe-intoxication, any additional Fe intake from nutraceuticals will therefore increase the risk for this sub-population.

Median daily Co intake was for algae-based samples 1.4 µg, garlic 37 ng, yeast 80 ng, fish oil 15 ng and krill oil 10 ng (range 0 - 53 µg, Figure 2E). The only form of Co which is known to be essential is vitamin B12. Vitamin B12 can only be synthesized by certain bacteria; all other life forms requiring vitamin B12 depend on vitamin B12 synthesized by these. Plants are not known to produce biochemically active B12, but may produce non-active relatives of B12 like pseudovitamin B12. No other biochemically important forms of Co for humans are known so far. Cobalt-ions ingested in any other form may have detrimental health effects and are carcinogenic (IARC, 1991). ATSDR (2004a) calculated a MRL of 0.01 mg/kg/d (= 0.7 mg/d for 70 kg person) for Co. Assuming all Co present was in inorganic form the daily intake from any supplement was below 10 % of the MRL. According to the distributors of A4-A6, the algae contain between 1.8 and 2 µg vitamin B12/serving equivalent to about 0.08 µg Co assuming an average molecular weight of 1349 g/mol for vitamin B12 (specific molecular form not specified on label). The determined Co intake per

serving ranged from 1.1 to 17 μg indicating that considerable amounts of Co would still be present in inorganic form, in case any of the Co present is indeed present in form of biological active vitamin B12 (Dagnelie et al., 1991). According to the label, Y2 contains 15 ng Vit B12/g (determined value via Co content 2 ng Vit B12/g). F20 was spiked with a vitamin preparation containing 2.5 μg Vit B12 ($\sim 0.11 \mu\text{g}$ Co) per serving. The determined amount of Co was about 0.3 μg , indicating either overdosing of vitamin B12 by the manufacturer (not uncommon according to EVM, 2003) or the presence of inorganic Co.

Median daily Cu intake was for algae-based samples 6.9 μg , garlic 2.3 μg , yeast 1.3 μg , fish oil 0.60 μg and krill oil 5.9 μg (range 0 - 103 μg , Figure 2F). The RDI for copper is 0.9-3 mg/d depending on organization setting it (SCF, 2003b, IoM, 2010). There are varying UL values for total Cu intake ranging from 5 – 11.2 mg/d (SCF, 2003b; EVM 2003; IoM, 2010). The daily intake from the supplements is below 6 % of the average RDI. Whereas most literature values were in the same range as found here, Garcia-Rico et al. (2007) found significantly more Cu in one mixed garlic product and Leblond et al. (2008) found significantly higher concentrations for unspecified krill based products, which may indicate natural variation, a significant difference between pure krill oil and other krill based supplements or potentially Cu-contamination during production.

Median daily Zn intake was for algae-based samples 19 μg , garlic 12 μg , yeast 35 μg , fish oil 1.8 μg and krill oil 2.0 μg (range 0 - 373 μg , Figure 2G). The Zn RDI is set between 7 and 11 mg (SCF, 2003c; IoM, 2010), the supplements studied here therefore did not contribute significant amounts to the daily Zn requirement (below 4 %). UL for Zn varies between 25 to 40 mg/d depending on organization setting the value (SCF, 2003c; IoM, 2010). As in the case of Cu, the Zn concentrations found by

Leblond et al. (2008) for krill were significantly higher than those found here in pure krill oils. All other concentrations were in line with available literature values.

Median daily Mo intake was for algae-based samples 1.2 µg, garlic 77 ng, yeast 102 ng, fish oil 22 ng and krill oil 10 ng (range 0 – 4.7 µg, Figure 2H). The AI for Mo is estimated to be between 45 and 100 µg/d with a UL of 600 µg/d (SCF, 2000b; IoM, 2010; NDA, 2013b). Four out of all studied supplements contribute more than 1 % to the AI, all others are well below 1 % of the AI. Mo was detectable in two out of 30 fish oil samples, both of which were fortified with vitamins and trace elements other than Mo. Three out of 19 krill oil samples contained low amounts of Mo.

3.3. Essential non-metallic trace elements

Among the non-metallic elements selenium, iodine and fluorine are known to be essential in traces to humans, bromine is not known to be essential. Of those fluorine was not determined in this study. Selenium is essential in form of Se-cysteine, forming the catalytic centre of several enzymes and iodine is essential for the production of thyroid hormones. Generally the use of algae based supplements resulted in the highest daily intake of all these elements and pure fish oils had the lowest contribution to the daily intake (Figure 3A-C, Table S8a and S8b). The spread within each supplement category is very wide with no correlation between the elements. No concentrations for iodine or bromine in dietary supplements of similar ingredients were found in the literature.

Median daily Se intake from none fortified supplements was for algae-based samples 177 ng, garlic 55 ng, yeast 115 µg, fish oil 75 ng and krill oil 362 ng (range 0 – 14 µg, Figure 3A). The RDI for Se is estimated to be between 26 and 55 µg/d with an UL estimated at 600 µg/d (IoM, 2010; SCF, 2000c). Se content in the same food

items can vary widely depending on geographical origin. Since it is suspected that Se-compounds have anti-cancer properties, it is permitted to use Se-enriched feed for animals in the US (ATSDR, 2003). IRIS classified Se-sulphide as carcinogenic (group B2) but no other Se-compounds (IRIS, 2015). ATSDR (2003) calculated the MRL for Se to be 0.005 mg/kg/d (=350 µg/d). All supplements are below this value. The yeast Se-supplement contributes 237 µg Se/d (~ 585 % of the average RDI of 40.5 µg/d). One fish oil (F10) supplemented with Se contained about 50 % of the average RDI, according to label it is supplemented with 25 µg Se / serving, the measured value was 20.5 µg/serving. Algae based products A4 and A5 contain 15 (resp. 35) % of the RDI and two krill oils (K9 and K10) contain about 10 % of the average RDI. All other supplements contained less than 6 % of the RDI. The Se content of the algae based supplements studied here was significantly higher than that found in the study of Avula et al. (2010) of similar material (max 0.19 mg/kg). This may reflect different geographic origins, but could also indicate that slightly varying growing conditions can have a significant impact on Se-content. Two Spirulina samples in this study came both from Hawaii (same producer) but their Se content was 1.5 and 0.66 mg/kg respectively. Originally it was expected that garlic based supplements would contain relatively high amounts of Se, since garlic is known to accumulate Se in a manner similar to sulphur. But with the exception of G2, none of the garlic based products contribute more than 2% to the RDI. Sivakumar et al. (2007) could not detect Se in fish oil sample above their detection limit (<0.08 mg/kg). Excluding the fortified sample F10 fish oil supplements studied here contained at most 1.9 mg/kg with the majority (18/30) containing undetectable levels of Se. Krill oil supplements on the other hand all contained between 0.1 and 1.5 mg/kg Se. No Se speciation has yet been done in these samples.

Median daily iodine intake was for algae-based samples 7.1 μg , garlic 0.11 μg , yeast 0.026 μg , fish oil 0.11 μg and krill oil 0.46 μg (range 0 – 674 μg , Figure 3B). RDI for I is set to 150 $\mu\text{g}/\text{d}$ (IoM, 2010; NDA, 2014) and a safe upper level is estimated to be 600 $\mu\text{g}/\text{d}$ (SCF, 2002; IoM, 2010). Fortification of food items, especially table salt, is widespread and in certain countries mandatory to reduce the risk of thyroid diseases. In the US, the amount of fortification and the required labelling is regulated by FDA rules (ATSDR, 2004b). For the UK, a guidance level for vitamin and mineral supplements of 0.5 mg/d was developed (EVM, 2003). A MRL of 0.01 $\text{mg}/\text{kg}/\text{d}$ (=7 mg/d) was developed by ATSDR (2004b). None of the studied supplements reaches this level. Three of the marine algae based products (A1, A3 and A7) supply increased amounts of I (186 to 450 % of the RDI), with A7 reaching 674 $\mu\text{g}/\text{d}$. A7 is based on fucus and distributed as support for weight control, with no reference to I made on the label. Iodine intake for A1 and A3 are similar to the level indicated on the labels. The marine algae derived supplements A3 and A7 contain enough iodine, that their use alone leads to I intake at or above the upper safe limit (0.6 mg/d) for I and this is not considering the intake from other sources. Prolonged use of these products may therefore cause thyroid problems for sensitive individuals. Organic iodine compounds may be the source for the relative high I content in fish and krill oil.

Median daily Br intake was for algae-based samples 21 μg , garlic 0.77 μg , yeast 12 μg , fish oil 0.30 μg and krill oil 11 μg (range 0 - 300 μg , Figure 3C). The element is not essential for humans, but naturally present, especially in marine products, accompanying iodine. The Br-species were not determined in the supplements, but are likely to be bromide and organo Br-compounds. Bromate is unlikely to be present naturally (Haag et al., 1983). For bromide an AI was set by JMPR of 0 – 1 mg/kg

body weight (WHO, 2011). There are some values set for organo-bromine /bromine chlorine compounds normally occurring during drinking water purification (e.g. bromoform tolerable daily intake: 17.9 µg/kg; WHO, 2011), but no MRLs for naturally occurring organo-bromine compounds are available. Assuming all bromine present to be bromide, all supplements are well below the acceptable maximal amount and daily intake does not present health problems.

3.4. Non-essential elements for humans

The following elements are not known to be essential to humans: boron, aluminium, arsenic, antimony, cadmium, lead, and were determined in this study. Among these B is known to be essential to plants.

The maximum daily intake for these elements was calculated to be: B 536 µg, Al 4.2 mg, A_{total} 35 µg, $A_{\text{inorganic}}$ 2.1 µg, Sb 243 ng, Cd 494 ng and Pb 1644 ng (Figure 4A-G, Table S9a and S9b). The spread within each supplement category is very wide with no or very little correlation between the content or daily intake of these elements. Of the non-essential elements certain molecular forms of As and Cd are known to be carcinogenic (IARC, 2012). As, Sb, Cd and Pb have relatively low toxicity thresholds in humans. The concentrations found and intakes calculated from these generally agree well with published values for the respective elements (Raman et al., 2004; Sivakumar et al., 2007; Garcia-Rico et al., 2007; Leblond et al., 2008; Dolan et al., 2003; Avula et al., 2010; Hight et al., 1993; Genuis et al., 2012; Heedegard et al., 2013), exceptions are mentioned in the paragraphs about the individual element.

Median daily B intake was for algae-based samples 66 µg, garlic 3.4 µg, yeast 0.92 µg, fish oil 0.77 µg and krill oil 0.70 µg (range < LOQ – 536 µg, Figure 4B). The MRL

for B exposure is estimated to be between 0.13 (Hasegawa et al., 2013) and 0.2 mg/kg/d (ATSDR, 2010). No comparable values for B content or intake from food supplements were found in the literature. Algae based supplements contained more B than other supplements. The use of none of the supplements results in B intake near the MRL levels (all below 6 %).

Median daily AI intake was for algae-based samples 56 µg, garlic 26 µg, yeast 8.0 µg, fish oil 5.4 µg and krill oil 2.7 µg (range 0 – 4.2 mg, Figure 4A). AI is not essential to humans and has in the past been implicated in but not proven as cause of Alzheimer disease (Greger JL, 1993; ATSDR, 2008). The FDA determined that AI used as a food additive and in medication is generally safe and that there is little risk of toxic effects arising from the consumption of individual supplements for healthy individuals (ATSDR, 2008). Negative health effects from a high intake of AI have been mostly observed in humans with kidney disease which can impair AI excretion (AFC, 2008). The use and amount of certain AI compounds for food supplements is regulated within the EU only as far as the levels should adhere to good manufacturing practice (AFC, 2008). MRL for AI from all sources is set by the World Health Organization (WHO) and EFSA to 1 mg AI/kg/week (AFC, 2008; WHO, 2007), level in the US: 7 mg/d and Australia 12 mg/d (Genius et al., 2012). Using a 10 mg/d value and setting a maximum of 10 % of this value as being acceptably coming from a single food supplement, three algae based supplements are significantly above this threshold. All other tested products contribute less than 0.2 % to the daily AI intake following WHO and EFSA guidelines. Compared to values found by High et al. (1993) in 3 fish or fish oil samples (between < 800 and 2900 mg/kg), the values found here are considerably lower.

Median daily As_{total} intake was for algae-based samples 11 μg , garlic 53 ng, yeast 61 ng, fish oil 50 ng and krill oil 5.0 μg (range < LOQ – 39 mg, Figure 4C), the median of $As_{\text{inorganic}}$ was for algae-based samples 834 ng, garlic 32 ng, yeast 54 ng, fish oil 5.0 ng and krill oil 73 ng (range < LOQ – 2.1 μg , Figure 4D). The toxicity of As is strongly dependent on the molecular species present, and not the total concentration. Inorganic As (sum of arsenite and arsenate) is a class I carcinogen (IARC 2012) and the primary As species of concern with regards to toxicity. MRLs for inorganic As have been set at between 0.3 μg and 8 inorganic As /kg/d for chronic exposure (ATSDR, 2007a; CONTAM, 2009b, 2014). Establishing limits for food and feed is complicated by the wide range of As species present depending on food category and their widely varying toxicity (Petursdottir et al. 2015). Attempts are being made to establish limits for inorganic As in certain foodstuffs. The subject is still under scrutiny in the US. In the EU, limits for polished rice of 200 $\mu\text{g}/\text{kg}$ and rice destined for infant food 100 $\mu\text{g}/\text{kg}$ were recently introduced (EU 2015a). WHO/FAO (Codex Alimentarius) set in 2014 a limit for inorganic As in polished rice of 0.2 mg/kg (WHO, 2014). China has implemented regulations for inorganic As in rice (CFSA, 2005) and the UL for dietary supplements was set to 0.3 mg/kg (CSA, 2012). Australia set limits of 1 mg total As / kg for cereals, and inorganic As in crustacean and fish is supposed to be below 2 mg/kg (1 mg/kg for molluscs and seaweed) (ANZ, 2015). ANSI states that raw materials used for the production of food supplements shall not contain more than 5 mg total As/kg and that inorganic As in the finished product should be below 10 μg per daily dose (ANSI, 2010). All studied supplements fulfil these requirements with regard to daily intake. The amount of inorganic As present in some algae based supplements reaches the level set by Australia for edible seaweeds and the level for dietary supplements set by China. This level is also

breached by one garlic based product. All products based on kelp, fucus and *Aphanizomenon Flos-Aquae* are above the limit suggested by ANSI for dietary supplements with regard to total As, but are below the limit for inorganic As per serving. Applying a 10 % limit of the MRL (2.1 µg) for chronic exposure to inorganic As, two of the algae products approach between 80 and 98 % of this value. Two further algae based products contained about 10 % of this value and one garlic based product contained nearly 25 % of this value. The amount of inorganic As in the other supplements was lower. Hedegaard et al. (2013) also detected inorganic As (by HPLC-ICP-MS) in three algae samples and found concentrations between 0.03 and 0.21 mg/kg resulting in a daily intake from 0.07 to 3.5 µg inorganic As, which are similar to our values. The As present in fish and krill oils may not be present be in inorganic form, but some organic lipophilic As may easily be metabolized to inorganic As. Little is as yet known about the metabolism of lipophilic As-compounds, except that there seems to be a very large number of different compounds present in marine biota (Amayo et al., 2013, Raab et al., 2013), some of which may have similar toxicity to inorganic As (Meyer et al., 2014a & b). Two of the 31 fish oil supplements contained detectable amounts of inorganic As. Of the 19 krill oil samples, only two did not contain detectable amounts of inorganic As. Both samples also contained significantly lower total As concentrations compared to the other krill oil samples.

Median daily Cd intake was for algae-based samples 193 ng, garlic 28 ng, yeast 5.2 ng, fish oil 10 ng and krill oil 10 ng (range < LOQ - 494 ng, Figure 4E). Cadmium is toxic especially when inhaled, and it is classed as carcinogenic upon inhalation (IARC, 2012). Oral exposure to Cd does not seem to cause cancer (IARC, 2012). Cd is highly concentrated in the liver and kidneys, where it can cause kidney failure. The

MRL estimates range from 0.1 to 0.8 $\mu\text{g}/\text{kg}/\text{d}$ (ATSDR, 2007b; CONTAM, 2009a; IRIS, 2015). Limits in Australia are set for specific food products resulting in an UL for dietary supplements of 15 $\mu\text{g}/\text{d}$ (ANZ, 2015). EU limits for food are set between 0.05 and 0.2 mg/kg (depending on category); for food supplements the limit is set to 1 mg/kg and for seaweed based material to 3 mg/kg (EC, 2015a). ANSI (2010) states that Cd in raw materials used for dietary supplements shall not exceed 300 $\mu\text{g}/\text{kg}$ and the daily uptake due to the supplement shall not exceed 6 μg . None of the supplements tested were above the legal limit set by the EU for food supplements. Algae based supplement A3 (a mixture of algae species) is at the ANSI limit for raw materials, but not for the finished product. When calculating the MRL for chronic intake using ATSDR's value, the maximum safe Cd intake is 7 $\mu\text{g}/\text{d}$ for a 70 kg person. Some of the algae based supplements tested were found to contribute up to 7 % of this value to the daily Cd intake. Cd values determined here were below values for comparable samples found in the literature.

Median daily Pb intake was for algae-based samples 383 ng, garlic 70 ng, yeast 18 ng, fish oil 28 ng and krill oil 37 ng (range < LOQ – 1644 ng, Figure 4F). No minimal risk levels or tolerable daily intake levels are set either in the USA, Europe or by WHO (ATSDR, 2007d; CONTAM, 2010; WHO, 2011), since Pb does not appear to have a threshold limit under which it can be considered safe. The main risks of elevated Pb intake are neurotoxic complications. Inorganic and organic lead has been categorized as group 2a resp. 3 by IARC (2006b). Lead is also one of the few elements where widespread regulation with regard to lead content in food exists in the EU (food supplements: 3 mg/kg) (EC, 2015a). Australia set limits for lead in food between 0.1 and 2 mg/kg depending on food type (ANZ, 2015). Lead concentrations below 10 mg/kg raw material are required to satisfy ANSI standard 173 (2010) and

the Pb intake from the finished product should be below 20 µg/d. None of the tested supplements had a Pb content of more than 21 % of the permissible level for food supplements as specified in Europe (EC, 2015a). Several of the supplements (especially the algae based ones) contained Pb at levels above the limit set for vegetables and fruit by Australia (food supplements are not specifically regulated by Standard 1.4.1). All supplements satisfy the ANSI requirements: the maximum daily Pb contribution from product A5 was less than 10 % of the level permitted by ANSI. Pb concentrations in algae based supplements were generally lower than those found in the literature.

Median daily Sb intake was for algae-based samples 24 ng, garlic 7.8 ng, yeast 23 ng, fish oil 10 ng and krill oil 5.0 ng (range < LOQ – 243 ng, Figure 4G). The MRL for Sb is estimated to be between 0.4 and 6 µg/kg/d (ATSDR, 1992; WHO, 2011). None of the tested supplements contributes significant amounts of Sb to the daily overall Sb intake.

4. Summary

Element content and intake in the studied supplements generally decreased in the order algae > garlic / krill oil > yeast > fish oil. The concentration of essential trace elements is in most cases insignificant. Use of these supplements therefore does not contribute significantly to the recommended daily intake of essential elements. There are some exceptions to this, the consumption of two of the algae based samples leads to Fe intake above the RDI for men and were near the maximum RDI for women, with the label of one of the samples indicating ~ 40 % less Fe than actually present. The use of two other products leads to iodine intake at levels above the RDI as indicated on the labels and the Se-supplement results in intake of about 4 times

the RDI. One algae based supplement intended as aid for weight loss does not indicate any trace element levels on the label, but does contain iodine at levels ~5 times above the RDI. The chromium intake of two algae based products was found to be significantly above the AI.

The daily intake of non-essential elements was for all supplements generally below the permissible limits. For inorganic As and total As some products based on marine algae and one garlic based product breached the limits set for food supplements in China resp. Australia. The use of three of the algae based supplements results in daily AI intake of more than 10 % of the daily safe level. All other elements are present in concentrations well below the MRL or UL.

6. Conflict of interest

The authors declare no competing financial interest.

5. Acknowledgements

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Captions

Figure 1: alkali and earth alkali elements; daily intake, RDI/AI and MRL where available for A) Rb, B) Cs, C) Mg, D) Ca, E) Sr and F) Ba; box whisker plot showing 25th and 75th percentiles, with a line at the median and error bars defining the 10th and 90th percentiles, outliers are marked by dots, x-axis: in brackets number of supplements above LOQ / total number of supplements in category.

Figure 2: essential metallic elements daily intake, RDI/AI, MRL and legal limits where available A) V, B) Cr, C) Mn, D) Fe, E) Co, F) Cu, G) Zn and H) Mo; box whisker plot showing 25th and 75th percentiles, with a line at the median and error bars defining the 10th and 90th percentiles, outliers are marked by dots, x-axis: in brackets number of supplements above LOQ / total number of supplements in category.

Figure 3: essential non-metallic elements daily intake, RDI, MRL and legal limits where available A) Se, B) I and C) Br; box whisker plot showing 25th and 75th percentiles, with a line at the median and error bars defining the 10th and 90th percentiles, outliers are marked by dots, x-axis: in brackets number of supplements above LOQ / total number of supplements in category.

Figure 4: non-essential elements daily intake, MRL and legal limits where available A) Al, B) B, C) total As, D) inorganic As, E) Cd, F) Pb and G) Sb; box whisker plot showing 25th and 75th percentiles, with a line at the median and error bars defining the 10th and 90th percentiles, outliers are marked by dots, x-axis: in brackets number of supplements above LOQ / total number of supplements in category.

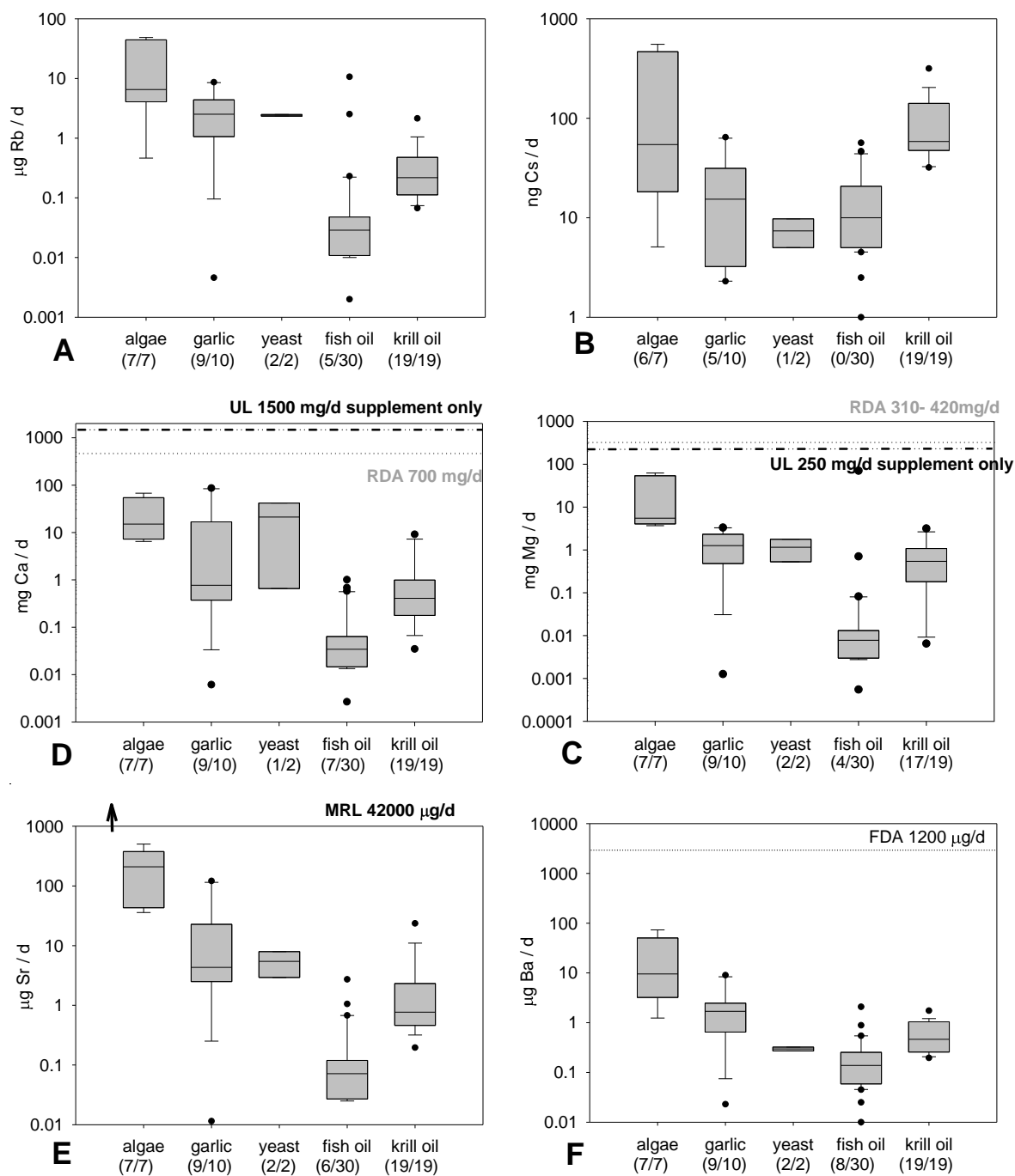


Figure 1

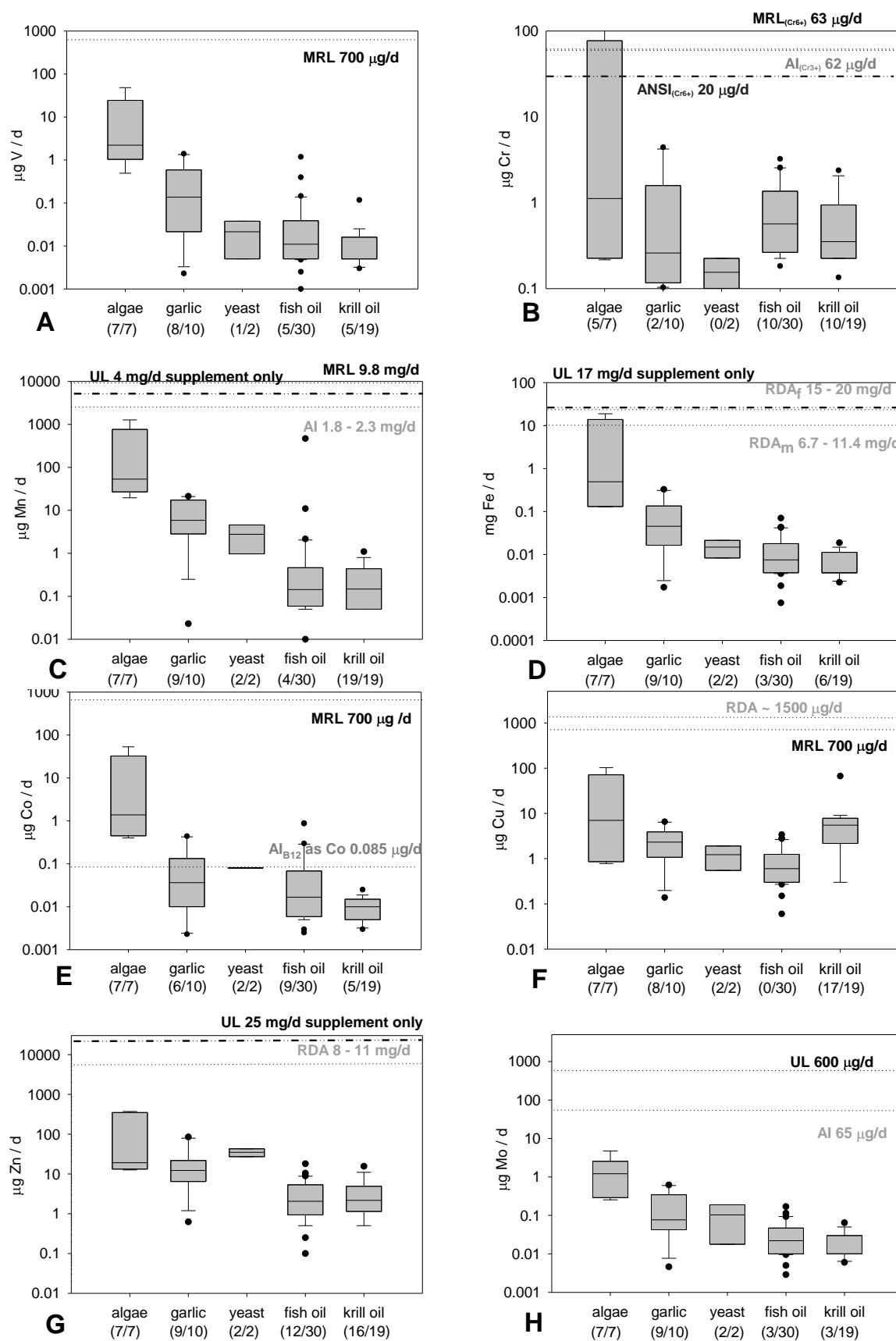


Figure 2

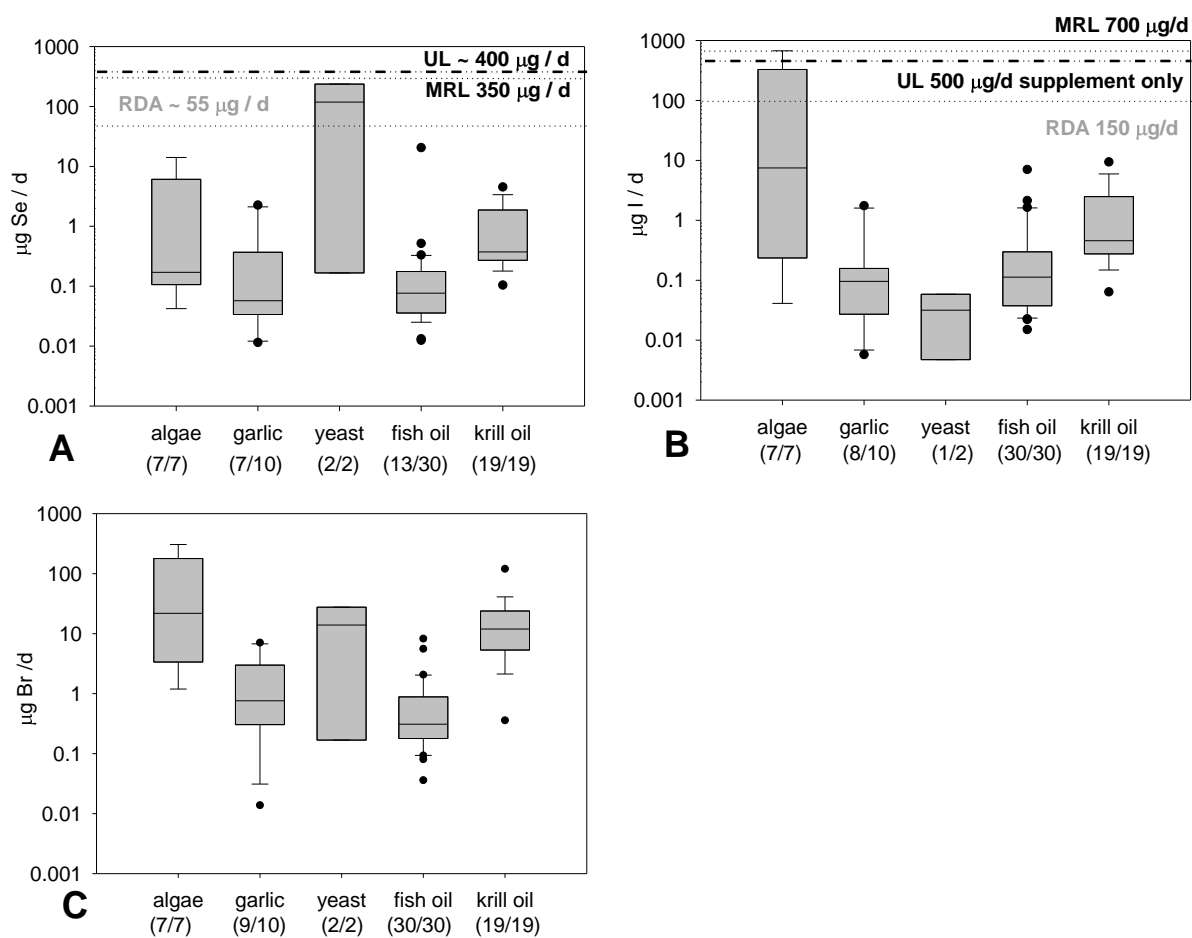


Figure 3

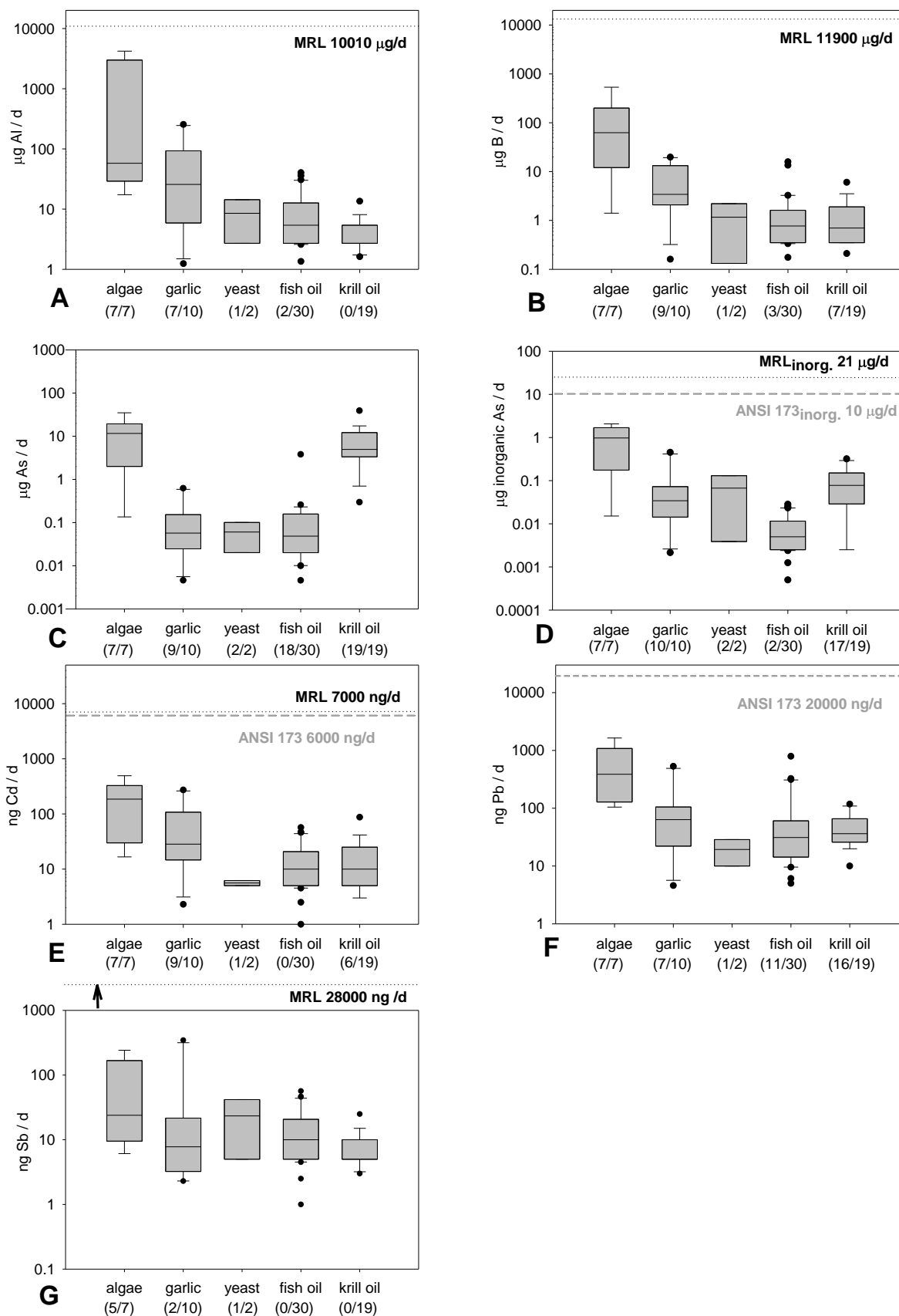


Figure 4