Correlations in the presence of contaminants in *Ulva lactuca* spp

Part of KB Project on Marine lower trophic systems (KB-34-2c-4)

S.T. van Tuinen, A. Gsell, E.J. Faassen, M.D. Klijnstra, A.A.A. Beerman

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Preface

- Seaweed is gaining popularity and economic importance as an ingredient in (novel) foods and is now commonly consumed across all population strata in the Netherlands. Moreover, seaweed may also provide a sustainable source of plant-based protein for human and animal provisioning.
- Food safety of seaweed is an important condition for bringing seaweed containing food products on the market. However, previous studies have shown that seaweeds can accumulate high concentrations of arsenic, although there is high variation among seaweed species, geographic location, harvesting season, seaweed metabolic activity, and cultivation method.
- Arsenic in seaweed is of concern due to its toxicity. Furthermore, literature and WFSR research has shown that arsenic concentrations in seaweed correlate with the availability of the nutrient phosphate. Furthermore, the flow in which the nutrients are added to the tanks may also influence the uptake and growth of seaweed.
- The aim of this project was to assess the effects of different nutritional conditions on the contaminant uptake in *Ulva lactuca spp* through: (1) adjustments in the water flow; (2) adjustments in the nutritional composition of the water flow. Furthermore, the project reviewed the comparison between the standard analysing method of ICP-MS and an alternative way using the XRF. Additionally, the correlations between various heavy metals and iodine within *Ulva* were reviewed.

Summary

Seaweed is now relatively commonly consumed across all population strata in the Netherlands and may provide a sustainable, plant-based protein source for humans and animals. Previous studies have revealed that seaweeds can accumulate high concentrations of iodine and heavy metals, although with high variation among seaweed species, geographic location, harvesting season, seaweed metabolic activity, and cultivation method. Food safety of seaweed is an important condition for bringing seaweed food products to the market. Better understanding on the uptake and metabolism of total arsenic is necessary for advising the industry towards a production of seaweeds that are safe for human consumption.

In the first three years of this KB project a broad screening of possible food safety risks on fresh seaweed and seaweed based animal feed has been performed. In the last year of the KB project the focus has been put on combining data collected in several projects within WFSR considering contaminants such as the heavy metals and iodine in *Ulva Lactuca spp*. This increased our knowledge about the ratio's found between the different contaminant levels, and contributes to understanding the complex uptake mechanisms of seaweeds in general.

Therefore, several experiments and data analysis exercises were performed within this project. One of the experiments concerned the manipulation of the nitrogen to phosphorus ratio available to *Ulva lactuca spp*, an experiment that exposed *Ulva lactuca spp* to two different water flow schemes. In the second one, an experimental test set-up was designed for future experiments with addition or depletion of nutrients, that can prove our current theories about the uptake of arsenic in seaweeds.

The experiments performed on *Ulva lactuca spp* give good insight in the possibilities of optimizing the set-up for future experiments which are foreseen for the brown seaweed species *Saccharina Latissima*, and where the uptake of arsenic and iodine is one of the show stoppers for a large scale market introduction in the Netherlands.

The different nutritional conditions of the *Ulva lactuca spp* in the mesocosms resulted only in slightly different concentrations of arsenic, heavy metals and iodine in the seaweed harvested. The scenario in which nutrients are added at a regular flow rate (2 L/min water) showed a statistically enhanced concentration for the analytes nickel, mercury, iodine and bromine. Moreover, iodine and mercury showed statistically enhanced concentrations within the scenario that the flow rate of the water was enhanced from 2 L/min to 25 L/min. Lastly, no statistical differences were found in the concentrations of the analytes when the high flow rate scenario (25 L/min) was compared with the scenario where nutrients were added. The higher flow of 25 L/min translates into a higher flow of nutrients that continuously passes through the tanks, which may level out the effect of the first scenario.

From the experiments performed it is concluded that before starting additional experiments an acclimatization period before starting the experiment of four to six weeks is advised. Besides this, the experimental set-up can be improved by monitoring weekly some relevant parameters like the weight of the seaweed growing in the mesocoms, and the concentrations of the nitrate and phosphate levels. The XRF technique can help to reduce costs during this experiments significantly. The experiments performed have resulted in an optimized monitoring strategy for future addition or depletion experiments. Finally, in future experiments it is advised to make use of triplicate test set ups for every addition or depletion experiment. This can lead to increased statistical relevance of the experiments.

Considering the data analysis part of the project reviewed the results obtained with standard analysing method of ICP-MS and an alternative way using XRF technique. Comparing the analysis results obtained with ICP-MS and XRF techniques linear correlations and uniform correlation slopes were found for the concentrations of arsenic, lead, mercury, cadmium versus the iodine concentrations. This provides great opportunities to develop a screening method with the XRF technique for certain analytes within

Ulva lactuca spp. It is expected that this will also be valid for other seaweed species, although additional experiments should confirm this.

The concentrations of the various heavy metals and iodine within *Ulva lactuca spp* were also evaluated on time dependency on time and on location dependency. Also here good correlations were observed: almost identical ratio's were found for heavy metals concentrations of arsenic, lead, mercury, cadmium when they are compared to the iodine concentrations. This means that when one of the concentrations of these elements (e.g. arsenic) is measured, the amount of other elements like lead, mercury, cadmium and iodine can be predicted quite well. These observations provide great insight in the uptake mechanisms of contaminants in *Ulva lactuca spp*, and can lead to lowering the operational costs for seaweed farmers significantly by analysing their seaweed during the growth on the presence of contaminants with the XRF technique. Also the moment that contaminant concentrations found in the seaweeds cultivated tend to increase significantly can be determined much more easily, which can result in the decision to harvest the seaweeds earlier or later than planned originally.

If the research performed could be expanded and extrapolated to other cultivation areas in national or international waters, the XRF technique could be used to predict the presence of heavy metals and iodine accurately and cost very effectively.

This could lead to a better and low-cost method for coping with possible food safety issues on several seaweed species, and a large scale market introduction of some edible seaweed species in and outside the Netherlands.

1 Introduction

1.1 Seaweed and seaweed consumption in the Netherlands

The term "seaweed" covers several taxonomic groups of marine, macroscopic photosynthetic algae. Based on their pigmentation, seaweeds are commonly classified into brown (Phaeophyceae), green (Chlorophyceae) and red (Rhodophyceae) seaweeds. Despite the overarching term "seaweed", there are considerable morphological and functional differences within and between these seaweed groups (Holdt and Kraan 2011). There are more than 12,000 species of seaweed, of which ca 220 are considered of commercial value (FAO 2018) which are either harvested from wild stocks or cultivated in aquaculture. Global seaweed production in aquaculture has increased steadily to 34.7 million tonnes in 2016 (including some microalgae production, FAO 2018) while harvesting of wild stocks has remained around 1.1 million tonnes (FAO 2022). Only about 10 seaweed species are cultivated intensively, with the brown algae *Saccharina japonica* and the red algae *Eucheuma* sp accounting for 66% of the global production in 2015 (Campbell et al. 2019). The major producers of cultivated seaweeds are China, Indonesia and further Asian countries, while European production is still emerging and contributes only about 1% to the global production (FAO 2018, 2022). The global market for algae-based products is expected to keep growing at an annual rate of 5.9%, reaching a projected USD 6.3 billion? by 2028 (based on a market research study by Credence Research 2022). This trend is also observed in Europe (CBI, 2023). One of the key drivers of this growth is the increasing demand for nutraceuticals and pharmaceuticals based on microalgae and seaweed biomass for human food supplements and animal feed additives (Credence, 2022, Van Hassel 2022). Hence, the sector is set to grow as the EU is one of the biggest importers of seaweed products globally, and the European Commission adopted a communication detailing a 23-actions plan to help the European seaweed sector to grow into a robust, sustainable and regenerative sector (European Commission, 2022).

Seaweeds have a long history as human food or animal feed. Human consumption of seaweeds has historically been most relevant in Asian countries, but has been increasing world-wide in recent years (Dawczynski et al., 2007; FSAI, 2020). Also in the Netherlands, the direct consumption of seaweed is now quite common across all socioeconomic strata and age groups of the population (Dinnissen et al. 2022). A survey by the RIVM detected that almost half of the respondents occasionally consumed seaweed in the form of (novel) food products containing fresh or dried seaweed, and the median daily intake in seaweed users amounted to 0.05 (95%-CI 0.04-0.06) g wet weight kg^{-1} human bodyweight, resulting in about 4g d^{-1} for an 80kg person (Dinnissen et al., 2022). In comparison, daily per capita seaweed consumption is estimated at 4g d^{-1} in Japan, 5.2g d^{-1} in China, and 8.5g d^{-1} in South Korea (Roleda et al., 2019). In the Netherlands, in addition to more established food products containing seaweed such as sushi, there is now is a wide range of novel(ty) food products available that are based on or contain seaweed. This products range from highly visible examples such as seaweed meat replacers and seaweed pasta, to products with seaweeds as flavouring agent such as seaweed cheese, seaweed mayonnaise, seasoning mixes, and even beers and liquor with seaweed (van den Burg et al., 2021, Banach et al., 2022).

Seaweeds also have a long history of use as livestock feed in the form of fodder of meal, particularly in coastal regions (Kadam et al., 2015). Generally, green and red seaweeds contain higher protein ratios but lower mineral ratios than brown seaweeds, although the specific composition strongly depends on time and location of harvest as well as on the environmental conditions during the growth phase (Banach, Hoek-van den Hill et al., 2020). Due to their relatively high protein contents, complex carbohydrates and the presence of polyunsaturated lipids, seaweeds in animal feed can contribute to the nutrient, protein and energy demands of livestock (Makkar et al., 2016). Moreover, prebiotic compounds produced by seaweeds (complex carbohydrates) has been shown to contribute to gastro-intestinal health and improve immune status of monogastric livestock, including pigs, chicken and fish (Makkar et al., 2016, FAO 2022). Supplementation of livestock diet with red or brown seaweeds can substantially reduce methane emissions in cows (Lean et al., 2021). The red seaweed *Asparagopsis taxiformis* has been highlighted as beneficial in this regard (Roque et al., 2020; Kinley et al., 2020), although there are yet challenges to be overcome as, for example, the active compound (bromoform) is known to be toxic and may be transferred to milk in lactating cows (Muizelaar et al., 2021).

Given the need to feed a growing world population and to mitigate the effects of climate change on food production, seaweed is perceived as a sustainable crop with a potential to support the blue carbon initiative and be part of the protein transition (FAO 2022, Banach et al., 2022). Generally, seaweed does not directly transfer carbon to the marine sediments, hence it remains unclear how large the contribution of increased seaweed farming can be on net carbon sequestration (Bindoff et al., 2019). However, seaweed farming or ocean afforestation may contribute to the mitigation of carbon emission when employed as alternative energy source, although full lifecycle analyses would be needed to assess upscaled, real-world net effects (Bindoff et al 2019). Moreover, (offshore) seaweed cultivation can promote healthy ecosystems by taking up nutrients (and thereby reduce the eutrophication levels in e.g. the North Sea), by providing habitat for invertebrates and young fish (thereby increasing biodiversity) and by oxygenating water and increasing the pH (thereby counteracting acidification). On the other hand, intensive farming can also have negative ecological impacts due to too much nutrient competition, increased traffic and risk formed by the seaweed cultivation installations themselves (Tonk and Jansen, 2019). Protein content varies widely across seaweed species and ranges from 0.7 to 45% of its dry weight, with red seaweeds showing the highest protein contents (Cherry et al., 2019, Banach et al., 2022). However, while these protein contents can be comparable to those of beef, the seaweed consumption is still comparatively rather small (Cherry et al., 2019). Moreover, the digestibility of proteins of raw seaweed is variable (Cherry et al., 2019) in some species it can even be relatively low and pre-processing before consumption may be necessary to make the proteins more bioavailable (Juul et al., 2022).

1.1.1 *Ulva* spp

Ulva is a genus in the green seaweeds that belongs to the division Chlorophyta and is also known under the common name "sea lettuce" (Figure 1). It grows worldwide, most species in this genus are marine but a few freshwater dwelling *Ulva* species also exist. Overall, *Ulva* is fairly tolerant to a wide range of light, temperature and salinity conditions (Mantri et al., 2020). When macronutrients are readily available, *Ulva* can reach growth rates of 30-50% per day (Mantri et al., 2020) and develop large biomasses within short time leading to green tides (Morand & Merceron, 2005). The plant is a flat, thin (two layers of cells thick) algae growing from a disc-shaped holdfast. In nature it often attaches to rocks or other algae in littoral or sublittoral zones. In cultivation settings it is seeded or clonally propagated into outdoor tank facilities or in the open sea (Mantri et al., 2020). Due to its fast growth potential and high protein to mineral contents, *Ulva lactuca* has been identified as a promising species for commercial farming to produce food, animal feed and other products such as refined proteins or fertilizer (Lubsch, 2019; Critchley & Ohno, 1998, Sahoo, 2002, Thangaraju, 2008, Holdt & Kraan, 2011). Moreover, it has also been used as biofilters in water treatment facilities and integrated as a component in multi-trophic aquaculture systems (e.g., Cohen & Neori, 1991, Neori et al., 2004).

Figure 1 *Ulva underwater (from https://www.nordicseafarm.com/ulva-farm).*

1.2 Arsenic in seaweed

Seaweeds have a documented capacity to accumulate potentially harmful contaminants, among which also arsenic (FAO 2022), warranting a need to better understand potential food and animal feed safety hazards of seaweeds (Banach et al., 2020). In seaweeds, the concentration of iodine and, to some extent of heavy metals varies with seaweed species, geographic location, harvesting season, seaweed metabolic activity, and cultivation method (Cherry et al., 2019, Karthick et al., 2012). Due to their capacity to accumulate metals, seaweeds have also been used as sentinel species to assess metal pollution in estuaries and coast lines (Morrison et al., 2008, Phillips, 2018) and are assessed as bioremediation agents to remove heavy metals from polluted locations (Znad et al., 2022). In general, heavy metal concentrations in seaweeds depend on the environmental concentrations of these elements and on the abiotic environmental conditions (e.g., salinity, light, temperature, etc), and on structural differences among seaweeds (Malea et al., 2015) that affect uptake capacity and storage of metals (Besada et al., 2009). Overall, green seaweeds have a lower metal-binding capacity than brown seaweeds and metal concentrations in seaweed tissues are lower during the active growth phase in summer and higher in periods of low metabolism in winter (Besada et al., 2009).

While there is European Union legislation regulating the maximum amounts of some of the heavy metals in seaweeds used in or as feed (organic and total arsenic, cadmium, mercury and lead) and in food supplements mainly made of seaweed (cadmium, lead and mercury), there is no European or Dutch legislation regarding food safety of seaweeds or definition of threshold concentrations for human consumption-safe seaweed (FAO 2022).

1.2.1 Total arsenic and inorganic arsenic species

Arsenic occurs in various forms, both organic and inorganic, whereby the inorganic forms are generally considered as the greater public health concern. Arsenic species in the marine environment have natural and human sources. Anthropogenic arsenic comes from diverse sources, such as mining activities, the use of pesticides, and wood preservatives. In seawater, arsenic predominantly presents as arsenate (As(V), (AsO_4^{3-})) and occurs generally in the range of a few µg L^{-1} (UNEP GESAMP, 1988; Li et al 2021). Additionally low concentrations of the organic arsenic species monomethylarsonic acid (MMA) or dimethylarsinic acid (DMA) are present through excretion from marine organisms (Li et al 2021). Arsenic can accumulate in seaweeds with concentrations up to 150 mg kg^{-1} in dry products (summarised in Cheyns et al 2017) or to 2000-5000 times greater than the surrounding seawater (UNEP GESAMP, 1988). In the brown seaweed *Hizikia fusiformis*, in particular, high inorganic arsenic concentrations have been reported, ranging from 30 to 117 mg kg^{-1} dw (summarised in Cheyns et al 2017, see also Besada et al 2009). Some of the inorganic arsenic species are classified as a known human carcinogens by the EPA (Hughes 2002) although the exact mechanism of arsenic carcinogenicity seems not yet to be understood.

1.2.2 Uptake mechanisms for arsenic

In higher plants, physiological studies have shown that arsenate and phosphate share the same transport pathway through transmembrane phosphate transporters, whereby the strongly conserved PHT1 family of phosphate transporters is localised across the plant cell membrane and involved in the acquisition of inorganic P from the environment (Wang et al 2017). To our knowledge, there are no physiological studies on the presence of these phosphate transporters in seaweeds, but given the highly conserved structure of PHT's across higher plants (Roch et al 2019), and their structural similarity to phosphate transporters in yeasts (Wang et al 2017), it is likely that the phosphate uptake in seaweed also occurs through transporters of the PHT family. The transporter family involved in phosphate uptake (Pht1) encompasses more than 100 transporters which vary in their uptake affinity and kinetics (Zhao et al, 2021). Due to the structural similarity between phosphate and arsenate, these transporters cannot discriminate between these two compounds and some have even been shown to have a higher affinity for arsenate than phosphate (Zhao et al 2020). Nevertheless, increased environmental phosphorus concentrations decrease accumulation of inorganic arsenic in seaweed although the underlying mechanism is not yet understood (Lin et al 2021). Uptake of arsenite (AS(III)) occurs mainly under anoxic conditions and therefore may play a smaller role in seaweeds cultivated in well oxygenated waters. Iron-rich plaques on the surface of plant cells have strong adsorption potential for both, phosphate and arsenate, and the thereby may increase the uptake of arsenate

(Al Mamun et al 2019). In addition to phosphate-dependent uptake pathways, there are also reports of phosphate-independent uptake of AS(V), suggesting the presence of additional, other uptake mechanisms in algae (see Al Mamun 2019). Arsenic disturbs ATP production and hence the plant's energy metabolism through multiple pathways. Amongst other pathways, arsenic competes with phosphate whereby arsenate uncouples the oxidative phosphorylation, inhibiting mitochondrial respiration and ATP synthesis, resulting in ATP depletion and cell necrosis (Hughes 2002). Seaweeds can detoxify arsenate, but when exposed to high levels of inorganic arsenic, their biotransformation capacity may reach a limit and arsenic accumulates in the organism (Cheyns et al 2017). In higher plants, once taken up, AS(V) is reduced to AS(III), methylated to MMA and finally presents as (non-toxic) arsenosugars (Zhao et al, 2021). This detoxification pathway can, however, be disturbed at too high environmental arsenic concentrations or under low environmental phosphorus concentration conditions.

1.2.3 Ecological stoichiometry and changes in environmental N:P ratios.

Phosphorus is an essential macro-element for life. Plant growth depends on the availability of inorganic dissolved phosphate (DIP) which plays a key role in many crucial processes such as reproduction (shown in Figure 2) and somatic growth (DNA, RNA), cellular compartmentalization (membrane lipids), energy metabolism (ATP), and phosphorylation-based signaling mechanisms (book Elser and Sterner).

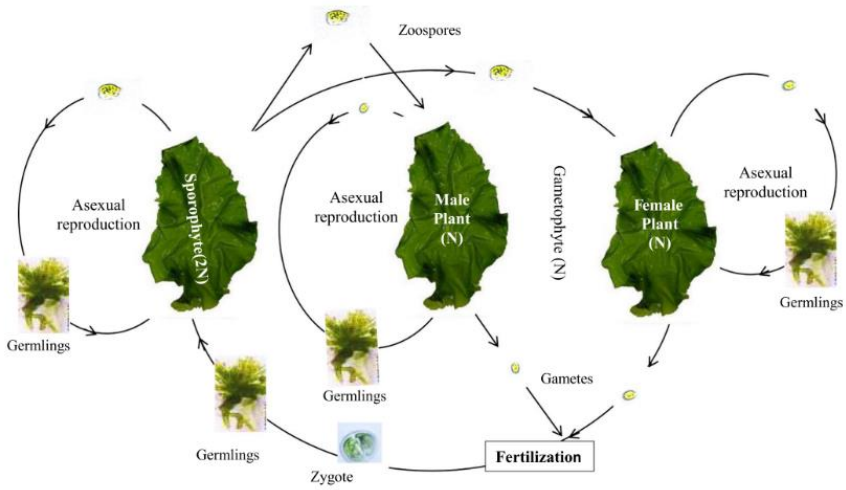

Figure 2 *Lifecycle of Ulva with asexual and sexual reproduction (Mantri et al 2020).*

Organisms have a preferred elemental ratio for optimal functioning, described by the Redfield Ratio (Redfield 1948). For somatic growth, a plant needs the elemental building blocks but also all building blocks that allow regulation and execution of protein synthesis. Seaweed tissues consist, on average, of about 10-50% carbon, 0.2-4.2% nitrogen, and 0.1-0.5% phosphorus per unit dry weight, resulting in a high C:N:P ratio of 800:49:1 (Duarte 1992), although these values strongly vary with species, age, tissue type and environmental conditions. Notably, the seaweed demands for balanced growth differ markedly from the overall C:N:P supply of seawater (106:16:1; Redfield, 1934). Overall, nitrogen is the most limiting nutrient in seawater, usually followed by phosphorus (Tyrrell 1999).

Oceans have traditionally been considered nitrogen limited, but this view has been challenged in recent years by reports of phosphorus limitation in various marine environments (Burson et al 2016). Despite its abundance in the environment (ranked as the 11th most abundant element), phosphorus is neither easily accessible nor evenly distributed in oceanic and coastal surface waters (Martiny et al 2019). Large scale spatial patterns of DIP distributions show higher DIP in high latitudes, low DIP in subtropical gyres and intermediate DIP levels in coastal and equatorial upwelling regions (Martiny et al 2019). In coastal regions, local DIP concentrations are further influenced by run-off waters of rivers carrying DIP from anthropogenic land use activities (Lubsch 2019). Phosphate rich waters at the coast can cause mass developments of opportunistic seaweeds such as *Ulva* spp, also called "green tides". Such green tides can cause several environmental issues, including rotting of beached seaweed biomass on shores, hindering shore-based activities (Lubsch 2019). Moreover, as seaweed biomass sinks and degrades, it can cause hypoxia in the water and promote microbial production of hydrogen sulfide with potentially dramatic consequences for the benthic and pelagic life (Lubsch, 2019). In the North Sea, the distribution of phosphorus is strongly determined by riverine inputs of dissolved nutrients from wastewater and agricultural run-off (Burson et al 2016). From the 1960s to mid-1980s, riverine inputs delivered high loads of DIN and DIP leading to coastal eutrophication and associated environmental problems (Burson et al 2016). Due to measures to reduce river nutrient loads since the mid-1980s, total P inputs were reduced by 50-70% while total N inputs were only reduced by 20-30%, leading to a shift in nutrient ratios in the coastal waters (Burson et al 2016). Winter DIP concentrations over 2004-2016 show that DIP concentrations are higher along the coastal regions with strong riverine influence than further offshore (Figure 3, OSPAR). However, since riverine DIN inputs are not reduced at the same rate as DIP inputs, the observed inorganic nitrogen to phosphorus (N:P) ratio in spring is increasing and showing a strong offshore gradient from 375:1 nearshore to wards 1:1 N:P in the Central North Sea (Burson et al 2016).

Despite the health risks associated with arsenic and potential for animal and vegetable seafood borne exposure to arsenic, there is little documentation of arsenic concentrations and dynamics in the North Sea, for example, arsenic is not included in the OSPAR North Sea Quality Status Reports. However, waterborne inputs of heavy metals cadmium, lead and mercury have been strongly decreasing between 1990-2006 (https://qsr2010.ospar.org/en/ch05.html), making it probable that also riverine arsenic inputs may have been decreasing in this period.

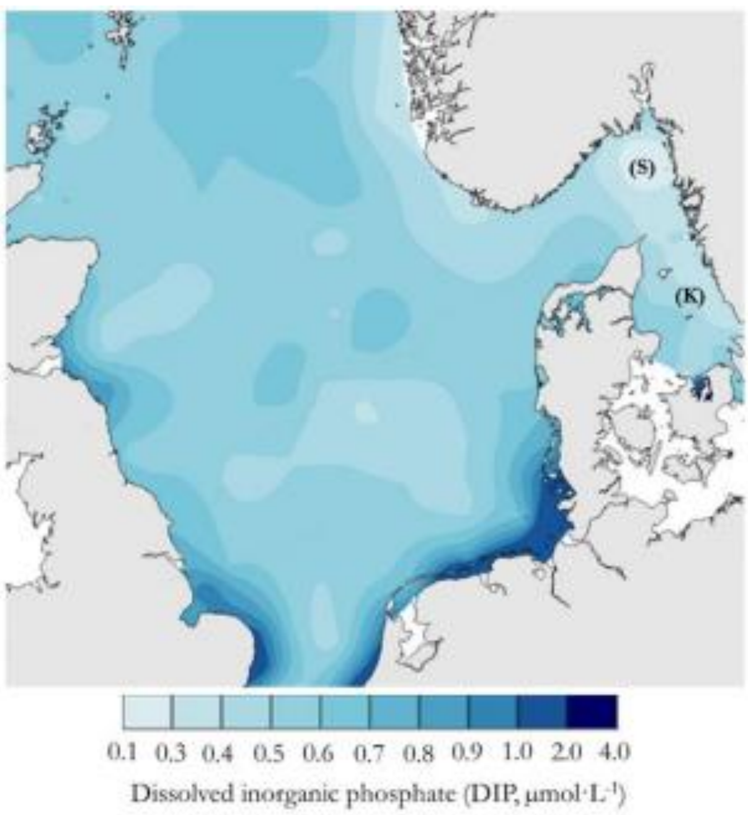

Figure 3 *Distribution of average winter DIP concentrations (µM) in the North Sea (2004-2016). Source: https://oap.ospar.org/en/ospar-assessments/intermediate-assessment-2017/pressures-human-activities/eutrophication/nutrients-concentrations/.*

1.3 Research questions and approaches

Within the framework of the KB project "Circular and climate neutral society" Wageningen Food Safety Research (WFSR) works on the topic "Food Safety by Design". From field experiments and environmental observations it is known that the accumulation of heavy metals, iodine and arsenic in fresh seaweed material and consumer products can be considerable, raising concerns over the food safety of seaweed based products. Since the uptake of arsenic seems to depend on the uptake and availability of phosphorus in the environment, this may provide an opportunity to design seaweed cultivation in a way that reduces the accumulation of arsenic. In this design ecological knowledge is used about how changes in the environmental conditions (decreasing absolute amounts of phosphorus in conjunction with changing elemental rations) can possibly affect the accumulation of arsenic and other elements in the green seaweed *Ulva lactuca spp.*

Here we compile the results of two experimental studies manipulating the nitrogen to phosphorus ratio available to *Ulva lactuca spp* and a comparison between elemental analysis methods. The first experiment exposed *Ulva lactuca spp* to two different water flow schemes, whereby faster water flows allow higher refreshment of nutrients and therefore result in a higher nutrient availability to *Ulva lactuca spp*. The samples of this experiment were measured with the standard method using an ICP, but also with X-ray fluorescence (XRF).

The research questions to be tackled here are as follows:

- Do the different nutritional conditions of the *Ulva lactuca spp* in the mesocosms result in different concentrations of arsenic, heavy metals and iodine in the seaweed?
- Do the heavy metal and iodine concentrations show linear correlations and uniform correlation slopes?
- What are the optimal monitoring conditions of cultivation experiments to be able to explain the underlying uptake mechanisms of nutrients and contaminants?

Ideally, our results will allow to assess how nutrient conditions, namely an increase in absolute amounts of N and/or P as well as changes in N:P ratios, affect the observed concentrations of arsenic, heavy metals and iodine in the green seaweed *Ulva lactuca* spp. Moreover, our results will indicate whether elements within *Ulva lactuca spp* show homeostasis (retain the same ratio) across nutrient treatments and across time.

2 Experimental set ups

2.1 Premisses of the two experiments

Here we report on two separate seaweed growth experiments that assessed the influence of growth conditions on the concentration of contaminants in the seaweed. In both experiments, the green seaweed *Ulva lactuca spp* was exposed to different nitrogen (N) and phosphorus (P) regimes:

a. Cultivation experiments under contrasting flow regimes:
   Velocity of water flow affects how fast nutrient are replaced and thereby are a way to regulate nutrient ratios. Of particular interest would be linear relationships between contaminants that would allow prediction of contaminant occurrence and concentrations through observation of indicator or sentinel contaminants.
b. Cultivation experiments under contrasting nutrient ratios:
   Through addition of concentrated N and P stocks to mesocosms allows manipulation of absolute amounts and ratios of nutrients.

2.2 Cultivation experiments under contrasting flow regimes

2.2.1 Background

Seaweeds have repeatedly been shown to take up and accumulate contaminants such as arsenic in concentrations that are far above the observed environmental levels. Moreover, as uptake of phosphate and arsenate occur through the same uptake pathways, the accumulation of arsenate is likely closely linked to uptake of phosphate. Some studies suggest that arsenate uptake is higher under low environmental phosphate conditions and relatively lower in high environmental phosphate conditions. Moreover, little is known about relationships between in-plant concentrations of contaminants.

Plants, as all other organisms, need carbon, nitrogen, and phosphorus to maintain photosynthesis as well as growth, hence regulating production (Roleda and Hurd 2019, Sterner and Elser book). To achieve optimal growth, seaweeds require nutrients in certain ratios (ecological stoichiometry) which is often markedly different from the nutrient ratios encountered in the seawater, resulting in an imbalance between supply and demand. According to Liebig's law of the minimum the nutrient available in the smallest quantity respective to the demands of the seaweed will limit its growth (see Roleda and Hurd 2019). However, if the limitation by a particular nutrient is resolved through increased supply, then a different nutrient may become limiting, leading to higher uptake efforts by the seaweed. For example, alleviating nitrogen limitation in the brown seaweed *Fucus vesiculosus* triggered an increase in the uptake of phosphate (Perini & Bracken 2014), but this co-limitation was not observed in the green seaweed *Ulva lactuca* (Lubsch and Timmermans, 2017). Since arsenic is taken up through the same pathway as phosphate, changes in the absolute amounts of phosphate but also in the nitrogen-to-phosphate ratio may influence the uptake and storage of arsenic in seaweeds depending on group and species.

Several abiotic factors can change the rates of nutrient uptake, notably water motion, light, temperature, salinity, and desiccation. Water motion is a major driver of nutrient supply. Increased water flow provides increased supply of nutrients as nutrient depleted water is replaced by nutrient replete water. Moreover, increased flow also affects the thickness of the diffusion boundary layer around the seaweed surface as increased shear lowers the thickness of the boundary layer and increases supply of nutrients to the plant (Roleda and Hurd 2019). Additionally, also biological factors affect nutrient uptake rates, amongst others life stage and age class of the seaweed, with older fronds and lamina of seaweeds showing lower nutrient uptake rates reflecting their generally low physiological activity (Roleda and Hurd 2019).

In addition to these macronutrients, plants need a range of further elements for growth and further also accumulate elements that are of no added value or even detrimental to plant health. Particularly for this last group there is little to no information of the absolute and relative ratios in seaweed tissues.

2.2.2 Experimental design and sampling

At the Wageningen Marine Research (WMR) site in Yerseke, six open mesocosms were installed on land. In each of the mesocosms, 1 kg of *Ulva lactuca spp* was planted. *Ulva lactuca spp* was obtained from an external party and was retrieved from the waters of the Eastern Scheldt in Zeeland.

Three treatments were applied to these six mesocosms, three different nutritional scenarios were applied in duplicate:
- Two mesocosms with a flow of 2 L min^{-1} (control);
- Two mesocosms with a flow of 25 L min^{-1} (high flow);
- Two mesocosms with a flow of 2 L min^{-1} with addition of nitrogen (N-addition).

The amount of N added to the N-addition treatment corresponded to the amount of N available in the high flow treatment. The *Ulva lactuca spp* from each mesocosm was sampled five times during the growth period of *Ulva* spp. from 10.08.2020 to 30.09.2020. Sampling was performed by WMR within a project of Wageningen Plant Research (WPR).

2.2.3 Chemical and statistical analysis

The *Ulva* samples were dried before further processing. Subsequently, test samples (i.e. pooled material from one mesocosm) from two mesocosms per time point were analyzed individually for weeks 33, 34, 36, 39 and 40.5 on several nutritional components by WPR, such as crude protein (based on the nitrogen content), starch, dietary fiber, individual protein amino acids, and ash.

In 2021 these test samples were transported to WFSR for analysis on heavy metal content and iodine levels, namely Ni, total As, inorganic As, Cd, Hg, Pb, Br, and I. Since these levels are assumed not to be influenced by changes of temperature and time it was still possible to analyze these samples one year later (NEN, 2021).

The raw results were compiled and analysed with Anova.

2.3 Intercomparison ICP-MS and XRF on flow regime experiment samples

2.3.1 Background

The contamination of seaweed with lead, arsenic, cadmium, mercury and iodine can raise questions about the food safety of seaweed. The current standard measurement method is based on an inductively coupled plasma mass spectrometry (ICP-MS) approach, which is relatively time consuming and costly, but is confirmatory, highly sensitive and accurate. To improve speed and cost effectiveness of risk assessment, there is interest in the development of faster screening methods. Here, an X-ray fluorescence (XRF) approach can be interesting to explore. XRF is a non-destructive method to assess elemental concentrations in a matrix. It is fast, mobile and relatively in-expensive, but less accurate and sensitive than an ICP-MS based method.

Strongly simplified, the ED-XRF method exposes a sample to X-rays from a radiation source at a specific wavelength. The elements in the sample absorb this specific energy and subsequently emit energy at a longer wavelength (with less energy than the absorbed energy). This emitted radiation is received by a detector. Since every element has a specific energy potential in its shell, the emitted energies are element specific. Based on this principle, the different elements can be detected and quantified in one measurement.

The method is sensitive to the matrix within which the elements of interest are embedded and its thickness. While XRF instruments can partially correct for these influencing factors, the preparation and presentation of the sample can influence the outcome of the measurement. Preparation methods include homogenising through drying (Masson et al., 2018), milling (Guimarães, Praamsma and Parsons, 2016) and compressing into a pellet (Pessanha et al., 2018), (Mir-Marqués et al., 2014). Moreover, further parameters can influence the outcome of the measurement, namely, total measurement time, filter time, measuring mode, interference from the container, sample density, sample thickness (Otaka, Hokura and Nakai, 2014b) and sample homogeneity (Tertian and Claisse, 1982).

Here, samples of the green seaweed *Ulva* sp. from the flow regimes experiment (see 2.2) were reanalysed using a portable ED-XRF (energy dispersive x-ray fluorescence) instrument for heavy metals, inorganic arsenic and iodine. These results were then compared to the results obtained by the standard, ICP-MS based method. Specifically, this study aimed to answer following questions:
- What detection limits can be achieved for different elements in dried and fresh *Ulva*?
- With what precision can ED-XRF measurements be performed?
- How should the *Ulva* samples be prepared for ED-XRF analysis?
- How do the detection limits of ED-XRF relate to other measuring systems, like ICP-MS?

2.3.2 Method and experimental design

2.3.2.1 Determining measurement and filter times for accurate detection of elements

To determine which measurement and filter times result in accurate measurements, a certified reference material (CRM, 1,25 Cr 0,5 Mo/ UNS K11572 alloy) was measured with different filter times and for different total analysis times on a Thermo Fisher Scientific XL3 analyser version 7.0.1. The excitation filters, of which the times could be adjusted, served to get a clearer signal in the detector. Excitation filters are physical filters that block specific wavelengths, which means that a range of elements does not get excited. This reduced the strain on the detector, which resulted in a clearer reception of the signal. As the CRM did not contain any of the elements from the research questions. Therefore, the elements copper, molybdenum, chrome and manganese were measured.

The CRM was measured up until 240 seconds, in intervals of 30 seconds. The different filter times used were 5, 10, 15, 30 and 60 seconds. These times were chosen because similar research used a filter time of 100 seconds (Bull, Brown and Turner, 2017) and the standard setting of the XRF instrument was 30 seconds. By choosing these specific filter times it was possible to determine whether prolonging or shortening the filter time would actually influence the measurement result.

2.3.2.2 Selecting an optimal sample container

To protect the XRF-instrument from sample residue, samples are placed into a container or film during measurements. However, this container or film can cause interference and influence the quality of the elemental measurement. Therefore, a range of container and film options was tested to determine the option with the highest recovery (%) of the CRM’s elements. The tested films and containers included parafilm, cling film, weighing paper, plastic weighing dishes and plastic petri-dishes. The films and containers were tested with the metal CRM and a plastic CRM in granulate form for recoveries of the CRM’s elements. The container with the highest recovery was then used during the measurements with the seaweed samples.

2.3.2.3 Determining an effective pellet preparation using *Saccharina latissima*

Samples can be presented in different ways for measurement. A common method is pressing finely milled and homogenised material into a pellet. These pellets can be pressed under different pressures and into different weights and densities. Here, powder of the brown seaweed *Saccharina latissima* with a grain size <250 micrometres and known concentrations of contaminants was available for testing pellet parameters.

Multiple pellets were pressed, of different weights at different pressures, resulting in different pellet heights using a combination of an International Crystal Laboratories KBR pellet press and a 13 mm diameter die set. The die set was placed in the press and determined the shape and size of the pellets. Pellets were pressed for weights of 300, 400 and 500 milligrams. Of each weight 3 pellets were pressed in machines settings of 5000, 6000 and 7000 PSI. Initially two pellets were tested in 3 measuring modes, to determine the most appropriate mode. After the suitable measurement mode was determined, all pellets were measured at the most suitable filter and most suitable total measurement time.

2.3.2.4 Measurements of *Ulva lactuca* pellets

In total 29 different seaweed samples were pressed into pellets. The dried seaweed was first milled finely using an IKA tube mill, Identification Number: 0004180000. Then, the powder was sieved to select for grain size below 500 micrometres. The pellets were pressed according to above method. Measurements were performed at increasing the total measurement time, the previously established optimal filter time was maintained. Measurements on a sample were performed repeatedly. Based on the results of the these experiments the total time of the measurements would be increased or decreased. The amount of times a sample was measured was changed accordingly, to keep the total measuring time practically attainable.

2.3.2.5 Determining LOQ’s

For the determination of LOQ’s an internal WFSR guideline was used, SOP A-0906. This document is a guideline for validation of methods and part of that is determining LOQ’s. The LOQ was defined as 6 times the standard deviation. The standard deviation was determined through 8 measurements of the same sample.

2.3.2.6 Determining linearity, significance and prediction error of the measurements

To assess the validity for the ED-XRF based measurements, the recoveries for the elements arsenic, bromine and lead were calculated. Recoveries ranging within 80 and 120 percent are accepted in keeping with WFSR validation guidelines. Another criterium for the use of data for calculations, was that a measuring result would only be classified as valid in case all five measurements resulted in a numerical value and hence above LOD. From 5 measurements per pellet the average was determined. To the averages of the pellets a regression model was fitted with the least squares method (Longnecker and Ott, 2017) and the coefficient of determination (R^2) was determined. The residual standard deviation was determined (Longnecker and Ott, 2017). The two sigma value was used as a measure for a 95% prediction interval for the estimate. (Pukelsheim, 1994). Normality for the multiple measurement results per sample was assumed.

2.3.3 Sample preparation

The samples were stored at room temperature before analyses were performed. The average sample weight was about 5 grams. Before the samples were analysed with the ICP-MS and the XRF they were sieved and milled to a grain size <500 micrometres.

After the sample preparation the homogenized samples were split into two portions of about 2.5 grams. The first portion of 2.5 grams was prepared for analysis on the ICP-MS measuring equipment. The second portion was prepared for analysis with the XRF instrument.

2.3.4 ICP-MS measurements

The different analyses have been performed according to standard operating and accredited procedures of WFSR. Element analysis was performed according to SOP-A-1331, inorganic arsenic according to SOP-A-108, and the bromine and iodine concentrations according to SOP-A-1341.

2.3.5 XRF measurements

The XRF instrument available at WFSR is a portable Thermo Fisher Scientific XL3 analyser version 7.0.1. With this instrument fluorescence spectra can be obtained, from which element composition is derived by spectral software. The optimal sample preparation and measuring configuration has been studied extensively in 2021 (Kerkvoorde, 2021).

2.4 Cultivation experiments under contrasting nutrient ratios

2.4.1 Background

Seaweeds have repeatedly been shown to take up and accumulate contaminants such as arsenic in concentrations that are far above the observed environmental levels. Moreover, as uptake of phosphate and arsenate occur through the same uptake pathways, the accumulation of arsenate is likely closely linked to uptake of phosphate. Some studies suggest that arsenate uptake is higher in low environmental phosphate conditions and relatively lower in high environmental phosphate conditions. Moreover, little is known about relationships between in-plant concentrations of contaminants.

2.4.2 Experimental design and sampling

The aim of the addition experiments was to assess the effects of nutrient conditions on the uptake and the correlations of contaminants. The experimental design was performed in triplicates and included:

- a control treatment (no nutrients added);
- a +N treatment (addition of NO_3^-); and
- a +P treatment (addition of PO_4^{3-}).

The used experimental set-up is displayed in Figure 4. The mesocosms were situated outdoors on the facilities of Wageningen Marine Research, Yerseke, NL and the experiment ran from October 25th to November 22nd, 2021. The mesocosms contained 1,000 L seawater, no sediment and no shading from light or precipitation. Temperature was not controlled but strongly influenced by the temperature of the inlet water from the Eastern Scheldt. As stated before, the experiments were conducted in triplicate and were divided into three groups: one with phosphate addition, one with nitrate addition and one control group, which were placed in a random order in the experimental set-up. In the experimental set-up, water from the Eastern Scheldt was continuously passed through the mesocosms at a flowrate of 2 L min^{-1} to ensure simulation of the normal growth conditions. Furthermore, additional pumps were used for continuous nutrient supply at a flowrate of 15 mL min^{-1} to the assigned mesocosms.

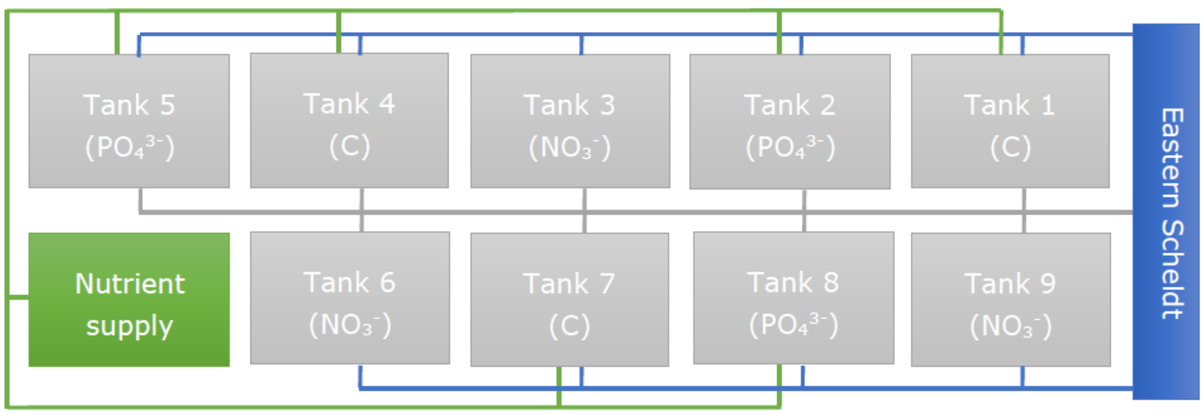

Figure 4 *Experimental set-up field experiments Ulva lactuca spp. Waterflow from the Eastern Scheldt to the mesocosms is illustrated in blue, waterflow from the mesocosms to the Eastern Scheldt is illustrated in gray. Nutrient supply to the mesocosms is illustrated in green. Mesocosms 1, 4 and 7 were used as the control. Mesocosms 2, 5 and 8 were supplied with additional phosphate. Mesocosms 3, 6 and 9 were supplied with additional nitrate. (by Amber Beerman).*

The +N and +P treatments aimed at doubling the NO_3^- or the PO_4^{3-} concentrations in the water of the respective mesocosms. With these treatments, the following nutritional scenarios were studied: (1) regular flow (of 2 L per minute) with no nutrient additions, serving as reference tank; (2) changed N:P ratio; (3) changed P:As ratio. The seawater NO_3 and PO_4 concentrations in the inlet water from the Eastern Scheldt were estimated using colorimetric test kits (Nitrate Pro (NO_3) Comparator Test kit and Red Sea Phosphate

Pro (PO_4) Comparator Test kit, Red Sea, Israel). With these tests the nitrate concentration was estimated around 0.375 mg/L and the phosphate concentration was estimated around 0.03 mg/L. to double the input of nutrients, the additional pumps supplied a nutrient concentration of 0.375 mg/L nitrate and 0.03 mg/L phosphate. After correcting for the water supply from the Eastern Scheldt (2 L/min), nutrient supply (15 mL/min) and counter-ions of the chemicals, the stock concentrations for the nutrient supply were 68.54 mg/L and 5.98 mg/L for sodium nitrate and sodium phosphate dibasic respectively.

The set-up of the experiment was based on theoretical and experimental insights that were obtained through several WUR-projects (e.g. by WFSR, WMR and WPR) over the last few years. Preferably, the set-up would have been tested with *Saccharine latissima*, as the uptake dynamics of this seaweed have been studied by WFSR. However, the growth period of *Saccharina latissima* is restricted to the winter, therefore, conducting experiments with this seaweed species was unattainable. Accordingly, the experimental set-up was tested with a different seaweed species, *Ulva lactuca spp*, which grows during the summer. *Ulva lactuca spp* was obtained from an external party and was retrieved from the waters of the Eastern Scheldt in Zeeland. At the start of the experiment, 1 kg of *Ulva lactuca spp* was placed in each mesocosm. This experiment allowed for thorough testing of the set-up, to ensure that all the necessary parameters were in place to study the uptake mechanisms of contaminants in macroalgae in relation to nutrient levels.

Throughout the experiment various parameters were monitored within the 9 cultivation mesocosms at the WMR site in Yerseke, which are described in Table 1. The parameters were monitored on a weekly basis. Water and seaweed samples were taken on October 25th, November 5th, November 15th and November 22nd. The temperature, pH and oxygen levels were directly determined on site. The nutrient levels in the water samples were analysed by the Royal Netherlands Institute for Sea Research (NIOZ) using a Bran & Luebbe TRAACS 800 autoanalyzer. The seaweed samples were internally analysed at WFSR.

Table 1 *Measured parameters field experiments Ulva lactuca spp and methods that were used for the analysis.*

Parameter		Matrix	Method
Temperature		Seawater	HACH HQ 40D multi device
pH		Seawater	HACH HQ 40D multi device
Oxygen level		Seawater	HACH HQ 40D multi device
Nutrient levels:	Phosphate	Seawater and seaweed	NIOZ and spectrophotometric analysis phosphate
	Ammonium	Seawater	NIOZ
	Nitrite	Seawater	NIOZ
	Nitrate	Seawater and seaweed	NIOZ and spectrophotometric analysis nitrate
	Totalnitrogen	Seawater	NIOZ
	Silicium	Seawater	NIOZ
Contaminant levels:	Arsenic	Seaweed	Elemental analysis with ICP-MS
	Lead	Seaweed	Elemental analysis with ICP-MS
	Cadmium	Seaweed	Elemental analysis with ICP-MS
	Mercury	Seaweed	Elemental analysis with ICP-MS

2.4.3 Chemical and statistical analysis

The chemicals and materials that were used within the experiments are described in Table 2 and 3.

Table 2 *Used chemicals field experiments Ulva lactuca spp.*

Chemical	Purity	Purchased from
Sodium nitrate	≥99.0%	Sigma Aldrich
Sodium phosphate dibasic	≥99.0%	Sigma Aldrich

Table 3 *Used materials field experiments Ulva lactuca spp.*

Testkit	Detection limit	Purchased from
Red Sea Nitraat Pro (NO_3) Comparator Test kit	0.125 PPM	Ocean store
Red Sea Fosfaat Pro (PO_4) Comparator Test Kit	0.02 PPM	Ocean store

3 Results

3.1 Contaminants flow regimes experiment

3.1.1 Experiment 1 flow regimes: ICP-MS results

The results of the ICP-MS analysis are presented in Annex 1. Figure 5 shows the results of the analysis of iodine and cadmium, respectively. Statistical evaluation has been performed on the analytical results with the aim to conclude whether a correlation can be found between the different nutritional situations and the concentrations of the heavy metal, inorganic arsenic and iodine concentrations during the growing period of the *Ulva lactuca spp*. This evaluation led to the results shown in Table 4.

Table 4 showed an increasing concentration for all analytes during growth. Furthermore, the scenario in which nutrients are added at a regular flow rate (2 L/min water) showed a statistically enhanced concentration for the analytes nickel, mercury, iodine and bromine. Moreover, iodine and mercury showed statistically enhanced concentrations within the scenario that the flow rate of the water was enhanced from 2 L/min to 25 L/min. Lastly, no statistical differences were found in the concentrations of the analytes when the high flow rate scenario (25 L/min) was compared with the scenario where nutrients were added.

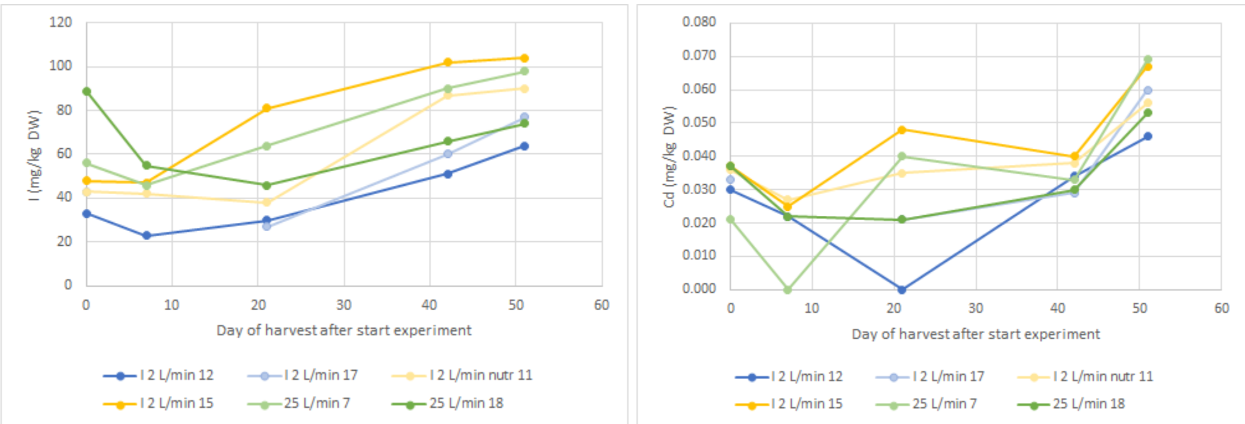

Figure 5 *Results flow experiment. Left: Iodine (mg/kg) versus day of harvest. Right: Cadmium (mg/kg) versus day of harvest.*

Table 4 *Evaluation flow experiment.*

Analyte	Effect of harvesting moment	Difference (2 L/min) vs (2 L/min + nut	Difference (2 L/min) vs (25 L/mir	Difference (2 L/min + nutr) vs (25 L/min)
Ni	build-up	more build-up at 2 L/min + nutr	no difference	no difference
As	build-up	no difference	no difference	no difference
Cd	build-up	no difference	no difference	no difference
Hg	build-up	more build-up at 2 L/min + nutr	more build-up at 25 L/min	no difference
Pb	build-up	no difference	no difference	no difference
iAs	build-up	no difference	no difference	no difference
I	build-up	more build-up at 2 L/min + nutr	more build-up at 25 L/min	no difference
Br	build-up	more build-up at 2 L/min + nutr	no difference	no difference

3.2 Intercomparison ICP-MS and XRF on data flow regimes experiment

Figure 6 (A-C) represents the correlation between the XRF and the ICP-MS for the concentrations of lead, arsenic and bromine. All figures show linear correlations between the measurements performed with the XRF and the ICP-MS. Since the XRF used at WFSR is not able to report the iodine concentrations, the results of iodine found with the ICP-MS are plotted against the bromine concentrations found with the XRF in Figure 6 D. From Figure 6 D it can be concluded that bromine and iodine concentrations show a linear correlation. Furthermore, the bromine concentrations are about a factor 5 higher than the iodine concentrations. Therefore, the XRF instrument could possibly be valuable in measuring relatively low iodine concentrations.

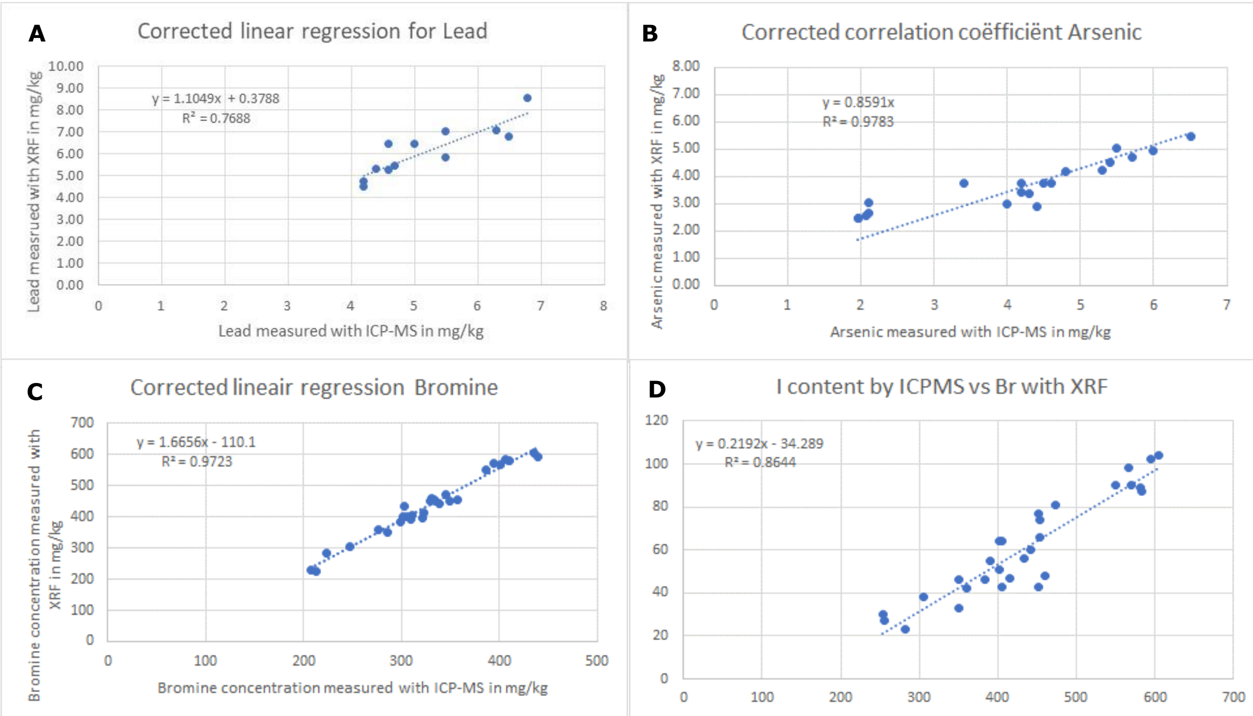

Figure 6 *Intercomparison between XRF and ICP-MS data. A: Intercomparison Lead (mg/kg). B: Intercomparison Arsenic (mg/kg). C: Intercomparison Bromine (mg/kg). D: Intercomparison Iodine (mg/kg) with ICP-MS and Bromine (mg/kg) with XRF.*

3.3 Contrasting nutrient addition experiment

To review the proposed hypothesis, a correlation matrix was constructed (Figure 7) that included the following 16 variables that were monitored during the experiment: the different mesocosms (represented as tank), nutrient concentrations in the water (e.g., WNO3), nutrient and contaminant concentrations in the seaweed (e.g., SNO3, Sas) and the temperature, pH and oxygen levels in the water of the test tanks. The expected correlations – arsenic concentration related to the presence of phosphate – were not found during the addition experiments on *Ulva lactuca spp.* Most likely, the uptake mechanism for arsenic is different than for *Saccharina latissima*. Besides this, it should be noted that the experiments were performed after the growing season of *Ulva lactuca spp*, which could have also influenced the results.

However, finding the predicted correlations between the phosphate and arsenic concentrations was not the main aim of the experimental set-up, as the main goal included obtaining the best possible experimental set-up for future experiments on *Saccharina latissima*. Furthermore, a data-evaluation was performed at the relationships between the heavy metals arsenic, lead, cadmium and mercury within the several *Ulva lactuca spp* samples. The results are represented in Figure 8 (taken from Beerman, 2022). The results show good correlations among several heavy metals.

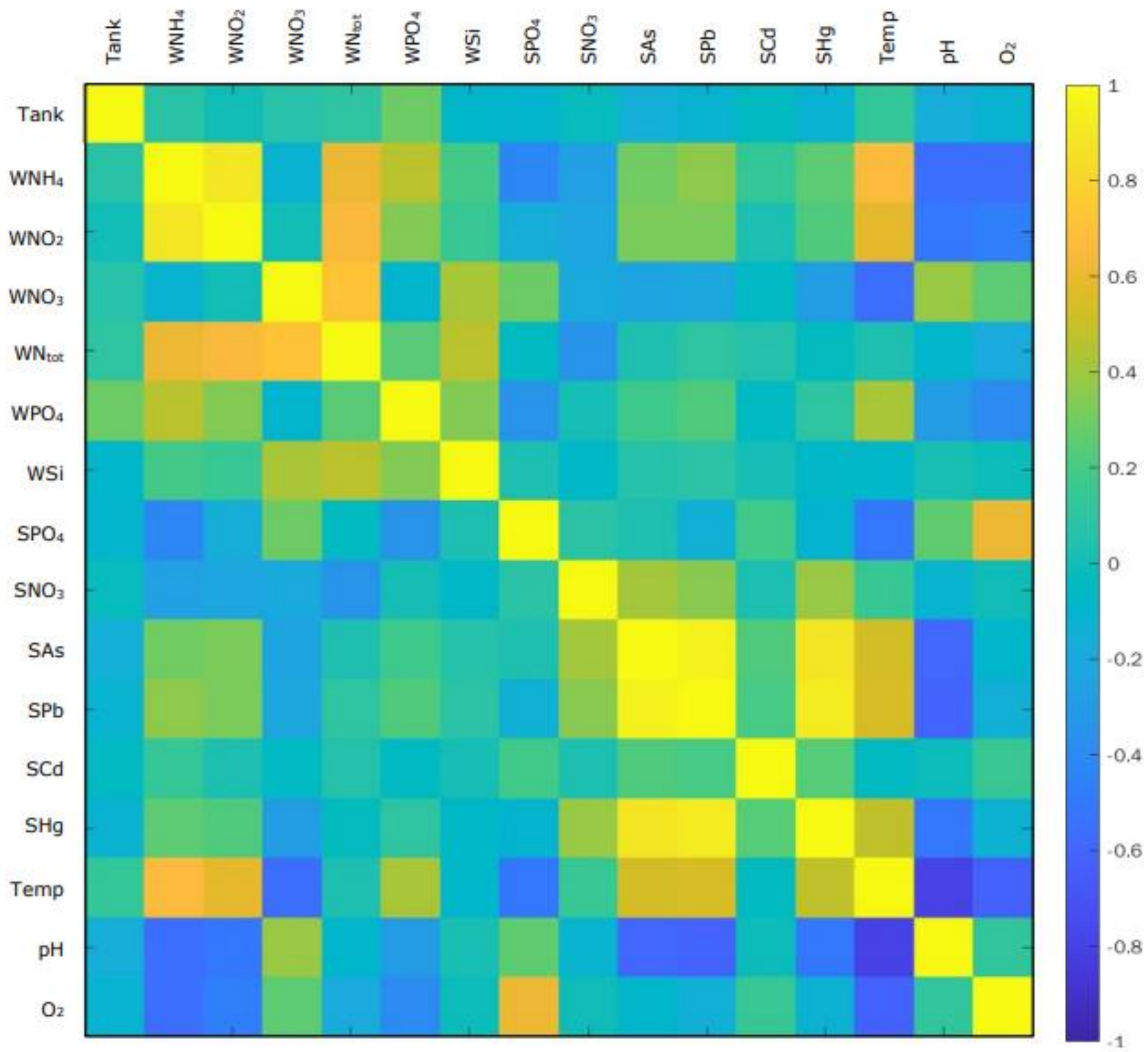

Figure 7 *Correlation matrix variables field experiments Ulva lactuca spp. WNH_4 – ammonium conc. In water; WNO_2 – nitrite conc. In water; WNO_3 – nitrate conc. In water; WN_{tot} – total nitrogen conc. In water; WPO_4 – phosphate conc. In water; WSi – Silicium conc. In water; SPO_4 – phosphate conc. In seaweed; SNO_3 – nitrate conc. In seaweed; SAs – arsenic conc. In seaweed; SPb – lead conc. In seaweed; SCd – cadmium conc. In seaweed; SHg – mercury conc. In seaweed. Constructed in Matlab (taken from Beerman, 2022).*

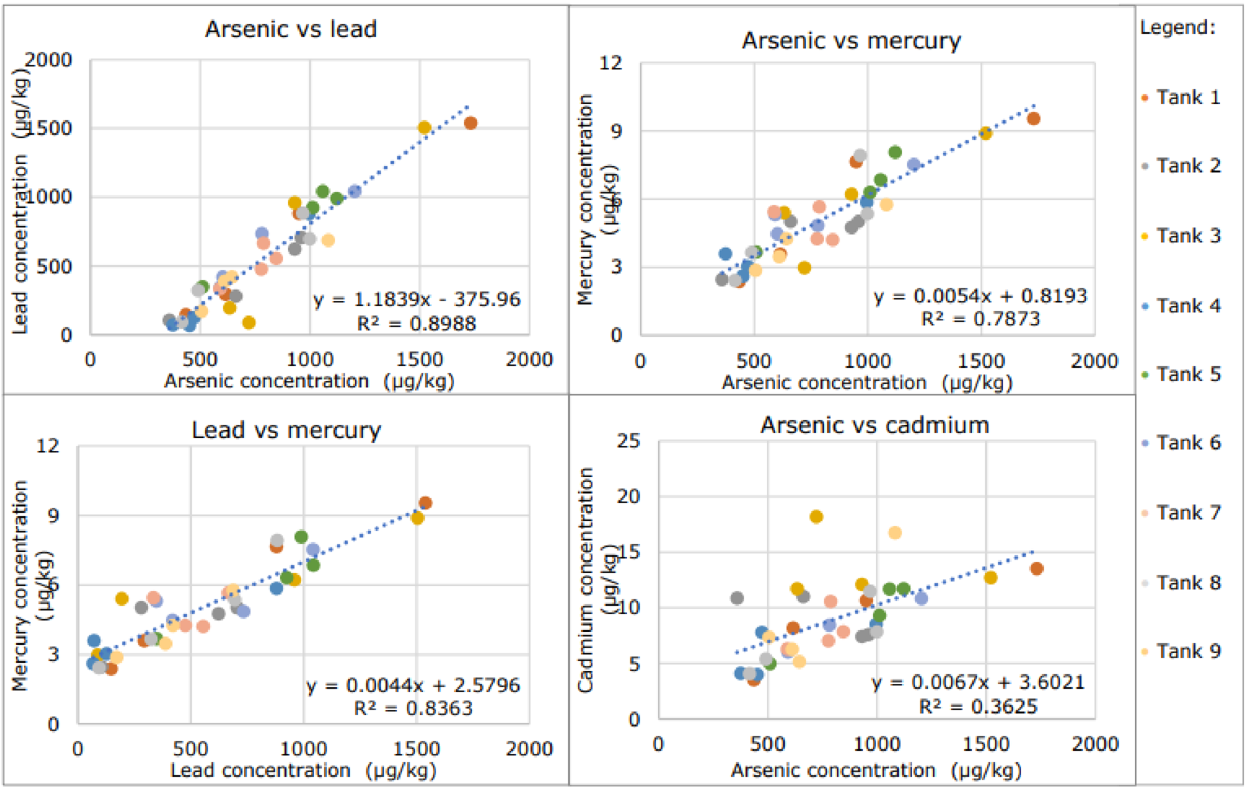

Figure 8 *Identified correlations between the heavy metals in Ulva lactuca spp. Control group: tank 1,4,7; phosphate additions: tank 2,5,8; nitrate additions: tank 3,6,9 (By Beerman, 2022).*

4 Discussion & recommendations

4.1 Contaminants in contrasting flow regimes

4.1.1 Influence of flow regime

Within the results section a number of scenarios were compared to one another. Within this section it was stated that the scenario in which nutrients are added to a flow rate of 2 L/min resulted in the buildup of the following contaminants: nickel, mercury, iodine and bromine. Furthermore, it was stated that no statistical differences were found in the buildup of contaminants between the scenario in which nutrients were added to a flow rate of 2 L/min and the scenario in which the flow rate was increased to 25 L/min. The higher flow of 25 L/min translates into a higher flow of nutrients that continuously passes through the tanks, which may level out the effect of the first scenario. Moreover, it was also stated that the buildup of iodine and mercury were statistically enhanced when the flow rate was increased from 2 L/min to 25 L/min.

Unfortunately, a number of parameters were not monitored during this experiment, including the growth, nutrient content within the seaweed, temperature and the concentration of the studied contaminants within the water-flow, which makes it difficult to draw conclusions from these experiments. Furthermore, the experiment only included 5 harvesting moments over a span of 50 days and the experiments were only performed singularly and not in duplicate or triplicate. All the above stated factors make it difficult, if not impossible to take strong conclusions from this experiment.

4.1.2 Recommendations

The experimental set-up of this experiment was relatively similar to the experiment described in section 4.2. Therefore, the recommendations for improving the experimental set-up are described in detail in section 4.2 and include an acclimatization period, weekly weighing's of the seaweed and frequent sampling of the seaweed and seawater. Furthermore, for this specific experiment it is recommended to increase the datapoints, perform the experiment in triplicate and monitor all analytes of interest in both the seawater and seaweed.

4.2 Contaminants in contrasting nutrient additions

4.2.1 Influence of nutrient addition

From the correlation-matrix (Figure 7), it became evident that the expected correlations were not found. This could have been caused by the inconclusiveness of the water data, as the data showed that the nitrate concentration did not only increase in the nitrate addition group, but also in the other two groups (phosphate addition and control). Furthermore, the data also showed that the phosphate concentration decreased over time in all mesocosms. Therefore, it is possible that the addition experiments were not successful. During the experiment, various problems were observed with respect to the pumps that were used for the nutrient additions. These issues were primarily related to the consistency of the flowrate of the used pumps. The inconsistency of the used pumps could be an explanation for the significant differences that were observed between the nutrient content of the addition test groups and the control group.

A second explanation for the insufficiency of the addition experiments could include the lack of an acclimatization period. Due to the lack of time, it was not possible to include an acclimatization period, in which the nutrient levels of the nitrate and phosphate addition groups could stabilize. Furthermore, it is important to consider that *Ulva lactuca spp* is a seaweed species that grows during the summer. Therefore, this seaweed species requires high temperatures for efficient growth. Towards the end of the test period the temperatures decreased, through which the *Ulva lactuca spp* likely stopped growing and started to die off, which may explain why the expected correlations were not found. Lastly, it should be considered that the expectations were not based on *Ulva lactuca spp*, and that the uptake mechanisms within *Ulva lactuca spp* and *Saccharina latissima* might differ from one another.

Even though no correlation was found between the addition of phosphate and the uptake of arsenic, interesting correlations were found among the heavy metals lead, arsenic, cadmium, and mercury (Figure 8). In the study, a strong correlation was found between arsenic and lead. These results coincided with other studies that were conducted on *Ulva lactuca spp*, which found a very comparable relationship (Faassen and van Tuinen, 2020). Furthermore, strong correlations were found between mercury and the above stated heavy metals, arsenic, and lead. The identified heavy metal concentrations differed strongly among the analysed seaweed samples. The highest heavy metal concentrations were observed in the seaweed samples that were sampled at the start of the experiment. The heavy metal content fluctuated throughout the experiments. The fluctuation seemed random and was difficult to explain, considering the heavy metal content was not measured in the water.

Overall, the heavy metal content decreased towards the end of the experiment, which represent the lower measurement points in the figure. The reduction in the heavy metal content can most likely be attributed to the period in which the *Ulva lactuca spp* was grown. As stated before, the temperature decreased towards the end of the experiment, which likely had a negative effect on the growth and resulted in the deterioration of the seaweed. Nevertheless, it was evident that these three heavy metals had a strong positive correlation with one another. Interestingly, no strong correlations were found between the above stated heavy metals and cadmium. A potential explanation for the lack of correlation includes the sand that was observed after the digestion of the seaweed samples. *Ulva lactuca spp* was cultivated in mesocosms that were continuously supplied with water from the Eastern Scheldt, through that, considerable amounts of sludge were also carried into the mesocosms. Since part of the samples contained sand, it is possible that additional cadmium was digested from the sand, as sand generally contains a considerable amount of cadmium (Anderson et al., 2002). This could have led to inaccurate data, which could explain the low correlation between cadmium and the remaining heavy metals.

The primary aim of this experiment was to test the set-up for future experiments with *Saccharina latissima*. Through this, the study proved to be of great value, as the test set-up was thoroughly tested and important suggestions could be made for the experiment with regards to *Saccharina latissima*, which will be discussed later. Even though the expected correlations were not found, other important correlations were identified. Lastly, the linear relationship between the heavy metals could be of great value, as this could make it easier to predict heavy metal concentrations in *Ulva lactuca spp* and possibly other seaweed species in the future.

4.2.2 Recommendations for set up experiment

It is recommended to conduct future field experiments on *Saccharina latissima* and focus on the hypothesized competition mechanism between the uptake of phosphate and arsenic, in addition to the effects of nitrate addition on the protein content in *Saccharina latissima*. Preferably, the set-up should include multiple scenarios in triplicate, in which various N:P-ratios and As: P-ratios could be tested. Furthermore, it is recommended to include an acclimatization period of four to six weeks. In this period, weekly testing of the nitrate and phosphate levels in the water should take place to review whether the desired levels are reached. During this period, it is important that the test mesocosms only contain seawater and do not yet contain seaweed, as the seaweed uptake of nutrients influences the results. It is also recommended to include weekly weighing's of the seaweed, to determine the effect of the scenarios on the growth. Lastly, it is recommended to review possibilities that can replace the weekly sampling of seaweed and seawater. This could include non-destructive analysis of the components in seaweed through a hand-held XRF device and real time monitoring of the nitrate, phosphate, and arsenic levels in the water.

4.3 Intercomparison ICP-MS and XRF measurements

4.3.1 Comparison of results

As stated in the results section, a great correlation was found between the ICP-MS and the XRF data for the analytes lead, arsenic and bromine. Furthermore, the iodine content by the ICP-MS was compared to the bromine content measured with the XRF, which also showed great correlation between one another. This comparison was made as iodine could not be detected by the used XRF-device. Fortunately, the XRF-devices are improving quickly, and new benchtop XRF-models have shown to be able to quantify iodine. Furthermore, the correlation between the analytes shows a great potential for the XRF to become a screening method for certain contaminants in seaweed.

4.4 Contaminants in *Ulva* compiled data 2018-2021

Since again rather linear correlations are found in the *Ulva lactuca spp* samples, WFSR results of several projects of the last years considering *Ulva lactuca spp* samples from different locations and different periods in the Netherlands (Faassen & van Tuinen, 2020; Faassen et al, 2022). So, the dataset contains samples during all the phases of the grow of the *Ulva lactuca spp*. Important to consider that linearity may be there, but slopes may be dependent on treatment, species, season, etc. Therefore, it is not a straightforward recalculation of one slope fits all scenarios. Figure 9 shows the correlations for different analytes in all *Ulva lactuca spp* samples analysed in the period 2018 – 2021.

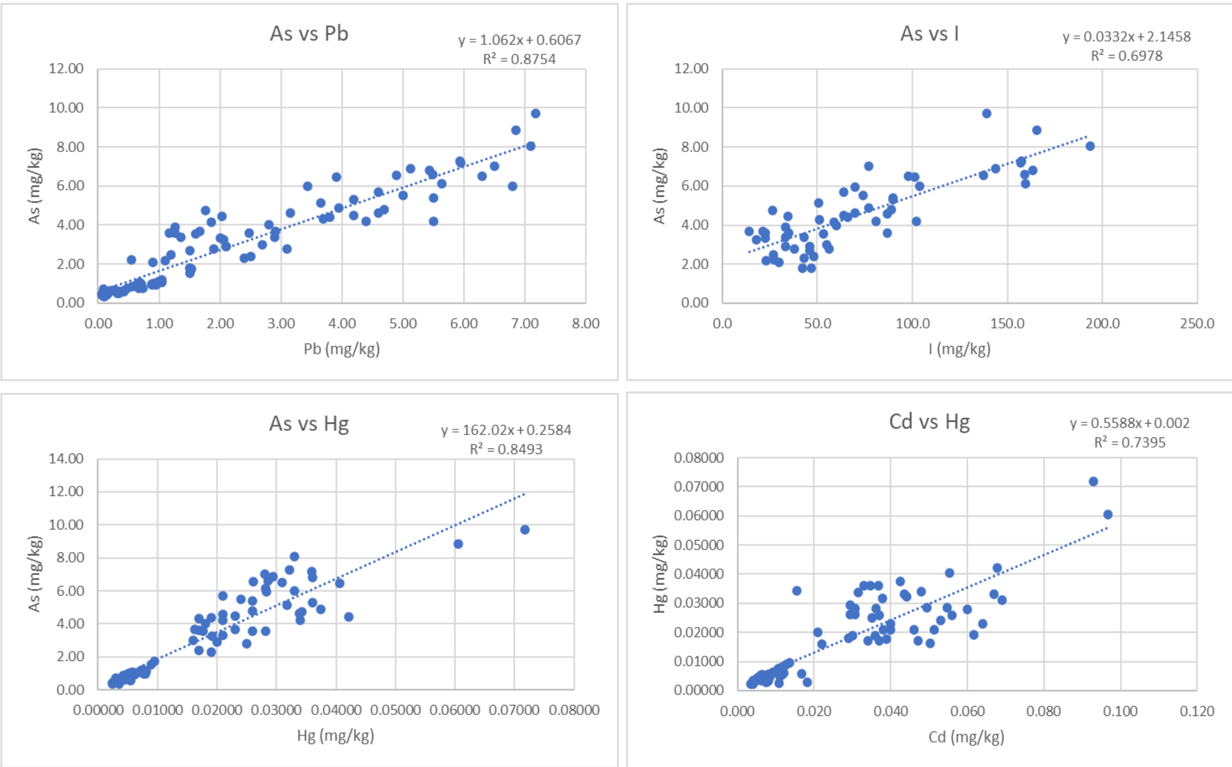

Figure 9 *Correlations between different contaminants in the period of 2018 – 2021.*

5 Conclusions

Several nutrition addition experiments and data analysis exercises have been performed on the green seaweed *Ulva lactuca spp* with the aim to answer the research questions of this project.

The experiments performed on *Ulva lactuca spp* give good insight in the possibilities of optimizing the set-up for future experiments which are foreseen for the brown seaweed species *Saccharina Latissima*, and where the uptake of arsenic and iodinie is one of the show stoppers for a large market introduction in the Netherlands.

The different nutritional conditions of the *Ulva lactuca spp* in the mesocosms resulted only in slightly different concentrations of arsenic, heavy metals and iodine in the seaweed harvested. The scenario in which nutrients are added at a regular flow rate (2 L/min water) showed a statistically enhanced concentration for the analytes nickel, mercury, iodine and bromine. Moreover, iodine and mercury showed statistically enhanced concentrations within the scenario that the flow rate of the water was enhanced from 2 L/min to 25 L/min. Lastly, no statistical differences were found in the concentrations of the analytes when the high flow rate scenario (25 L/min) was compared with the scenario where nutrients were added. The higher flow of 25 L/min translates into a higher flow of nutrients that continuously passes through the tanks, which may level out the effect of the first scenario.

From the experiments performed it is concluded that before starting additional experiments an acclimatization period before starting the experiment of four to sixs weeks is advised. In this way the seaweed can obtain a new equilibrium between the nutrients available in the mesocoms and its growth. Besides this, the experimental set-up can be improved by monitoring weekly some relevant parameters like the weight of the seaweed growing in the mesocoms, and the concentrations of the nitrate and phosphate levels. The XRF technique can help to reduce costs during this experiments significantly. The experiments performed have resulted in an optimized monitoring strategy for future addition or depletion experiments. In future experiments it is advised to make use of triplicate test set ups for every addition or depletion experiment. This can lead to increased statistical relevance of the experiments.

Comparing the analysis results obtained with ICP-MS and XRF techniques linear correlations and uniform correlation slopes were found for the concentrations of arsenic, lead, mercury, cadmium versus the iodine concentrations. This provides great opportunities to develop a screening method with the XRF technique for certain analytes within *Ulva lactuca spp*. It is expected that this will also be valid for other seaweed species, although additional experiments should confirm this.

The concentrations of the various heavy metals and iodine within *Ulva lactuca spp* were also evaluated on time dependency on time and on location dependency. Also here good correlations were observed: almost identical ratio's were found for heavy metals concentrations of arsenic, lead, mercury, cadmium when they are compared to the iodine concentrations. This means that when one of the concentrations of these elements (e.g. arsenic) is measured, the amount of other elements like lead, mercury, cadmium and iodine can be predicted quite well. These observations provide great insight in the uptake mechanisms of contaminants in *Ulva lactuca spp*, and can lead to lowering the operational costs for seaweed farmers significantly by analysing their seaweed during the growth on the presence of contaminants with the XRF technique. Also the moment that contaminant concentrations found in the seaweeds cultivated tend to increase significantly can be determined much more easily, which can result in the decision to harvest the seaweeds earlier or later than planned originally.

If the research performed could be expanded and extrapolated to other cultivation areas in national or international waters, the XRF technique could be used to predict the presence of heavy metals and iodine accurately and cost very effectively.

This could lead to a better and low-cost method for coping with possible food safety issues on several seaweed species, and a large scale market introduction of some edible seaweed species in and outside the Netherlands.

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Annex 1 IC-PMS data flow regimes experiment

MNr	Tank	Behandeli	datum	day	DW	LIMS	Ni	As	Cd	Hg	Pb	iAs	I	Br
1	12	2L_a	10-8-2020	0	85,9	200629692	3,3	2,9	0,03		2,1	0,47	33	322
3	12	2L_a	17-8-2020	7	38,7	200629694	1,9	2,2	0,022		1,1	0,26	23	223
5	12	2L_a	31-8-2020	21	52,1	200629695	1,6	2,1			0,9	0,22	30	213
7	12	2L_a	21-9-2020	42	53,3	200629697	3,9	4,3	0,034	0,017	3,7	1,2	51	306
9	12	2L_a	30-9-2020	51	167,2	200629699	4,9	5,7	0,046	0,021	4,6	1,6	64	302
2	17	2L_b	10-8-2020	0	56,2	200629693	3,5	3,4	0,033		2,9	0,7	43	357
4	17	2L_b	17-8-2020	7	18,9									
6	17	2L_b	31-8-2020	21	45,6	200629696	1,9	2,5	0,021		1,2	0,32	27	207
8	17	2L_b	21-9-2020	42	52,3	200629698	3,5	4	0,029	0,018	2,8	0,96	60	339
10	17	2L_b	30-9-2020	51	213,9	200629700	6,5	7	0,06	0,028	6,5	1,7	77	329
11	11	2L_Nut_a	10-8-2020	0	56,5	200629701	3,6	2,3	0,036	0,019	2,4	0,44	43	349
13	11	2L_Nut_a	17-8-2020	7	29,3	200629703	2,9	1,8	0,027		1,5	0,24	42	277
15	11	2L_Nut_a	31-8-2020	21	52,4	200629705	3,2	2,8	0,035	0,025	3,1	0,63	38	248
17	11	2L_Nut_a	21-9-2020	42	52,8	200629707	5,3	4,6	0,038	0,021	4,6	1,3	87	407
19	11	2L_Nut_a	30-9-2020	51	153,2	200629709	6,4	5,4	0,056	0,026	5,5	1,3	90	386
12	15	2L_Nut_b	10-8-2020	0	116,8	200629702	4,2	2,4	0,037	0,017	2,5	0,5	48	331
14	15	2L_Nut_b	17-8-2020	7	32,6	200629704	3,4	1,8	0,025		1,5	0,24	47	323
16	15	2L_Nut_b	31-8-2020	21	32,6	200629706	5,5	4,2	0,048	0,034	5,5	0,86	81	345
18	15	2L_Nut_b	21-9-2020	42	48,6	200629708	5,1	4,2	0,04	0,021	4,4	1	102	440
20	15	2L_Nut_b	30-9-2020	51	125,6	200629710	7,4	6	0,067	0,033	6,8	1,5	104	436
21	7	25L_a	10-8-2020	0	109,4	200629711	2,7	2,8	0,021		1,9	0,47	56	303
23	7	25L_a	17-8-2020	7	25,7	200629713	2,1	2,7			1,5	0,39	46	299
25	7	25L_a	31-8-2020	21	42,3	200629715	4,2	4,5	0,04	0,023	4,2	0,95	64	311
27	7	25L_a	21-9-2020	42	54,1	200629717	5,1	5,3	0,033	0,036	4,2	1,5	90	395
29	7	25L_a	30-9-2020	51	75,6	200629719	7,1	6,5	0,069	0,031	6,3	1,6	98	401
22	18	25L_b	10-8-2020	0	38,8	200629712	5,5	4,8	0,037	0,026	4,7	1,1	89	410
24	18	25L_b	17-8-2020	7	28,1	200629714	3	3	0,022	0,016	2,7	0,54	55	310
26	18	25L_b	31-8-2020	21	37,8	200629716	2,6	2,9	0,021	0,02	2,1	0,55	46	286
28	18	25L_b	21-9-2020	42	52,9	200629718	4,5	4,4	0,03	0,019	3,8	1,1	66	334
30	18	25L_b	30-9-2020	51	160,4	200629720	6	5,5	0,053	0,024	5	1,4	74	335

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The mission of Wageningen University & Research is "To explore the potential of nature to improve the quality of life". Under the banner Wageningen University & Research, Wageningen University and the specialised research institutes of the Wageningen Research Foundation have joined forces in contributing to finding solutions to important questions in the domain of healthy food and living environment. With its roughly 30 branches, 7,600 employees (6,700 fte) and 13,100 students and over 150,000 participants to WUR's Life Long Learning, Wageningen University & Research is one of the leading organisations in its domain. The unique Wageningen approach lies in its integrated approach to issues and the collaboration between different disciplines.

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