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Cultivation of *Ulva fenestrata* using herring production process waters increases biomass yield and protein content

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Ulva spp. (sea lettuce) has recently gained attention as a sustainable protein source due to its high productivity and many nutritional properties interesting for the food industry. In this study, we explored a possible industrial symbiosis between herring production processing industries and *Ulva fenestrata* cultivation. We show that *U. fenestrata* cultivated in herring production process waters had four to six times higher biomass yields (27.17 - 37.07 g fresh weight vs. 6.18 g fresh weight) and three times higher crude protein content (> 30% dry weight vs. 10% dry weight) compared to *U. fenestrata* cultivated in seawater. Along with the elevation of protein, the herring production process waters also significantly increased levels of all essential amino acids in the seaweed biomass. The content of some heavy metals (arsenic, mercury, lead, and cadmium) was well below the maximum allowed levels in foodstuff. Therefore, quantities of biomass around 100 g dry weight could be consumed daily following the US Environmental Protection Agency's reference doses. Combined, the results show that cultivation of *U. fenestrata* in herring production process waters has great potential to produce sustainable proteins for the growing world population. At the same time, nutrients of currently discarded process waters are circulated back to the food chain.

KEYWORDS

arsenic, wastewater, proteins, aquaculture, seaweed, macroalgae, process water, food production

Introduction

The demand for protein rich and nutritious food is steadily increasing, and it is evident that we need more sustainable food production systems to feed the growing human population (Aiking, 2014; Gephart et al., 2021). Seaweed aquaculture is considered one of the most environmentally friendly aquaculture practices, with a net uptake of both nitrogen and phosphorus (Buschmann et al., 2017; Gephart et al., 2021). The green seaweed *Ulva* spp. (sea lettuce) has received attention by the aquaculture sector due to its high productivity and high environmental tolerance (Bolton et al., 2016; Nardelli et al., 2019; Steinhagen et al., 2021). *Ulva* biomass has many nutritional properties interesting for food and feed applications (Bolton et al., 2016). Its protein content is often relatively high (10 – 20% of dry weight (dw)) (Fleurence, 2016; Toth et al., 2020; Steinhagen et al., 2021) compared to most vegetable staple foods, but still not as high as in, for example, soybean biomass (35 - 40% dw) (Grieshop and Fahey, 2001; Thakur and Hurburgh, 2007). Further, its proteins have a high proportion of essential amino acids (Machado et al., 2020). However, *Ulva* can increase growth and alter biochemical properties by responding to changing environmental conditions and higher nutrient availability (Bolton et al., 2016; Toth et al., 2020; Steinhagen et al., 2022). Recently, we found that *Ulva fenestrata* Postels & Ruprecht increases both its growth and crude protein content when cultivated in a wide variety of food production process waters (e.g. from shrimp, herring, and oat-milk production) (Stedt et al., 2022b), and there is a great potential to cultivate *U. fenestrata* with many different kinds of nutrient rich waters (Arumugam et al., 2018; Stedt et al., 2022a).

Water is used in almost every step of food processing, and for some seafood up to 50 m³ process water is used per tonne of final product (Forghani et al., 2020a; Forghani et al., 2020b). These process waters contain a wide range of nutrients and can be used for downstream recovery of macronutrients or as feed stock e.g. for microorganisms while they are still in a food grade state (Osman et al., 2015; Forghani et al., 2020a; Forghani et al., 2020b; Forghani et al., 2022). By integrating seaweed cultivation with industries that generate large quantities of nutrient rich process waters, some of the major challenges for land-based seaweed cultivations such as seawater intake and costly

Abbreviations: HPPW(s), herring production process water(s); TUB, tub water from in-house pre-processing storage of whole herring at a primary processor; SAL, salt brine from maturation of herring fillets at a secondary processor; SW, seawater control; US EPA, United States Environmental Protection Agency; EFSA, European Food Safety Authority; RfD, reference doses for heavy metals set by US EPA; MLs, maximum allowed levels of heavy metals in foodstuff set by the European Union's Commission Regulation (EC) No. 1881/2006 (version 03/05/2022).

infrastructure, can be reduced. The possibility for an industrial symbiosis between food processing industries and seaweed cultivation is thus high.

Despite all the promising health benefits of eating seaweeds, it is important to determine possible toxic elements in the biomass to estimate potential health risks associated with its consumption (Almela et al., 2002; Mæhre et al., 2014; Neveux et al., 2016; Arumugam et al., 2018). To date, there are no general regulations from the European commission regarding maximum levels of heavy metals in seaweeds intended as food (European Commission, 2018). But as the interest for consuming seaweeds is growing outside of Asia, it is increasingly important to assess the seaweeds' contribution to human exposure of heavy metals on the Western market. To establish the true potential of *U. fenestrata* as a food source, it is therefore important to quantify and evaluate heavy metals in the biomass originating from different cultivation settings, and to report realistic values of how much biomass that can safely be consumed each day.

Our goal in this study was twofold, firstly we wanted to assess if the increased growth and crude protein content of *U. fenestrata* cultivated in two herring production process waters (HPPWs) that we previously reported (Stedt et al., 2022b) were maintained when the cultivation setting was up-scaled. Secondly, we wanted to establish if *U. fenestrata* cultivated in these two HPPWs can become a nutritionally sound and safe protein source for the future. We answered these questions in two experiments. In the first experiment we investigated how growth, crude protein content, and content of some health concerning toxic elements (commonly grouped as “heavy metals”: arsenic, mercury, lead, and cadmium) in *U. fenestrata* were affected by cultivation for 28 days in the two different HPPWs. Based on the results from the first experiment, we ran a second experiment for 14 days where we cultivated *U. fenestrata* in the two HPPWs, this time monitoring growth, crude protein content and amino acid composition of *U. fenestrata*.

Materials and methods

Algal material and taxonomic identification

Algal material were collected from a long-term indoor tank culture at Tjärnö Marine Laboratory (TML, 58°52'33.7'' N, 11° 08'44.9'' E), where gametophytes of *U. fenestrata* were cultivated in 90 L cultivation tanks, at 14°C, under 16:8 h (L:D) light cycle, at an irradiance of 100 μmol m⁻² s⁻¹ and in seawater supplemented by half strength Provasoli Enriched Seawater (PES) (Provasoli, 1968; Redmond et al., 2014). Molecular identification of the algal material used in the present study has been described by Toth et al. (2020) (GenBank accession numbers: MN240309, MN240310, MN240311).

Herring production process waters

The two herring production process waters (HPPWs) used in the experiments were collected in a food grade state in February 2021. The process waters included: (i) tub water (TUB) from in-house pre-processing storage of whole herring in 3% NaCl at a primary processor (Sweden Pelagic AB, Ellös, Sweden), and (ii) saturated salt brine (SAL) from maturation of herring fillets at a secondary processor (Klädesholmen Seafood AB, Rönning, Sweden). A detailed explanation of the HPPWs has been described by Stedt et al. (2022b). After collection, the waters were filtered to remove coarse particles (> 300 μm) and stored at - 60°C. The HPPWs were quantified for ammonium, nitrate and nitrite using commercial enzymatic kits following the method described in Stedt et al. (2022b), while the inorganic phosphorus/orthophosphate content was measured with a standard curve made with monopotassium phosphate as reported by Qvirist et al. (2015). The HPPWs were diluted with filtered (0.2 μm + UV-light treated) deep-sea (40 m) seawater to 25 μM NH_4^+ in both experiments (Table 1).

Experimental setup

The effect of HPPWs as cultivation media on growth and chemical composition of *U. fenestrata* was explored in two different land-based laboratory experiments. Both experiments used nine 14 L tanks filled to 10 L at 14°C, under 16:8 h (L:D) light cycle, and at an irradiance of 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (light source: INDY66 LED 60 W 4000 K 6000 lm). The experiments included the two HPPWs TUB and SAL diluted to 25 μM NH_4^+ , as well as a seawater control (SW) set up in triplicates (Table 1). Both salinity (WTW MultiLine 3420, Xylem Analytics) and pH (Mettler Toledo FiveEasy bench meter) remained stable throughout the experiments in all treatments (34.0 ± 1.0 ppt and 7.9 ± 0.2 respectively, mean \pm SD). Every second day the tanks were cleaned, and the respective waters were renewed to avoid nutrient depletion and microbial spoilage. The tanks were spatially interdispersed to avoid position biases.

In the first experiment, lasting 28 days, *U. fenestrata* (approximately 7 cm^2) were cleaned and divided into the tanks at a density of 0.3 g L^{-1} fresh weight (fw). The total fw

of the *U. fenestrata* in each tank was measured every seventh day, crude protein content was measured bi-weekly, while heavy metal content was measured at the end of the experiment.

Based on the significant differences in fw and crude protein content between the seawater control and the HPPWs recorded after 14 days in the first experiment, the second experiment lasted for only 14 days. The *U. fenestrata* were cleaned and cut into 30 cm^2 discs and added to the tanks at a density of 0.3 g L^{-1} fw. The total fw and crude protein content of the *U. fenestrata* in each tank were measured on day 7 and 14. The amino acid composition of *U. fenestrata* was measured at the end of the experiment.

Growth

The biomass yield (fw) in each tank was determined in a standardized way by weighing the *U. fenestrata* on a lab-scale (Sartorius TE1502S, Göttingen, Germany), after removing the excess water with a salad spinner.

Crude protein content

Tissue samples from each tank were haphazardly collected, frozen, freeze-dried and homogenized to a fine powder. Total nitrogen content was analyzed using combustion GSL elemental analyzer coupled to an isotope-ratio mass spectrometer (EA-IRMS, 20 - 22, Sercon Ltd., Crewe UK). The nitrogen content was converted to crude protein content using a conversion factor of five (Angell et al., 2016).

Heavy metal content and exposure assessment

For the first experiment, random freeze-dried, homogenized tissue samples (0.5 g dw) from two tanks per HPPW treatment were analyzed for heavy metal content. Total arsenic (tAs), mercury (Hg), lead (Pb), and cadmium (Cd) were quantified at the Swedish Food Agency, Uppsala, Sweden, using inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7700x)

TABLE 1 Characterization of the seawater (SW) that was used as the control and to dilute the herring production process waters (HPPWs), as well as the undiluted HPPWs SAL and TUB (mean \pm SEM, n = 3) collected in February 2021.

	pH	Ammonium (mM NH_4^+)	Nitrate (mM NO_3^-)	Nitrite (mM NO_2^-)	Inorganic phosphorus (mM P)	N:P ratio	Dilution factor with SW to reach 25 μM NH_4^+
SW	7.9	< 0.0004	< 0.006	n.d.	< 0.0005	12:1	
SAL	5.8	8.0 ± 0.5	n.d.	n.d.	25.9 ± 0.2	1:3	321
TUB	6.8	2.3 ± 0.2	n.d.	n.d.	22.1 ± 0.1	1:9	92

n.d., non detectable.

following the procedures of NMKL no. 186 and EN 15763:2009, which includes total microwave acid digestion of the samples using a mixture of concentrated nitric acid and hydrochloric acid (6 + 1 mL). The determination of inorganic arsenic (iAs) was performed at the Swedish Food Agency, Uppsala, Sweden, according to the European Standard EN 16802:2016. The method is based on extraction with dilute nitric acid and hydrogen peroxide in hot water bath, followed by analysis with strong anion exchange HPLC-ICP-MS (Agilent 1260 Infinity Quaternary LC and Agilent 7700x ICP-MS). The method performances are described in Kollander et al. (2019). The heavy metal contents were compared to the maximum allowed levels in foodstuff (MLs) set by the European Union's Commission Regulation (EC) No. 1881/2006 (version 03/05/2022). Furthermore, a simple exposure assessment was performed to establish how much unprocessed dried seaweed biomass that can be consumed without exceeding the reference doses (RfD) set by US Environmental Protection Agency (US EPA):

$$\text{Eq.1} \quad \text{Maximum intake} = \frac{\text{RfDi} \times \text{bw}}{C_i}$$

Where RfDi is the reference dose for heavy metal i ($\mu\text{g kg bw}^{-1} \text{ day}^{-1}$) (US EPA, 2007), bw is reference body weight for adults (63.3 kg) (EFSA, 2017), and C_i is the content of heavy metal i in dry seaweed biomass ($\mu\text{g g dw}^{-1}$). Because iAs is the most toxic species of arsenic, RfD values are only established for this form of As (US EPA, 2007). The US EPA RfD was chosen over the EU Tolerable daily intake (TDI) in order to simplify comparisons with other publications in the area.

Amino acid composition and human requirements

Total amino acids (TAA) of *U. fenestrata* in the second experiment were analyzed following the method in Trigo et al. (2021), with minor modifications. All measurements were made in triplicates. First, 100 mg of the homogenized seaweed tissue samples and 4 mL of 6 M HCl were mixed in a screw-cap glass tubes, after which the air in the tubes was replaced with nitrogen. The samples were hydrolyzed at 110°C for 24 h using a heat block, and the samples were then diluted with 0.2 M acetic acid and filtered (0.22 μm ; Fisher Scientific). Two μL of each sample were run in a LC/MS (Agilent 1100 HPLC) with a Phenomenex column (C18 250 μm x 4.6 μm x 3 μm), coupled to an Agilent 6210 quadrupole in the SIM positive mode (Agilent Technologies). Separation at 0.7 mL min^{-1} for 40 min was performed using different ratios of mobile phase A (3% methanol, 0.2% formic acid and 0.01% acetic acid) and mobile phase B (50% methanol, 0.2% formic acid and 0.01% acetic acid). A mix of 17 amino acids (Thermo Scientific) was used for the calibration curve. Due to the use of acidic hydrolysis, (i) tryptophan and cysteine could not be recovered, and (ii)

glutamine and asparagine were co-determined with glutamic and aspartic acid, respectively. MassHunter Quantitative Analysis software (version B.09.00, Agilent Technologies) was used to analyze the data.

The required consumption of dried seaweed biomass per day, in order to receive all the essential amino acids, was established with the following equation:

$$\text{Eq.2} \quad \text{Required consumption} = \frac{\text{rEAA}_i \times \text{bw}}{\text{EAA}_i}$$

Where rEAA $_i$ is the essential amino acid requirement per day ($\text{mg kg bw}^{-1} \text{ day}^{-1}$) provided by World Health Organization (WHO/FAO/UNU, 2007), bw is the reference body weight for adults (63.3 kg) (EFSA, 2017), and EAA $_i$ is the amino acid content of amino acid i in dry seaweed biomass (mg g dw^{-1}).

Statistical analysis

All statistical analyses were performed in RStudio (v. 1.2.5001). All data were visually checked for homogeneity and normality with diagnostic plots (density-, normality- and QQ-plots). Furthermore, normality was checked with Shapiro Wilks test for each combination of factor levels, and Levene's test was used to test for homogeneity of variance at each level of time. Growth and crude protein content were analyzed using a two-way mixed model ANOVA with day as within (repeated measures) and treatment as between subject factors. Comparisons between treatment and day were analyzed *post-hoc* with pairwise paired t-test (Bonferroni corrected). Amino acids were analyzed with one-way ANOVA followed by Tukey's *post-hoc* test for pairwise comparison.

Results

Growth

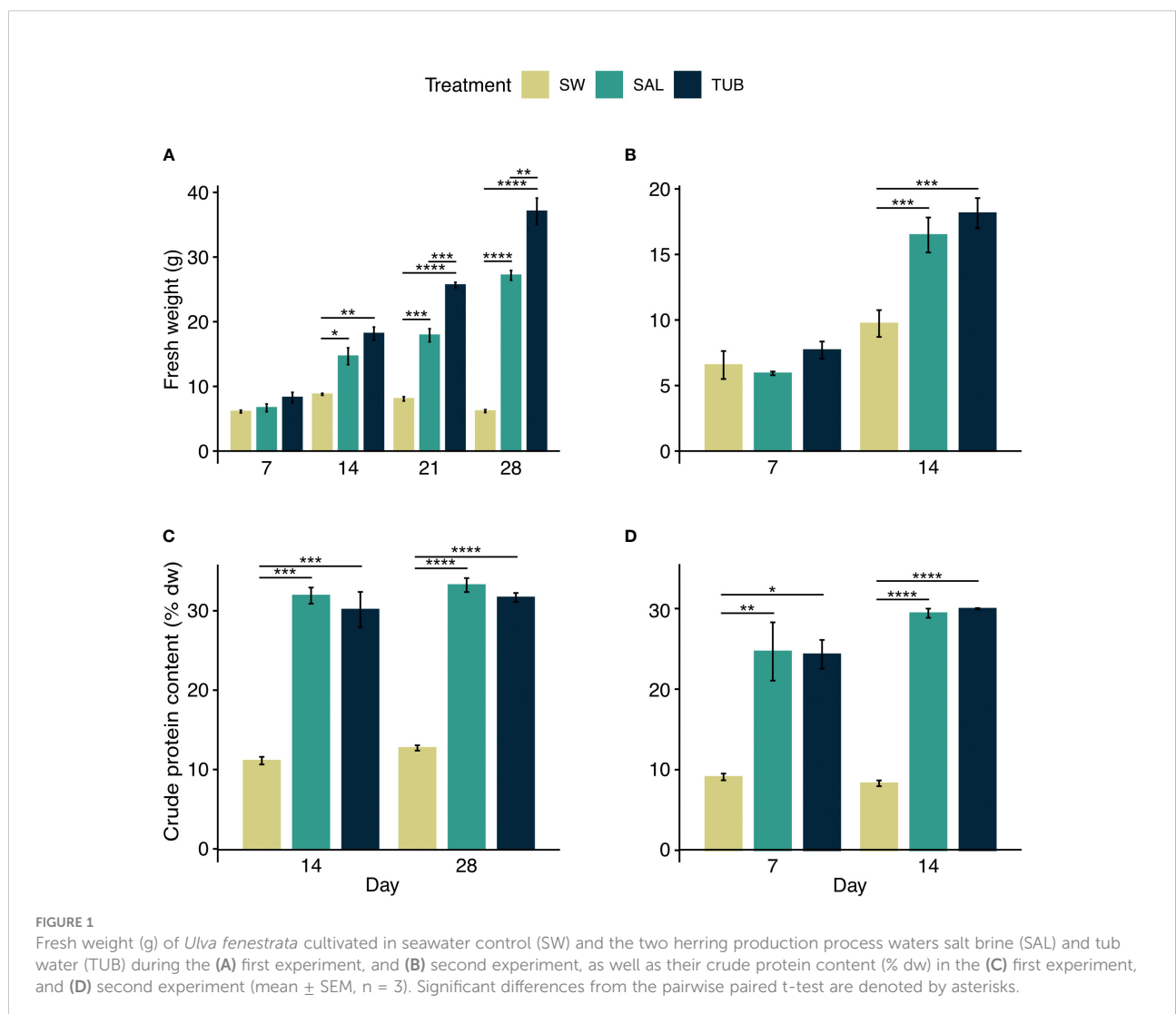
In the first experiment there was a statistically significant interaction between treatment and time in explaining growth (Table 2). After 28 days the biomass yield had increased 13 times in TUB ($37.07 \pm 2.05 \text{ g fw}$), nine times in SAL ($27.17 \pm 0.75 \text{ g fw}$), but only two times in SW ($6.18 \pm 0.21 \text{ g fw}$, mean \pm SEM) (Figure 1A). This led to four to six times higher biomass yield in the HPPWs compared to in the seawater control. The effect of treatment was significant at day 14, 21, and 28 ($p < 0.005$), and the pairwise comparisons show that the mean fw was significantly higher in TUB and SAL compared to SW on day 14, 21 and 28 ($p < 0.05$). TUB had significantly higher fw compared to SAL on day 21 and 28 ($p < 0.005$).

In the second experiment, there was a statistically significant interaction between treatment group and time in explaining

TABLE 2 Two-way mixed model ANOVA (Type III) of fresh weight (g) and crude protein content (% dw) in the (A) first, and (B) second experiment of *Ulva fenestrata* cultivated in the two different herring production process waters (SAL and TUB), as well as the seawater control (SW).

	Fresh weight (g)						Crude protein content (% dw)					
(A) First exp.												
Effect	SS	MS	df ₁	df ₂	F	<i>p</i>	SS	MS	df ₁	df ₂	F	<i>p</i>
Treatment	1225.8	612.9	2	6	256.6	< 0.001	630.4	315.2	2	5	226.38	< 0.001
Day	1269.3	423.1	3	18	177.1	< 0.001	8.0	8.0	1	5	5.01	0.08
Treatment x Day	723.6	120.6	6	18	50.5	< 0.001	0.08	0.04	2	5	0.09	0.92
(B) Second exp.												
Treatment	13.2	6.6	2	6	7.30	< 0.05	1325.4	662.7	2	6	79.3	< 0.001
Day	292.2	292.2	1	6	324.0	< 0.001	46.2	46.2	1	6	5.5	0.06
Treatment x Day	53.8	26.9	2	6	29.9	< 0.001	36.6	18.3	2	6	2.2	0.19

Significant *p*-values are indicated with italics. Data are visualized in Figure 1.



growth (Table 2). The biomass yield had increased six times in TUB (18.15 ± 1.15 g fw), five times in SAL (16.49 ± 1.33 g fw), and three times in SW (9.73 ± 1.02 g fw, mean \pm SEM) after 14 days (Figure 1B), which led to double the biomass yield in the HPPWs compared to in the seawater control. The effect of treatment was significant after 14 days ($p < 0.005$), and the pairwise comparisons show that the mean fw was significantly higher in TUB and SAL compared to SW ($p < 0.05$).

Crude protein content

In the first experiment, seaweeds reached crude protein contents of 31.67 ± 0.57 , 33.24 ± 0.88 , and $12.72 \pm 0.34\%$ dw (mean \pm SEM) in TUB, SAL, and SW, respectively (Figure 1C). There was no statistically significant interaction between treatment group and time explaining crude protein content (Table 2). The statistical analysis showed that treatment was the factor influencing the crude protein content, and that there was no statistically significant effect of days (Table 2). The crude protein content of the seaweed was nearly three times higher in both the HPPWs compared to in the seawater control after both 14 ($p < 0.001$) and 28 days ($p < 0.001$).

In the second experiment, seaweeds reached crude protein contents of 29.98 ± 0.08 , 29.42 ± 0.56 , and $8.31 \pm 0.36\%$ dw (mean \pm SEM) in TUB, SAL, and SW, respectively (Figure 1D). There was no statistically significant interaction between treatment group and time explaining crude protein content (Table 2). The statistical analysis showed that treatment was the factor influencing the crude protein content, and that there was no statistically significant effect of days (Table 2). The crude protein content of the seaweed was almost three times higher in both the HPPWs compared to in the seawater control after seven days ($p < 0.05$), and four times higher after 14 days ($p < 0.001$).

Heavy metal content and exposure assessment

The heavy metal content of *U. fenestrata* cultivated in TUB and SAL from the first experiment were all below the EU maximum levels (MLs) in foodstuff. For TUB the Hg content of the biomass was below the method's quantification limit ($0.036 \mu\text{g g dw}^{-1}$) (Table 3). The tAs of *U. fenestrata* cultivated in TUB ($106.95 \pm 6.14 \mu\text{g g dw}^{-1}$) was higher than when cultivated in SAL ($4.85 \pm 0.10 \mu\text{g g dw}^{-1}$, mean \pm SEM). However, the percentage of iAs was only 0.02% of tAs for biomass cultivated in TUB, and 0.32% in SAL. The level of iAs in both HPPWs was $0.02 \mu\text{g g dw}^{-1}$, which is well below the EU MLs for iAs in food ($0.1 - 0.3 \mu\text{g g}^{-1}$, Table 3).

The simple exposure assessment indicates that it is possible to eat approximately 90 g dw of *U. fenestrata* cultivated in SAL (limiting heavy metal Hg), and 160 g dw of *U. fenestrata* cultivated in TUB (limiting heavy metal Cd) daily before exceeding the RfDs of US EPA (Table 4).

Amino acid composition and human requirements

All amino acids (AA) were present at significantly higher levels in *U. fenestrata* cultivated in the HPPWs compared to in the seawater control (Table 5), with no difference between TUB and SAL. Most AA were two to three times higher in seaweeds cultivated in HPPWs compared to in SW ($p < 0.05$), except for lysine and glutamic acid, which were four times higher ($p < 0.05$), and methionine and arginine which were six to eight times higher ($p < 0.05$). As a result of the high amount of some non-essential AA such as glutamic acid and arginine in biomass cultivated in the HPPWs, the relative TEAA was lower in the HPPWs compared to in the SW ($p < 0.05$).

TABLE 3 Heavy metal content ($\mu\text{g g dw}^{-1}$) in *Ulva fenestrata* cultivated in the two herring production process waters salt brine (SAL) and tub water (TUB) after 28 days (mean \pm SEM, $n = 2$), as well as the maximum allowed levels in foodstuff (MLs) set by the European Union's Commission Regulation (EC) No. 1881/2006 (version 03/05/2022).

	Content in biomass* $\mu\text{g g dw}^{-1}$		MLs in foodstuff $\mu\text{g g}^{-1}$
	SAL	TUB	
Total arsenic (tAs)	4.85 ± 0.10	106.95 ± 6.14	Not established
Inorganic arsenic (iAs)	0.016 ± 0.001	0.018 ± 0.001	0.1** - 0.3
Mercury (Hg)	0.21 ± 0.01	< 0.036	0.1 - 1
Lead (Pb)	1.45 ± 0.09	0.97 ± 0.15	0.02** - 3***
Cadmium (Cd)	0.21 ± 0.01	0.39 ± 0.01	0.01** - 3***

*The amount of biomass from the seawater control were not sufficient for the determination of the heavy metals.

**Food destined for babies and young children (for iAs rice intended for baby food, Pb cereal based food, and for Cd young children formulae).

***Food supplements as sold, consisting exclusively or mainly of dried seaweed, products derived from seaweed, or of dried bivalve mollusks.

TABLE 4 Exposure assessment indicating how much dried biomass of *Ulva fenestrata* cultivated in the two herring production process waters salt brine (SAL) and tub water (TUB) that can be consumed before exceeding the reference dose (RfD) for each heavy metal, as indicated by the US Environmental Protection Agency (US EPA, 2007).

	RfD $\mu\text{g kg bw}^{-1} \text{ day}^{-1}$	Max. seaweed biomass intake per day	
		SAL g dw day^{-1}	TUB g dw day^{-1}
Inorganic arsenic (iAs)	0.3	1220	1030
Mercury (Hg)	0.3	90	> 480
Lead (Pb)	3.6*	160	240
Cadmium (Cd)	1	300	160

*In 2004 US EPA decided against a reference value for lead, and according to EFSA, 2010, the tolerable daily intake of lead is $0.63 \mu\text{g kg bw}^{-1} \text{ day}^{-1}$. The maximum biomass intake per day is calculated by Eq. 1 and the body weight is based on the reference body weight for adults (63.3 kg) (EFSA, 2017). The maximum biomass intake is indicated with red color.

Discussion

Land-based cultivation of *Ulva* has several advantages over sea-based cultivations, such as full control of environmental cultivation conditions and easy accessibility and harvest of the biomass (Hafting et al., 2012). However, even if there are some commercial land-based cultivations (such as Algalplus, Monterey Bay Seaweed, Blue Evolution, and Nordic Seafarm), they are often costly to operate and maintain, which has limited the commercial interest of the systems (Titlyanov and Titlyanova, 2010; Hafting et al., 2012; Hafting et al., 2015). In this study, we show that one way to maximize the value of land-based produced seaweed biomass is to integrate *U. fenestrata* cultivation with high-nutrient food production process waters from the herring processing industry.

Proteins and amino acids

We show that *U. fenestrata* responds quickly to the addition of the process waters (HPPWs) by increasing its crude protein content and TAA significantly. After only seven days the *U. fenestrata* in HPPWs have reached crude protein contents of 24 - 25% dw compared to 9% dw in the seawater control. These values are already within the high range for *U. fenestrata* (Holdt and Kraan, 2011; Toth et al., 2020; Steinhagen et al., 2021), but after another seven days the crude protein content was over 30% dw. This crude protein content is comparable to soybean (35 - 40% dw) (Grieshop and Fahey, 2001; Thakur and Hurburgh, 2007), and pulses such as lentils, chickpeas and beans, in which crude protein makes up 17 - 30% dw (Boye et al., 2010). The rapid increase in crude protein content indicates that the biomass from these cultivation settings can become an alternative protein source for human consumption, comparable to high-protein terrestrial crops.

Most seaweed species contain all the essential amino acids (Fleurence, 2004), and the amino acid profile of the *U. fenestrata* cultivated in our experiment revealed that cultivation in HPPWs

further increased the levels of all amino acids along with the buildup of proteins. The lower TAA content compared to the crude protein content can possibly be explained by the selected method for the amino acid analysis, in which the acidic hydrolysis prevents the recovery of tryptophan and cysteine, and the use of single hydrolysis time may destroy some amino acids (Angell et al., 2016). For *U. fenestrata* cultivated in the seawater control, the limiting amino acid was methionine, which is generally perceived as a limiting amino acid in seaweed proteins (Holdt and Kraan, 2011; Thiviya et al., 2022). However, when cultivating *U. fenestrata* in the HPPWs, methionine increased over six times and was no longer limiting. Lysine, another important EAA that is often limiting in proteins from seaweeds (Holdt and Kraan, 2011; Thiviya et al., 2022), follows the same pattern as methionine and was not limiting in *U. fenestrata* cultivated in the HPPWs. To receive all required EAA only from *U. fenestrata*, a person would need to eat around 230 g dw of the HPPW-treated seaweeds and 1510 g dw of the control seaweeds per day. These amounts are very high compared to the average consumption of seaweed biomass in Japan ($4.0 \text{ g dw day}^{-1}$), China ($5.2 \text{ g dw day}^{-1}$), and South Korea ($8.5 \text{ g dw day}^{-1}$) (Chen et al., 2018). Nevertheless, when compared to the amount of soybean intake to reach the EAA recommendations (170 g dw, based on average soybean AA profile from Brazil, China, and the US) (Grieshop and Fahey, 2001), seaweeds should be considered an interesting future protein source. Furthermore, recent studies on *Ulva* show that its proteins can be concentrated by different extraction processes (Harrysson et al., 2019; Trigo et al., 2021; Juul et al., 2022), in a similar manner as is currently done with soy, which further enhances the prospects of *U. fenestrata* biomass as a protein source for human consumption.

Biomass yields

Land-based *Ulva* cultivations often use fertilizers to obtain high growth rates, as *Ulva* species are known to respond with increasing growth when cultivated in nutrient rich waters. Our

TABLE 5 Amino acid composition (mg g dw⁻¹) of dried biomass of *Ulva fenestrata* cultivated in seawater control (SW) and the two herring production process waters salt brine (SAL) and tub water (TUB) (mean ± SEM, n = 3) after 14 days, as well as the biomass (g dw day⁻¹) consumption needed to reach the recommended daily intake of essential amino acids (EAA) (Eq. 2, based on a body weight of 63.3 kg) as provided by the World Health Organization (WHO/FAO/UNU, 2007).

Amino acid	Amino acid content mg g dw ⁻¹ (% of TAA)			Biomass consumption to reach EAA requirement g dw day ⁻¹		
	SW	SAL	TUB	SW	SAL	TUB
Glycine	5.77 ± 0.38 ^b (7.28)	14.71 ± 0.19 ^a (6.12)	14.12 ± 0.34 ^a (6.29)			
Alanine	8.38 ± 0.45 ^b (10.58)	20.67 ± 0.25 ^a (8.60)	20.61 ± 0.37 ^a (9.19)			
Serine	5.03 ± 0.37 ^b (6.35)	13.47 ± 0.29 ^a (5.61)	12.40 ± 0.52 ^a (5.53)			
Proline	3.71 ± 0.34 ^b (4.69)	13.78 ± 0.55 ^a (5.74)	13.88 ± 0.64 ^a (6.19)			
Valine	5.16 ± 0.43 ^b (6.52)	14.05 ± 0.26 ^a (5.85)	13.21 ± 0.31 ^a (5.89)	318	117	125
Threonine	5.63 ± 0.40 ^b (7.10)	14.29 ± 0.23 ^a (5.95)	13.34 ± 0.35 ^a (5.95)	169	66	71
Isoleucine	3.01 ± 0.30 ^b (3.80)	9.01 ± 0.27 ^a (3.75)	8.26 ± 0.24 ^a (3.68)	420	140	153
Leucine	6.71 ± 0.41 ^b (8.47)	14.62 ± 0.58 ^a (6.09)	14.06 ± 0.29 ^a (6.27)	368	168	175
Aspartic acid*	11.05 ± 0.85 ^b (13.95)	28.99 ± 0.49 ^a (12.07)	27.67 ± 0.78 ^a (12.33)			
Lysine	2.59 ± 0.35 ^b (3.27)	11.71 ± 0.31 ^a (4.88)	10.59 ± 0.49 ^a (4.72)	733	162	179
Glutamic acid*	10.56 ± 0.80 ^b (13.33)	44.53 ± 2.63 ^a (18.54)	40.93 ± 1.55 ^a (18.24)			
Methionine	0.42 ± 0.11 ^b (0.53)	3.54 ± 0.31 ^a (1.47)	2.82 ± 0.11 ^a (1.26)	1508	179	224
Histidine	1.06 ± 0.04 ^b (1.34)	2.82 ± 0.07 ^a (1.17)	2.67 ± 0.09 ^a (1.19)	595	225	237
Phenylalanine	4.21 ± 0.43 ^b (5.32)	11.08 ± 0.15 ^a (4.61)	10.54 ± 0.26 ^a (4.70)	376	143	150
Arginine	2.09 ± 0.13 ^b (2.64)	15.81 ± 1.07 ^a (6.58)	12.44 ± 1.01 ^a (5.54)			
Tyrosine	3.82 ± 0.06 ^b (4.83)	7.11 ± 0.14 ^a (2.96)	6.79 ± 0.14 ^a (3.03)			
TAA (% dw)	7.92 ± 0.57 ^b	24.02 ± 0.21 ^a	22.43 ± 0.67 ^a			
TEAA (%)	36.27 ± 0.44 ^a	33.78 ± 0.73 ^b	33.66 ± 0.09 ^b			

TAA total amino acids, TEAA total essential amino acids.

*Glutamine and asparagine were co-determined with glutamic and aspartic acid, respectively.

Significant differences between treatments are denoted by superscript letters, EAA are indicated by bold font, and the biomass consumed to reach the EAA requirement is indicated with red color.

results correspond well with previously reports on cultivation of *Ulva* spp. in nutrient rich side streams from the aquaculture sector (Ashkenazi et al., 2019; Nardelli et al., 2019). Extrapolating our results, using a dw:fw ratio of 20:80, indicates a production of 14 - 19 t dw ha⁻¹ y⁻¹ when cultivating *U. fenestrata* in the HPPWs. This is lower than estimates from fertilized tank cultivation of *Ulva* spp. (46 t dw ha⁻¹ y⁻¹) (Gadberry et al., 2018), but several folds higher than estimated average ocean-based *Ulva* spp. farming yields (4 t dw ha⁻¹ y⁻¹) (Savvashe et al., 2021; Nordic Seafarm, 2022) and soybean yields (< 3 t dw ha⁻¹ y⁻¹) (Ainsworth et al., 2012; FAO, 2021). It is important to note that this study did not focus on

obtaining optimal biomass yields, and the start density used in this experiment (0.3 g L⁻¹ fw) was very low compared to possible cultivation densities (> 12 g L⁻¹ fw; Mata et al. (2010); Al-Hafedh et al. (2015); Gadberry et al. (2018), and personal experience). The biomass yields could therefore be optimized, further increasing the productivity of the cultivation system. However, the results need to be confirmed in an upscaled setting where a continuous supply of process water is ensured.

The decrease in fw between day 14 and 28 for *U. fenestrata* in the seawater control can be explained by a sporulation event that was observed during this experiment. When seaweeds sporulate,

most of their energy is allocated from growing to releasing swimmers (in *Ulva* either zoids or gametes) (Han et al., 2003). Interestingly, we did not observe sporulation events in *U. fenestrata* cultivated in the HPPWs. It can only be speculated why the *U. fenestrata* did not sporulate in the HPPWs, as sporulation events are often triggered by changes in environmental conditions or nutrient availability (Balar and Mantri, 2019). Sporulation events can be detrimental to commercial *Ulva* cultivations, as large amounts of biomass are lost. Identifying potential sporulation inhibitors would further increase the value of the HPPWs as cultivation media.

Heavy metals

Seaweeds may accumulate toxic elements, and there have been concerns about the risk of elevated levels of the carcinogenic inorganic form of arsenic (iAs) in seaweed biomass (Duinker et al., 2020; Blikra et al., 2021). In this study we show that iAs constitutes $0.02 \mu\text{g g dw}^{-1}$ of the *U. fenestrata* cultivated in HPPWs, which is below the EU maximum levels (MLs) in rice and rice products ($0.1 - 0.3 \mu\text{g g dw}^{-1}$) (European Commission, 2006; European Commission, 2022). However, it is important to note that the MLs reflect the levels, size of consumption, and toxicity in foodstuff occurring at the market. New food items on the market and/or changed consumption patterns will eventually call for new risk assessments and new MLs (EFSA, 2005). The heavy metals limiting the seaweed biomass consumption were according to US EPA reference doses mercury in the biomass cultivated in SAL and cadmium when cultivated in TUB. To reach these reference doses, as much as 90 g dw of *U. fenestrata* biomass cultivated in SAL and 160 g dw cultivated in TUB can be consumed each day. It should be noted that the US EPA decided against a reference value for lead in 2004, and according to the European Food Safety Authority (EFSA) the tolerable daily intake is $0.63 \mu\text{g kg bw}^{-1} \text{ day}^{-1}$ (EFSA, 2010). Using the EFSA reference value results in seaweed biomass consumption being limited by lead in both HPPW-treatments. Since both US EPA (2007) and EFSA (2010) stress the toxicity of lead, the intake should be kept as low as possible. When cultivating seaweeds in tanks on land it is possible to select for the best quality of water, and make sure there are no leakage of lead from pipes, while lead levels may also be reduced using ion exchange methods as a cleaning pre-step prior to using the process water for food production (EFSA, 2012; Lalmi et al., 2018; European Commission, 2022).

It should be noted that our estimations are based on the EFSA reference body weight for adults (63.3 kg), which is low compared to the average weight in America, Australia, and Europe (above 75 kg) (worlddata.info, 2022), and that all the heavy metals are

bioavailable. When compared to the average seaweed biomass intake in Japan, China, or South Korea, and the level of heavy metals in *U. fenestrata* cultivated in HPPWs, the potential health risk for humans can be considered low. Furthermore, processing of the biomass, such as blanching, washing, and boiling, reduces the content of some heavy metals in seaweed biomass by up to 40 - 60% (Hanaoka et al., 2001; Stévant et al., 2018; Ownsworth et al., 2019; Blikra et al., 2021), and protein extraction processes may decrease the level of heavy metals which are not bound to proteins. Together, this further increases the possible amount of biomass that can safely be consumed.

Conclusion

In conclusion, our study shows that herring production process waters (HPPWs) can effectively be used as *U. fenestrata* cultivation media. In 7 - 28 days, we managed to produce *U. fenestrata* biomass with four to six times higher biomass yields (27.17 - 37.07 g fw vs. 6.18 g fw) and almost three times higher crude protein content (31.57 - 33.24% dw vs. 12.72% dw) when cultivated in HPPWs compared to in seawater. The increased protein content also translated to significantly higher EAA content with e.g. methionine content over six times higher in HPPWs compared to in seawater (2.82 - 3.54 mg g dw⁻¹ vs. 0.42 mg g dw⁻¹). Furthermore, the heavy metal content in the biomass was below the maximum allowed levels in foodstuff set by the commission of the European communities. Combined, our results show a great potential for the herring processing industry and the cultivators of *U. fenestrata* to jointly produce sustainable proteins for the future.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Author contributions

KS, conceptualization, formal analysis, investigation, methodology, project administration, supervision, validation, visualization, writing – original draft, and review and editing. OG, conceptualization, investigation, methodology, writing – review and editing. BK and IU, funding acquisition, resources, writing – review and editing. GT, supervision, validation, writing – review and editing. HP, funding acquisition, resources, supervision, validation, writing – review and editing. All authors contributed to the article and approved the submitted version.

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