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Cultivation of seaweeds in food production process waters: Evaluation of growth and crude protein content

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ABSTRACT

There is an increasing demand for sustainably produced, protein-rich, and nutritious food. Seaweeds are promising protein sources for the future if their protein content can be optimized, something which can be achieved by cultivation in elevated nutrient concentrations. Cultivation of seaweeds in integration with fish farms have received much attention lately, but using nutrient-rich process waters from other food industries as feed stock for seaweed has rarely been studied. Here, we demonstrate a simple and sustainable strategy to answer the increasing world demand for food rich in plant-based proteins by connecting food production process waters with seaweed cultivation. We compared growth rates and crude protein content of four different seaweed species, the brown species *Saccharina latissima*, and the green species *Ulva fenestrata*, *Ulva intestinalis*, and *Chaetomorpha linum*, when cultivated in two dilutions (providing 20 and 200 μM ammonium) of eight different process waters emerging from recirculating salmon aquaculture systems as well as from herring, shrimp and oat processing. Growth rates of the green seaweeds were up to 64% higher, and crude protein content was almost up to four times higher when cultivated in the food production process waters, compared to seawater controls. Growth rates were generally higher in presence of 20 μM compared to 200 μM ammonium, while crude protein content was either unaffected or positively affected by the increasing ammonium concentration. This study indicates the potential for cultivating seaweeds with food production process waters to generate additional protein-rich biomass while nutrients are being circulated back to the food chain. A new nutrient loop is thus illustrated, in which the costly disposal of food production process waters is instead turned into value by seaweed cultivation.

1. Introduction

The world population growth increases the demand for protein-rich and nutritious food that is sustainably produced [1,2]. Seaweeds have high productivity compared to many terrestrial crops such as wheat, seeds, and soybean [3], while also having a favorable amino acid profile for human consumption [4]. Based on existing studies, seaweed species like *Porphyra yezoensis* can reach up to 47% protein on a dry weight (dw) basis in extreme cases, but more commonly reported levels for seaweeds fall within 5–25% protein (dw) [5,6]. Therefore, to make seaweeds competitive protein sources there are incentives to raise their growth rates and protein content further.

Several studies have reported benefits of cultivating seaweeds in association with both land-based and sea-based aquaculture [7–9]. For example, *Gracilaria chilensis* had 81% higher growth, and 15% higher nitrogen content when cultivated 100 m compared to 7000 m (control) from a salmon farm [10], while *Ulva rigida* had almost three times higher growth and nitrogen content when cultivated in sea bream cultivation wastewater compared to in seawater [11]. Cultivation of seaweeds in such integrated multi-trophic aquaculture (IMTA) systems has been widely studied in recent years (e.g. [12–14]), while little attention has been given to other types of nutrient-rich industrial side streams. Some studies have cultivated seaweed in waters with nutrient concentrations simulating those of industrial process waters [15], however, cultivation in waters actually emerging from industrial practices is little explored, but needed as their complex characteristics may affect the seaweed differently than simulated waters. To date, there are no reported studies on seaweed cultivation in outlet waters from the food processing industry [9], although these provide a wide range of nutrients and can be tapped off while they are still in a food grade state [16–18].

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Water is used in almost every step of food processing and are often rich in high-value compounds such as micronutrients, proteins, and long chain n-3 fatty acids [18,19]. The proteins and lipids can be recovered by a coagulation-flocculation technique [16,17]. However, the remaining dissolved inorganic nutrients such as nitrogen and phosphorus are still lost from the food processing industries in large quantities every year [16]. Microalgae have successfully been cultivated in different food industry process waters to minimize the discharge of nutrients [20,21], but the process of harvesting microalgae is difficult and energy consuming [22]. Cultivation of seaweed in food industry-derived process waters provides a chance to recycle the nutrients, while at the same time generating biomass yields with increased protein content which are easier and less costly to harvest than microalgae.

The disposal of process waters constitute a significant cost for many food companies but by integrating land-based seaweed cultivation with these waters it can be utilized and not only treated as waste. Land-based cultivation also creates an opportunity to cultivate high value species with morphologies not suited for ocean-based cultivation [23], and to control the production cycle and biomass composition independent of the season; altogether yielding highly productive systems [23,24]. For example, the green seaweeds *Derbesia tenussima* and *Ulva ohnoi* reached productivities of 56 and 138 t dw ha^{-1} y^{-1}, respectively, when cultivated on land with fish broodstock wastewater [3], while *Ulva lactuca* reached productivities of 84 t dw ha^{-1} y^{-1} when cultivated with abalone farming wastewater [25]. These productivities are several folds higher than some sea-based kelp cultivations (<1.5 t dw ha^{-1} y^{-1}) [26], and average commercial soybean yields (<3 t dw ha^{-1} y^{-1}) [27,28].

In this study we explored the hypothesis that growth rates and protein content of different species of seaweed would increase by using process waters from food production industries as cultivation media. The selected waters were from salmon aquaculture as well as from production of peeled shrimps, marinated herring and oat-based products. We tested the brown kelp *Saccharina latissima*, and the three green seaweeds *Ulva fenestrata*, *Ulva intestinalis*, and *Chaetomorpha linum*. *Saccharina latissima* has successfully been cultivated in IMTA settings as a strategy to increase growth rates of the seaweed [8,29], while the *Ulva* and *Chaetomorpha* species are regarded as opportunistic 'green tide' species that easily incorporate nitrogen and grow rapidly [30–32]. Prior to cultivation, a subgoal was also to characterize total nitrogen and inorganic nutrients of the process waters, so that the seaweeds could be cultivated in dilutions based on the ammonium (NH_4^+) content of the process waters. Growth rates and crude protein content of the seaweeds were measured and compared between different process waters and dilutions, as well as with control seaweeds cultivated in untreated, and NH_4^+-enriched seawater. Furthermore, to give an indication of the physiological status of the seaweeds, their color was quantified by analyzing the three band colors red, green, and blue (RGB-values) [33]. We thus rely on manipulative experiments to (i) demonstrate the unexplored potential of food production process waters as cultivation media for seaweeds, and (ii) assess the growth and crude protein content potential of the different seaweed species in these cultivation settings.

2. Materials and methods

2.1. Collection of seaweed

Saccharina latissima were collected from indoor tank cultivation systems at Tjärnö Marine Laboratory (TML, 58°52′33.7″ N 11°08′44.9″ E). Whole sporophytes were collected from cultivation spools in a 1000 L aerated tank with filtered (0.2 μm + UV-light application) deep-sea (40 m) seawater enriched with half strength Provasoli Enriched Seawater (PES) [34,35] at 10 °C, 16:8 light regime at an irradiance of 100 μmol m^{-2} s^{-1}. Taxonomic identification of *Ulva* strains used in the present study was based on molecular identification of the *tufA* marker gene and followed the procedure as described by Toth, et al. [36]. Individuals of *U. fenestrata* originated from a long-term indoor tank cultivation and were molecularly identified in a previous study (GenBank accession numbers: MN240309, MN240310, MN240311) [36]. A population of *U. intestinalis*, pre-identified by morphological characters, was collected at the island Rossö located at the Swedish west coast (58°50′33.9″ N 11°09′06.6″ E). Three randomly chosen individuals were subsequently identified by molecular techniques to assess their taxonomic species affiliation. Resulting sequences of *U. intestinalis* were uploaded to GenBank and are publicly available (GenBank accession numbers: OK078880, OK078881, OK078882). *Chaetomorpha linum* was collected in intertidal rock ponds at Ursholmen, located at the Swedish west coast (58°49′57.6″ N 10°59′19.2″ E). The *Ulva* and *C. linum* were cultivated at 15 °C, 12:12 light regime at an irradiance of 80 μmol m^{-2} s^{-1}, until the start of the experiments. Filtered (0.2 μm + UV-light application) deep-sea (40 m) seawater enriched with half strength PES was used for the cultivation, and the salinity fluctuated between 30 and 34 PSU depending on the prevailing weather and seasonal condition. All seaweeds were kept in tank cultivation systems for an average of eight weeks prior to the experiments to expose them to the same nutrient concentrations. On this background, and based on previous experience, we assumed that the internal nutrient concentrations are similar at the start of the experiment. The seaweeds were rinsed in 0.2 μm filtered seawater before the start of the experiments.

2.2. Food production process waters

2.2.1. Collection

Process waters generated by food production industries were collected between October 2019 and September 2020. Table 1 provides a general overview of the origin of these waters. In brief, they comprised (i) three types of process waters from a primary herring processor; refrigerated sea water (RSW) from herring trawlers, tub waters from in-

Table 1
Types and acronyms of food production process waters used as media for seaweed cultivation, as well as controls. Number after each acronym corresponds to the final ammonium content (20 or 200 μM NH_4^+) after dilution with seawater. All seawater used is filtered (0.2 μm + UV-light application) deep-sea (40 m) seawater.

Type of water	Acronym	Origin	Provider	Water description
Control	C	–	–	Seawater control
Seawater enriched with NH_4Cl	NH4-20 NH4-200	–	–	Seawater enriched with NH_4^+
Refrigerated seawater	RSW-20 RSW-200	Herring	Sweden Pelagic AB	From on board refrigerated seawater (RSW) tanks
Tub water	TUB-20 TUB-200	Herring	Sweden Pelagic AB	From storage tubs with herring in 3% NaCl
Salt brine I	SBI-20 SBI-200	Herring	Sweden Pelagic AB	From pre-salting of headed/gutted herring in 5% NaCl
Salt brine II	SAL-20 SAL-200	Herring	Klädesholmen Seafood AB	From maturation of herring fillets in saturated salt brine
Spice brine	SPI-20 SPI-200	Herring	Klädesholmen Seafood	From maturation of herring fillets in spice brine
Shrimp boiling water	SBW-20 SBW-200	Shrimp	Bua Shellfish	From steaming of shrimps
Oat processing water	OAT-20	Oat	Oatly AB	From processing of oat to oat milk
Recirculated aquaculture system (RAS) water	RAS-20	Salmon	Nordic Aquafarms AS	Salmon RAS water after biofiltration-nitrification process

house storage of whole herring (TUB) and salt brine I (SBI) from pre-salting of headed/gutted herring, (ii) two types of process waters from secondary herring processing; salt brine II (SAL) and spice brine (SPI) from maturation of herring fillets in saturated salt brine and spice brine, respectively, (iii) shrimp boiling water (SBW) from steaming of whole shrimps, (iv) processing water from oat milk production (OAT), and (v) biofiltered recirculated aquaculture system water (RAS) from land-based salmon aquaculture. After collections, all waters were stored in plastic containers at −60 °C until further use. All process waters, except OAT and RAS, were tapped off in a food grade state.

2.2.2. pH, total nitrogen and inorganic nutrients

All the analyses were conducted in triplicates on the same biological sample for each type of food production process water.

2.2.2.1. pH and total nitrogen. The pH measurements were performed with a pH-meter (PHM 210, Meterlab, Hach, USA). Total nitrogen (N) was analyzed with a LECO Nitrogen Analyzer (TruMac N, LECO Corporation, USA) using EDTA 9.56 as standard.

2.2.2.2. Ammonium content. Ammonium concentration (NH_4^+) of all process waters was quantified using a commercial enzymatic kit (AA0100, Sigma, USA). Before starting the analysis, all samples were adjusted to a pH close to 7.5 and centrifuged (5000 ×*g*, 10 min) to remove coarse particles. Next, 20 μL of sample and 200 μL of ammonia assay reagent were mixed and left to incubate for 5 min. After that, 2 μL of L-glutamate dehydrogenase solution was added to the reaction mixture; followed by another 5 min of incubation. The absorbance was recorded at 340 nm after every incubation period and the NH_4^+ concentration calculated according to the manufacturer's instructions. All analyses were carried out in 96-well microplates.

2.2.2.3. Nitrate and nitrite content. Nitrate (NO_3^-) and nitrite (NO_2^-) concentration were determined with the help of a commercial enzymatic kit (Cat. No. 11746081001, Roche Diagnostics, Germany). Before determination, all samples were cleared with Carrez solutions I and II, followed by adjusting the pH to 8.0 ± 0.2. Thereafter, 0.5 mL of sample were mixed with 0.250 and 0.020 mL of co-factors and nitrate reductase solutions, respectively. The reaction mixture was incubated for 30 min and then two different color reagents were added (0.250 mL each). Afterwards, the mixture was incubated in darkness for 15 min. The absorbance was recorded at 540 nm after every incubation period and the total content of NO_3^- + NO_2^- was calculated. The NO_2^- concentration was determined similarly but without adding nitrate reductase and co-factors.

2.2.2.4. Inorganic phosphorus content. Inorganic phosphorus/orthophosphate (P) was measured as previously reported by Qvirist, et al. [37]. Prior to quantification, samples were centrifuged as described in Section 2.2.2.2. Ammonium content. Then, 0.5 mL of supernatant was mixed with 0.9 mL of 5% sodium dodecyl sulphate, followed by 1 mL of 1.25% of ammonium molybdate solution in 2 M HCl and 0.1 mL of 1 g L^{-1} of ascorbic acid. The final reaction mixture was incubated for 30 min followed by one absorbance reading at 700 nm. The P concentration was calculated through a standard curve made with monopotassium phosphate (1–20 mg L^{-1}).

2.3. Experimental setup

2.3.1. Preparation of the food production process waters to be used as cultivation media

In most of the process waters the predominant nitrogen source was NH_4^+ (Table 2). The process waters were diluted with filtered (0.2 μm + UV-light application) deep-sea (40 m) seawater to 20 and 200 μM NH_4^+. Due to low NH_4^+ content in OAT and RAS, these waters were only tested at the 20 μM concentration. The seawater average NH_4^+ concentration was 0.5 μM. The seawater control, NH_4^+-enriched seawater, and the diluted process waters were filtered (60 μm) to remove coarse particles, followed by autoclaving for 20 min at 1200 kPa. To assure that this thermal treatment did not influence the NH_4^+ concentration of the waters, the sample SAL was systematically evaluated, revealing no significant effect of the thermal treatment (8212 ± 733 vs. 8818.89 ± 531.67, mean ± SD, n = 3). The pH and salinity in the final waters were 7.8 ± 0.3 and 32.4 ± 1.9 PSU, respectively (mean ± SD).

2.3.2. Seaweed cultivation in process waters

All cultivation experiments were performed in aerated Petri dishes (100 mL) with one whole specimen in each (average size 47 ± 26 mm^2, 143 ± 73 mm^2, 414 ± 122 mg, and 476 ± 4 mg for *S. latissima*, *U. fenestrata*, *U. intestinalis*, and *C. linum*, respectively (mean ± SD)). Each water (seawater control, NH_4^+-enriched seawater, and process water) was tested in 6 replicate Petri dishes. Petri dishes were placed in a randomized order in a controlled temperature room at 12 °C, 12:12 light regime at an irradiance of 70 μmol m^{-2} s^{-1}. Due to a shortage of OAT and RAS water the *S. latissima* experiment was divided into two consecutive experiments. Each experiment ran for eight days, starting on the 22nd of January, and 8th of October 2020 for *S. latissima*, and on the 19th of February, 31st of March, and 20th October 2020 for *U. fenestrata*, *U. intestinalis*, and *C. linum*, respectively. The water was renewed every second day to avoid nutrient depletion and spoilage induced by seaweed-derived microorganisms.

2.4. Specific growth rate

The specific growth rates (SGRs) of *S. latissima* and *U. fenestrata* were evaluated with photo-scanning (see Supplementary material Fig. S1). All seaweeds were scanned at the start and end of the experiment using a Canon EOS400D digital camera (1/25, F22, ISO400), after placed on a lightning table with a glass slide on top to ensure that the seaweed's surface was flat. The seaweed area was then analyzed using image processing software (ImageJ V. 2.0.0-rc-69/1.52p). Due to their

Table 2
Characterization of pH, total nitrogen and inorganic nutrients of undiluted process waters to be used as seaweed cultivation media, and their corresponding dilution factors to reach 20 and 200 μM NH_4^+. Values are shown as mean ± SD (n = 3). Process water abbreviations are explained in Table 1.

Process water	pH	Total nitrogen (μM TotN)			Ammonium (μM NH_4^+)			Nitrate (μM NO_3^-)			Nitrite (μM NO_2^-)			Inorganic phosphorus (μM P)			Dilution factors 20/200 μM NH_4^+
RSW	7.0	35,842	±	3421	1166	±	51	6	±	<1		*n.d*		3524	±	72	58/5.8
TUB	6.8	46,207	±	1293	1399	±	123	9	±	1	3	±	2	5664	±	92	70/7.0
SBI	6.5	342,407	±	19,786	3585	±	41	16	±	0.6		*n.d*		33,576	±	107	179/17.9
SAL	5.8	332,950	±	1964	8212	±	733		*n.d*			*n.d*		27,321	±	94	411/41.1
SPI	5.9	361,893	±	7650	6285	±	72	12	±	<1		*n.d*		21,196	±	146	314/31.4
SBW	8.9	183,171	±	2135	8862	±	136	11	±	<1		*n.d*		410	±	2	443/44.3
OAT	9.7	10,671	±	271	26	±	6	6819	±	83	333	±	1	298	±	5	1.3/
RAS	7.8	1836	±	500	41	±	19	3059	±	28	17	±	<1	42	±	<1	2.1/

n.d. non detectable.

filamentous morphological characteristics the SGRs for *U. intestinalis* and *C. linum* were evaluated by weight instead of area. The wet weight (ww) was determined at the start and end of the experiment in a standardized way by pulling the seaweeds with forceps along the inside edge of a beaker for 5 s before weighing for *U. intestinalis*, and by gently shaking the seaweed for 5 s for *C. linum*. These methods had been tested before starting the experiment to yield the best R^2 value for the ww/dw ratio (R^2 = 0.94 for both methods). The SGR was calculated for all seaweeds according to the formula: SGR = [((Ln(A_t) − Ln(A_0)) / t) * 100],where A_t is the area/weight after t days and A_0 is the start area/weight.

2.5. Total nitrogen and crude protein content

After the experiment, the seaweeds were freeze-dried (16 h), ground into a fine powder, and analyzed for N content using combustion elemental analysis (Elementar vario MICRO cube, Elementar Analysensysteme, Germany). Due to technical failure of the elemental analyzer, some N samples for *U. fenestrata* (n = 20) and *U. intestinalis* (n = 22) were destroyed and could therefore not be included in the analyses. For N content analyses of *C. linum* the samples were dried at 60 °C (24 h) and then analyzed with a LECO Nitrogen Analyzer (TruMac N, LECO Corporation, USA) using EDTA 9.56 as standard. Nitrogen data were then converted to crude protein using a conversion factor of 5 [38].

2.6. Color measurements (RGB-values)

Images from the last day of the experiments were used for analysis of the three band colors red, green, and blue (RGB) (see Supplementary material Fig. S2). The images were analyzed using image processing software (ImageJ V. 2.0.0-rc-69/1.52p) to determine the mean of the three band colors separately.

2.7. 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 Q-Q plots). For each species, statistical difference in SGR and crude protein content between treatments was tested with one-way analysis of variance (ANOVA) using the *lm* function [39]. Significant difference between groups was tested a posteriori with Student-Newman-Keuls (SNK) post hoc test ($\alpha = 0.05$), using the SNK.test function in the *agricolae* package [40].

For each species, principal component analysis (PCA) was used to explore and analyze differences between RGB-values in seaweeds cultivated in the different treatments using *ggplot2* [41]. PCA was accompanied by PERMANOVA run with the euclidean method and 999 permutations using the *adonis* and *pairwise.adonis* functions in the *vegan* package [42]. The PERMANOVA tested the response of the dependent variables band colors (R, G, and B) to the fixed-factor treatment (seawater control, NH_4^+-enriched seawater at 20 μM, and 200 μM, and process waters at 20 μM, and 200 μM). Significant differences between treatments were tested a posteriori with the *pairwise.adonis* function in the *vegan* package, using the p.adjust.m function 'bonferroni' [42].

3. Results

3.1. Physiochemical characterization of the process waters

Table 2 provides a composition map over pH, total nitrogen and specific inorganic nutrients of the process waters. The native pH of herring-derived process waters ranged from 5.8 to 7.0, while the RAS had a slight alkaline pH of 7.9. The highest pH values, 8.9 and 9.7, were detected in SBW and OAT, respectively. In herring and shrimp-derived process waters, NH_4^+ content was higher than NO_3^- content by a factor ≥200. By contrast, OAT and RAS had 261 and 74 times more NO_3^- than NH_4^+, respectively. Overall, NO_2^- was not detected or present in negligible quantities when compared to NH_4^+ and NO_3^-. The total nitrogen values suggest herring and shrimp-derived process waters contained organic N as the major source of N, whereas inorganic N was the predominant nitrogen present in OAT and RAS. Finally, regarding inorganic phosphorus, herring-derived process waters showed the highest level (3524–33,576 μM), followed by SBW (410 μM), OAT (298 μM), and RAS (42 μM).

3.2. Specific growth rate, crude protein content, and color

3.2.1. Saccharina latissima

Apart from the seawater control, in which *S. latissima* had a positive SGR of 9.06 ± 0.74% d^{-1}, all waters resulted in negative SGRs in the first experiment (Fig. 1a). In the second experiment, SGR was also positive in the seawater control, and NH_4^+-enriched seawater (4.02 ± 0.90, 3.93 ± 1.12, and 3.29 ± 0.63% d^{-1}, respectively), but negative in all the process waters (Fig. 1b). All *S. latissima* cultivated in process waters had died at the end of the experiments. Therefore, it was not possible to perform any further analyses in terms of their crude protein content or color (RGB-values).

3.2.2. Ulva fenestrata

Ulva fenestrata grew in all the process waters as well as in the seawater control and NH_4^+-enriched seawater (Fig. 2a). There was a significant difference in SGR between treatments, and growth ranged from 2.17 ± 0.20% d^{-1} in OAT-20 to 14.25 ± 0.88% d^{-1} in TUB-20 (Table 3). There was a tendency towards higher growth in some process waters compared to the seawater control, but no statistical difference was found (SNK, $p > 0.05$), except for OAT-20 where the growth rate was significantly lower (SNK, $p < 0.05$). There was a general trend towards higher growth rates in 20 μM treatments compared to 200 μM treatments, however this observation was only statistically significant for SBI (SNK, $p < 0.05$).

Crude protein content in *U. fenestrata* cultivated in process waters ranged from 17.88 ± 0.64% dw in SPI-20 to 23.28 ± 0.85% dw in SBW-20 (Fig. 2b). There was a significant difference in crude protein content between treatments (Table 3), where seaweeds cultivated in process waters had significantly higher crude protein content compared to C (10.02 ± 0.62% dw) and NH4-20 (8.49 ± 0.40% dw) (SNK, $p < 0.05$). The two dilution levels of process water provided similar crude protein content values.

In the PCA performed on RGB data, the first main component (PC1) accounted for 92.6% of the data variation and the second main component (PC2) accounted for 7.3% (Fig. 3). Seaweeds cultivated in C

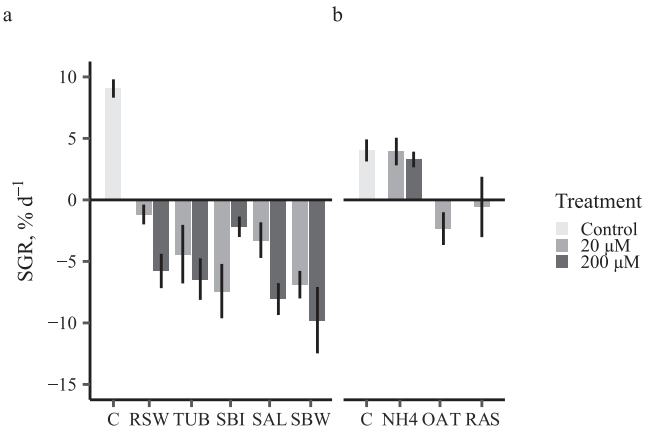

Fig. 1. Specific growth rate (SGR, % d^{-1}) of *Saccharina latissima* cultivated in seawater control, NH_4^+-enriched seawater, and process water on the 8th day of the (a) first and (b) second experiment (mean ± SE, n = 6).

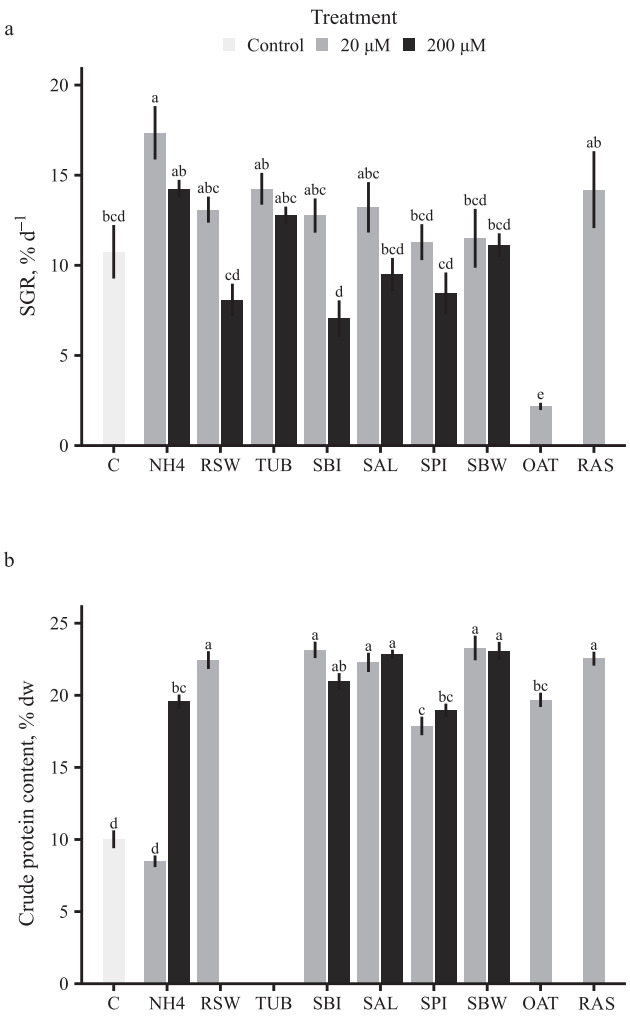

Fig. 2. (a) Specific growth rate (SGR, % d^{-1}) and (b) crude protein content (% dw) of *Ulva fenestrata* cultivated in seawater control, NH_4^+-enriched seawater, and process water, on the 8th day of the experiment (mean ± SE, n = 6 except in crude protein content for SAL-200 and SPI-20 where n = 5, and RSW-200, TUB-20, and TUB-200 where n = 0). Different letters denote significant difference (α = 0.05) in post hoc SNK test.

Table 3
Results of one-way analysis of variance (ANOVA) analyzing the effect of treatment on specific growth rate (SGR) and crude protein content for *Ulva fenestrata*, *Ulva intestinalis*, and *Chaetomorpha linum*.

		df	MS	F-value	p-Value
U. fenestrata	SGR	16	73.863	9.663	$3.52e^{-13}$
	Crude protein content	13	133.566	67.829	$<2.2e^{-16}$
U. intestinalis	SGR	16	8.962	1.454	0.137
	Crude protein content	16	144.545	23.548	$<2.2e^{-16}$
C. linum	SGR	16	42.269	16.481	$<2.2e^{-16}$
	Crude protein content	15	225.197	184.770	$<2.2e^{-16}$

and NH4-20 showed signs of losing its color towards the end of the experiment, whereas seaweeds cultivated in process waters stayed clear olive-green. In the PCA, seaweeds cultivated in C and NH4-20 are thus distinguishable from the other waters. The PERMANOVA analyses also showed that treatment had a significant effect on the band colors ($p < 0.001$). The color of the seaweeds cultivated in C and NH4-20 were significantly different from seaweeds cultivated in process waters at 20 and 200 µM NH_4^+ levels ($p < 0.05$).

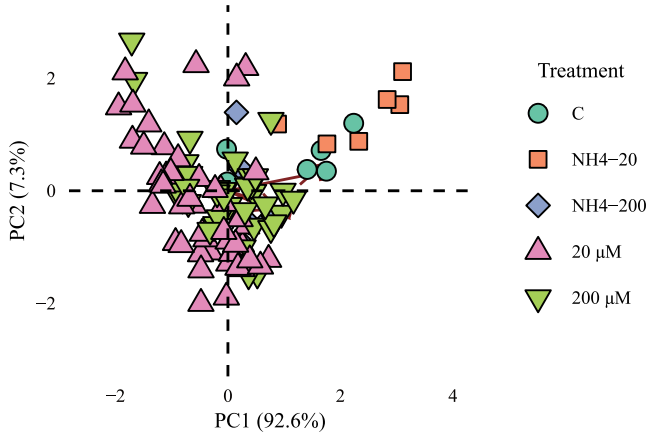

Fig. 3. Principal Component Analysis (PCA) of RGB-values for *Ulva fenestrata* in the different treatments on the 8th day of the experiment. The first main component (PC1) accounted for 92.6% of the data variation and the second main component (PC2) accounted for 7.3%.

3.2.3. *Ulva intestinalis*

Ulva intestinalis grew in all the process waters as well as in the seawater control and NH_4^+-enriched seawater (Fig. 4a). The SGR of *U. intestinalis* cultivated in the process waters ranged from 4.04 ± 0.69% d^{-1} in RAS-20 to 8.14 ± 0.65% d^{-1} in RSW-200, but no statistical difference was found between treatments (Table 3). Sporulation occurred in all the treatments at some point during the experiment, which could help explain the high variability in growth within treatments, and non-significant result between treatments.

Crude protein content in the seaweed cultivated in process waters ranged from 8.83 ± 0.52% dw in TUB-20 to 21.83 ± 0.97% dw in SBI-20 (Fig. 4b). There was a significant difference in crude protein content between treatments (Table 3), and all process waters, except for TUB-20, resulted in significantly higher content levels compared to C (6.13 ± 0.33% dw), NH4-20 (6.65 ± 0.19% dw), and NH4-200 (8.69 ± 0.83% dw) (SNK, $p < 0.05$). Significant difference between the 20 and 200 µM NH_4^+ treatments was only found in the RSW and TUB (SNK, $p < 0.05$).

In the PCA performed on RGB, the first main component (PC1) accounted for 90.1% of the data variation and the second main component (PC2) accounted for 9.3% (Fig. 5). Similar as for *U. fenestrata*, the C and NH4-20 are distinguishable in the analysis. *U. intestinalis* in these two waters were bright green, while in the other waters they appeared darker. The PERMANOVA analyses showed that treatment had a significant effect on the band colors ($p < 0.001$). The color of the seaweeds cultivated in C and NH4-20 were significantly different from seaweeds cultivated in process waters at 20 and 200 µM NH_4^+ levels ($p < 0.05$).

3.2.4. *Chaetomorpha linum*

Chaetomorpha linum grew in all the waters, except in SPI-200 where the growth was negative (Fig. 6a). There was a significant difference in SGR between treatments (Table 3), with process waters yielding SGRs ranging from −1.73 ± 0.51% d^{-1} in SPI-200 to 9.42 ± 0.58% d^{-1} in SPI-20. Seaweeds grew better in the seawater control than in the NH_4^+-enriched seawater (SNK, $p < 0.05$). Regarding the process waters, there was a trend towards higher growth rates in 20 µM NH_4^+ treatments compared to 200 µM NH_4^+ treatments, however, this observation was only statistically significant for RSW, SBI and SPI (SNK, $p < 0.05$).

Crude protein content of *C. linum* in the process waters ranged from 9.28 ± 0.31% dw in SPI-20 to 24.97 ± 0.63% dw in SBW-200 (Fig. 6b). There was a significant difference in crude protein content between treatments (Table 3), with seaweeds cultivated in process waters having significantly higher crude protein contents compared to seaweeds cultivated in the seawater control (SNK, $p < 0.05$). Seaweeds cultivated

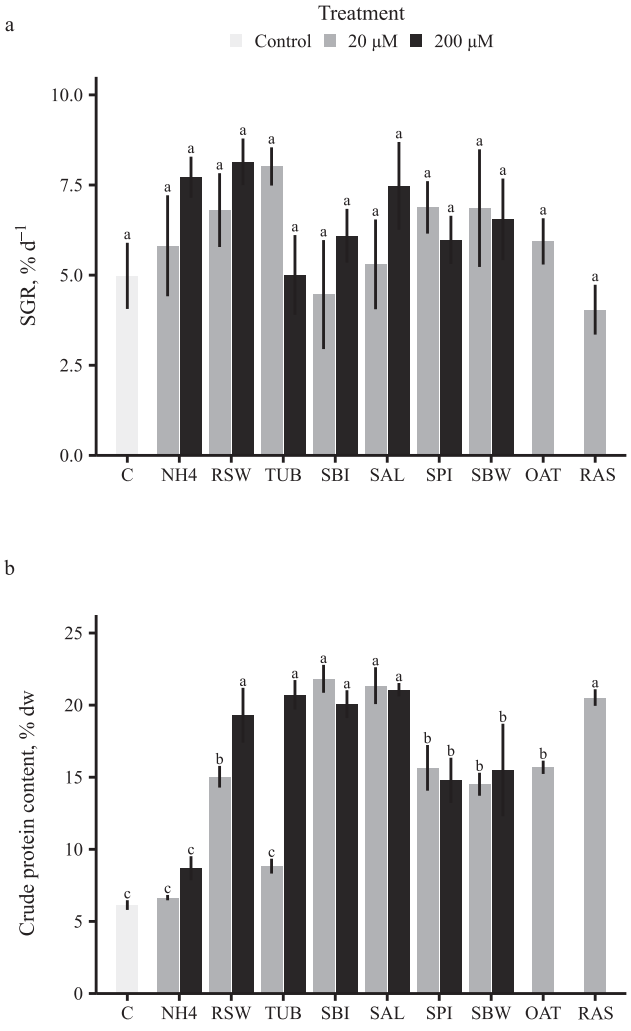

Fig. 4. (a) Specific growth rate (SGR, % d^{-1}) and (b) crude protein content (% dw) of *Ulva intestinalis* cultivated in seawater control, NH_4^+-enriched seawater, and process water on the 8th day of the experiment (mean ± SE, n = 6 except in SGR for SAL-20 where n = 5, and in crude protein content for NH4-20, NH4-200, SBI-200, SAL-20, SAL-200, and SPI-20 where n = 5, SBI-20, SBW-20, and OAT-20 where n = 4, SPI-200, and SBW-200 where n = 3, and RAS-20 where n = 2). Different letters denote significant difference (α = 0.05) in post hoc SNK test.

in the 200 µM NH_4^+ process water treatments had significantly higher crude protein contents compared to seaweeds cultivated in the 20 µM NH_4^+ process water treatments (SNK, $p < 0.05$).

In the PCA performed on RGB, the first main component (PC1) accounted for 94.1% of the data variation and the second main component (PC2) accounted for 5.2% (Fig. 7). The PCA distinguished one of the 200 µM NH_4^+ process water treatments in the analyses, and was identified as SPI-200. The seaweeds in SPI-200 lost all its color and turned grey during the experiment, which is also reflected in its negative SGR (Fig. 6a). The PERMANOVA analyses showed that treatment had a significant effect on the band color ($p < 0.001$), where seaweeds cultivated in SPI-200 were significantly different from seaweeds cultivated in any of the other treatments ($p < 0.05$).

4. Discussion

This study assessed the prospects of cultivating four different species of seaweeds in industrial process waters collected from different types of food production. Our results demonstrated a high potential of all the tested process waters as growth media for cultivation of *U. fenestrata*,

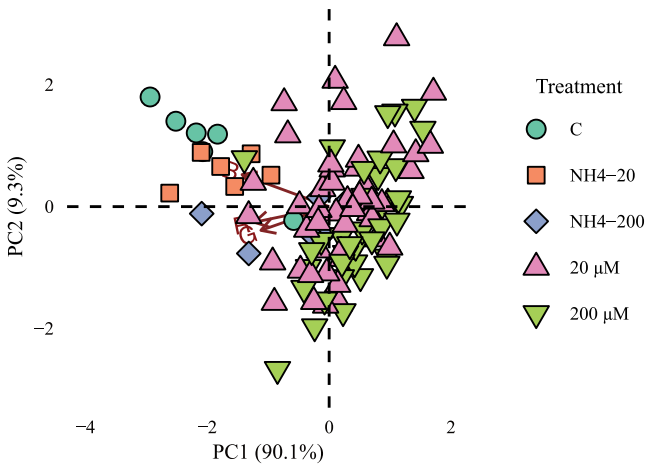

Fig. 5. Principal Component Analysis (PCA) of RGB-values for *Ulva intestinalis* in the different treatments on the 8th day of the experiment. The first main component (PC1) accounted for 90.1% of the data variation and the second main component (PC2) accounted for 9.3%.

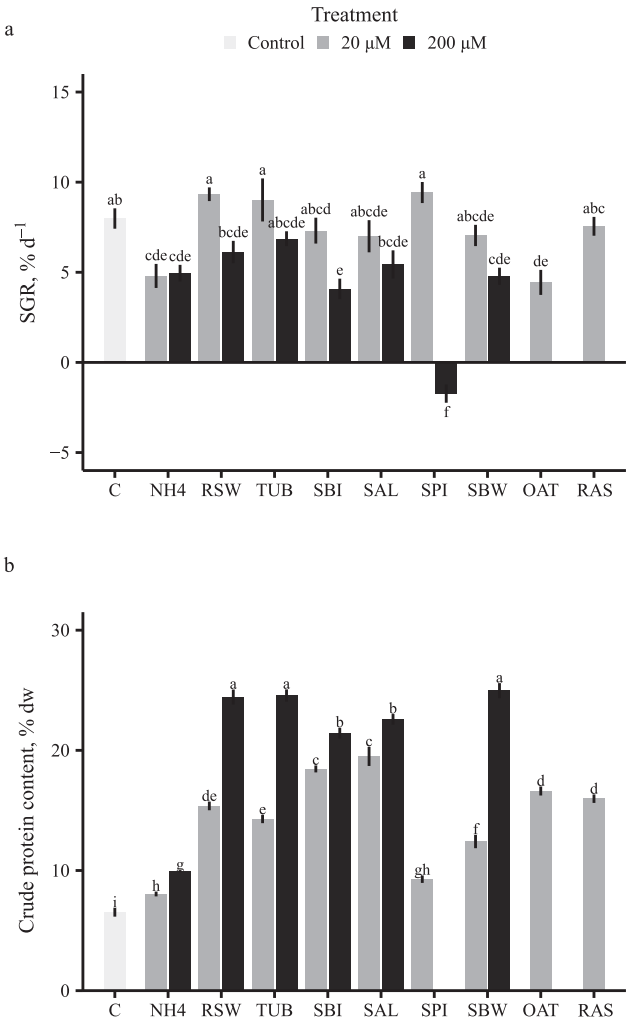

Fig. 6. (a) Specific growth rate (SGR, % d^{-1}) and (b) crude protein content (% dw) of *Chaetomorpha linum* cultivated in seawater control, NH_4^+-enriched seawater, and process water on the 8th day of the experiment (mean ± SE). Different letters denote significant difference (α = 0.05) in post hoc SNK test.

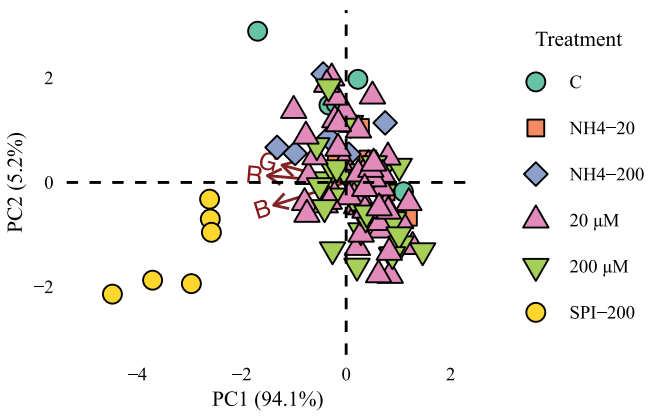

Fig. 7. Principal Component Analysis (PCA) of RGB-values for *Chaetomorpha linum* in the different treatments on the 8th day of the experiment. The first main component (PC1) accounted for 94.1% of the data variation and the second main component (PC2) accounted for 5.2%.

U. intestinalis and *C. linum*, but not for *S. latissima*. This was verified by multiple lines of evidence including (i) for the three green seaweed species, the growth rates in process waters were similar or higher than in seawater controls, while (ii) *S. latissima* had negative growth rates in all the process waters, (iii) the crude protein content of seaweeds cultivated in process waters was significantly higher than in seawater controls, and (iv) the color of the seaweed thallus was maintained or darkened with the food process waters, the latter possibly indicating higher chlorophyll concentration [33]. Combined, the results show that opportunistic 'green tide' species are promising candidates for cultivations in process waters from different food industries, paving the way for a new circular production route of vegan protein.

The optimal ratio of N and P for seaweed growth is 30N:1P with a range between 10N:1P and 80N:1P, indicating that N is often the limiting nutrient for seaweed growth [43,44]. Based on the observations that ammonium (NH_4^+) is often the preferred N source for seaweeds [45] and that it was the predominant nitrogen source in the process waters in our study (Table 2), we predicted it to be the nutrient affecting seaweed growth the most. We thus diluted the process waters to two different concentrations of NH_4^+ (20 and 200 µM) to assess it as cultivation media. Other species of nitrogen and phosphorus were not accounted for in the dilutions. Kelp species such as *S. latissima* sometimes favor nitrate (NO_3^-) as the nitrogen source [45,46], and high NH_4^+ concentrations could inhibit the NO_3^- uptake of the seaweed [46]. The fact that *S. latissima* grew in seawaters enriched with NH_4^+ indicates that it was not the high concentrations of NH_4^+ that inhibited growth in this species. Kelps have been shown to grow well in salmon cultivation wastewater and other waters elevated in nutrient concentration. For example, *S. latissima* deployed at a fish farm grew by 2.5–4% d^{-1} [8], while kelps cultivated in 20 µM NO_3^- grew by over 8% d^{-1} [47]. The total N concentration in the process waters used in our study was however higher than in any of the earlier cited studies, and also higher than in our seawaters enriched with NH_4^+. Kelps are not opportunistic species and grow naturally in much lower N concentrations [45], and it is possible that the high N concentration in the cultivation media in our experiment was a shock for the *S. latissima*, which may explain why they died [48,49], or, alternatively, the waters contained unidentified compounds that inhibited kelp growth.

U. fenestrata cultivated in process waters grew up to 32% faster and had over twice the crude protein content when compared to seawater controls. The relatively high growth rates in our controls may be explained by the use of young gametophytes that often grow faster than older individuals [50]. *U. fenestrata* grew in all waters, but growth was 6.5 times higher in TUB-20 (14.3% d^{-1}) compared to in OAT-20 (2.2% d^{-1}). Even though growth was low in OAT-20, its protein content was higher than the controls. In fact, all seaweeds cultivated in process waters had higher protein content compared to seaweeds cultivated in controls. This shows that some process waters were more suitable for cultivation of *U. fenestrata* than others. Our results resemble previously reported growth rates for *U. fenestrata* in NH_4^+-enriched media, e.g. Ale, et al. [51] showed maximum SGR of 16% d^{-1} when cultivated in 50 µM NH_4^+, while Sode, et al. [52] showed average SGR of 15% d^{-1} in 440 µM N *f/2* medium.

Even if growth for *U. intestinalis* was 64% higher in RSW-200 (8.14% d^{-1}) compared to the seawater control (4.98% d^{-1}), the high variability of growth within each treatment resulted in no statistical difference between growth rates in any of the waters. However, the crude protein content was up to 3.6 times higher in the *U. intestinalis* cultivated in process waters compared to the control. The same pattern as for *U. intestinalis* was seen for *C. linum*, where seaweed cultivated in process waters did not have statistically higher growth compared to the seawater control, while crude protein content was up to almost four times higher. The growth rates of *U. intestinalis* in our experiment (4.0–8.1% d^{-1}) were relatively low compared to previously reported growth rates of 12.6% d^{-1} in 100 µM NO_3^- [53] and 9.5–22.2% d^{-1} at different salinity, temperature, light, and culturing density [54]. One explanation for the low growth rates may be the sporulation events that happened in all the treatments. During such events, the seaweed allocates energy to releasing spores or gametes and usually stops growing [55]. Sporulation events can happen sporadically in 'green tide' species and are not yet fully understood, but is often a response to changing environmental conditions [50]. The sporulation event that happened in the *U. intestinalis* experiment was most likely a result of changing the nutrient availability.

Generally, *U. fenestrata* and *C. linum* grew better in 20 µM compared to 200 µM NH_4^+ treatments, whereas both NH_4^+ concentrations resulted in similar growth rates for *U. intestinalis*. High NH_4^+ concentration can have an inhibitory effect on growth for some seaweeds [45], and for *Ulva* spp. this threshold is indicated to be at around 60 µM [56,57]. Our results match those reported in previous studies on cultivation of green seaweeds where the optimum NH_4^+ concentration for growth of *U. fenestrata* cultivated in manure was found at 25 µM [57], while concentration above 80 µM did not stimulate further growth of *Enteromorpha linza* and *E. compressa* [32]. Alternatively, the higher growth in 20 µM NH_4^+ treatments may be the effect of unidentified growth inhibitory metabolites being more diluted in these treatments compared to in the 200 µM treatments. Regardless, high N concentrations increase the internal N content in the seaweed biomass, leading to improved assimilation of inorganic N into amino acids and proteins [43].

There was a positive effect of increased crude protein content in the seaweed biomass when cultivated in the food production process waters. These results confirm that the composition of nitrogen, and hence protein, in the biomass is directly influenced by the culturing media, which has also been shown in previous studies on green seaweeds [9,53,57]. However, it was only for *C. linum* that NH_4^+ concentration above 20 µM results in further increased protein content in the biomass. Similar effect between increased N concentration of the cultivation media and N tissue content of the seaweed has been reported for *C. linum* when cultivated in different dilutions of municipal wastewaters [58]. However, *U. lactuca* cultivated in effluents from marine fishponds reached highest N tissue content at around 10–20 µM NH_4^+ and then stagnated when the concentration was elevated further [59].

Both *Ulva* species showed a darker, olive-green color, at the end of the experiment in process waters compared to in controls. The color difference may be a result of availability of N in the water leading to accumulation of N-containing photosynthetic pigments such as chlorophyll [33]. Similar to our results, previous studies have found seaweed to become darker as a result of being exposed to aquaculture effluents [60,61]. The color of the *Ulva* species in our study matched the crude protein content in the thallus well, and color could therefore be used to help indicate nitrogen and protein content of green seaweed [33].

Similar methods have been shown to effectively indicate the status of nutrients in terrestrial crops such as quinoa and amaranth leaves [62], as well as for tomatoes [63]. Overall, there was no distinguishable difference in color between *C. linum* in process waters and controls; despite that the former had higher total N-content. This may be explained by N-containing pigments not playing an important role as N stores of *C. linum* [64].

5. Conclusion

In this study we, for the first time, show that 'green tide' species are suitable to culture in a wide variety of industrial food production process waters, resulting in increased growth and crude protein content of the seaweeds, at the same time as outlet nutrients are circulated back into the food chain. In the most promising cases, growth rates were up to 64% higher, and crude protein content was almost four-fold in food production process waters compared to in seawater controls (25% vs 6.5% dw). It should, however, be stressed that our experiments were small scaled (100 mL petri dishes), and the promising results need to be confirmed in an up-scaled setting where the economic feasibility of up-scaling is also evaluated. Furthermore, it was beyond the scope of this study to reveal which specific characteristics of the process water, besides $NH4^{+}$ concentration, that accounted for the positive response in the seaweeds. Still, the experimental design allowed us to clearly show the potential of the process waters as cultivation media for seaweeds. This opens the possibility for a novel nutrient loop in which the costly disposal of food production process waters can instead be turned into economic revenue by sustainably producing new protein-enriched raw materials via seaweed cultivation. In light of the ongoing dietary protein shift, high-protein seaweeds are very promising as an alternative food protein source.

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CRediT authorship contribution statement

Kristoffer Stedt: Conceptualization, Methodology, Software, Formal analysis, Investigation, Writing – Original Draft, Writing – Review & Editing, Visualization, Project administration.

João Trigo: Resources, Writing – Review & Editing.

Sophie Steinhagen: Formal analysis, Resources.

Göran M. Nylund: Writing – Review & Editing, Supervision.

Bita Forghani: Writing – Review & Editing.

Henrik Pavia: Validation, Resources, Writing – Review & Editing, Supervision, Funding Acquisition.

Ingrid Undeland: Conceptualization, Validation, Resources, Writing – Review & Editing, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

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