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Short communication

Effect of ammonium formate washing on the elemental composition determination in Nannochloropsis oceanica

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ABSTRACT

This study investigated the effect of salt presence and removal on the integrity and elemental determination of the marine microalgae *Nannochloropsis oceanica*. Cells were harvested by centrifugation and washed with ammonium formate five times and subjected to mineral analysis after each washing step. Supernatants from each washing step were also analysed. Ca, Cu, Fe, Mg, Mn, Na, P, K, S, Zn were determined by inductively coupled plasma – optical emission spectrometry (ICP-OES). Results showed that washing microalgal biomass twice is sufficient to eliminate equipment oversaturation, allowing for more accurate elemental analysis, by using matrix matching. Moreover, washing the biomass did not rupture the cells, removed cell culture debris and decreased the concentration of Na, K, Ca present in the leftover growth medium. This study provides a reliable protocol for elemental determination in marine microalgae saving time in sample processing and analysis.

1. Introduction

Microalgae are becoming an attractive organic mineral source in aquafeed. Minerals in microalgae are naturally abundant and predominantly present in an organic form, which allows for more efficient assimilation in the fish gut (Doucha et al., 2009). *Nannochloropsis* is a genus of photosynthetic marine microalga and it is considered of interest for aquafeed, due to the high total lipid content, of up to 60% per dry weight. Moreover, *Nannochloropsis* contain up to 5.2% of omega-3 eicosapentaenoic acid (EPA) which could also act as substitute for fish oil (Ashour et al., 2019; Ma et al., 2016; Zanella and Vianello, 2020). In this study we aimed at investigating the elemental content of *N. oceanica*. However, marine microalgal elemental analysis is an underexplored field and it is undermined by major challenges such as: the amount of sample required for elemental analysis, the accessibility to equipment and time to perform elemental analysis, the necessity for laborious sample pre-treatment (washing, digestion and dilution of samples), matrix interference (from sodium or other elements) (Olesik, 1991) and matrix matching technique (Boss and Fredeen, 2004).

Methods to determine elemental composition in microalgal biomass have changed extensively over the years, from single element analysers, such as Flame atomic absorption spectrometry (FAAS) to multi-element analysers, such as inductively coupled plasma (ICP). The machines detection limits are also constantly improving (Bolann et al., 2007; Chan et al., 1998). In 1974, ICP-optical emission spectroscopy (ICP-OES) instruments became commercially available (Olesik, 1991) which allowed for the detection of many elements at the same time. In 1983, ICP-mass spectrometry (ICP-MS) became available allowing for an even lower detection limit of elements, up to parts per trillion (nanomolar) range (Olesik, 1991; Wilschefski and Baxter, 2019), becoming the gold standard (Chan et al., 1998). Currently, microalgal elemental analysis is mostly performed with either ICP-MS or ICP-OES (Piccini et al., 2019). However, ICP-MS machines are often too expensive and require highly trained staff for operation (Miller-Ihli and Baker, 2001). Furthermore, high salt containing samples need to be diluted, since high salt concentrations (>20 mg Na/L) interfere with the measurement and cause oversaturation of the ICP-MS machine. On the other hand, ICP-OES has a detector based on light measurements and it can tolerate higher levels of dissolved salts without requiring additionally dilutions, which can make the process less laborious and less time consuming. Therefore, ICP-OES can be a more favourable way of measuring samples with high total dissolved solids (TDS), as it has much higher tolerance (up to 30%,

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compared to the 0.2% tolerance, for ICP-MS) (Tyler, 2001). Nevertheless, for both types of measurements, the amount of sample needed (100–1000 mg DW) for elemental analysis is still a limiting factor to perform extensive studies. Prior to mineral analysis, microalgal samples are microwave acid extracted and become the so called sample matrix (Grotti et al., 2003). The sample matrix composition influences the detection of the elements of interest and care should be taken to avoid matrix interference. Matrix interference can occur during analysis of, for example, samples with high concentration of sodium causing an under or overestimation of elements (Napan et al., 2015; Olesik, 1991). This interference can usually be avoided by different dilutions of the samples depending on the element concentration (Napan et al., 2015) or through the introduction of an internal standard that can compensate for matrix effects (Grotti et al., 2003). Although ICP is a multi-elemental technique, it is still limited to the type of sample matrix. Therefore, techniques such as matrix matching have been proposed to save time and efforts in preparing samples and standard mixes (Boss and Fredeen, 2004). In matrix matching, single element standards are combined into a tailored multi-element standard, thus avoiding serial dilutions of standard mixes, as well as several measurements of the same sample. This also allows the quantification of all elements in a single sample measurement. However, matrix effects and matrix matching remain unexplored in microalgae elemental analysis. Moreover, there have been no studies that combine the benefit of matrix matching with sample pre-treatment, as a strategy to reliably measure elements in the biomass of marine microalgae species.

Regarding sample pre-treatment, microalgal biomass is traditionally washed prior to dry weight determination (Zhu and Lee, 1997) or other biochemical analysis (Borges et al., 2016) and care is taken to avoid osmotic shock (Vonshak, 2017). Washing is used as a method to remove cell debris and residual media/buffer (Breuer et al., 2013). Typically, freshwater microalgae are washed with distilled or deionised water (Grimi et al., 2014; Mitra et al., 2015). Saltwater microalgae are commonly washed with NaCl (López et al., 2010), artificial seawater (Yao et al., 2013), isotonic solutions such as ammonium formate (Janssen et al., 2018) or phosphate-buffered saline (PBS) (Hounslow et al., 2016). A mineral-free washing agent is required to remove any salt precipitates that may interfere with the elemental analysis (Tokuşoglu and Ünal, 2003). However, to date there is no standard washing agent or information on how the washing steps affect the determination of microalgal elemental composition. This study is the first to address the effect of washing on cell recovery of *N. oceanica* and elemental composition. The concentration of the following elements was determined in the culture medium and biomass samples: Ca, Cu, Fe, Mg, Mn, Na, P, K, S, Zn. These elements are present in the medium composition, in a fixed formulation (artificial seawater) and preliminary trials did not show the presence of other elements such as selenium, lithium or cadmium (data not shown). The effect of washing on the oversaturation of the analytical equipment and matrix matching was also investigated.

2. Materials and methods

2.1. Microalgal strain, medium and growth conditions

Nannochloropsis oceanica (*N. oceanica*) CCAP (849/10) was cultivated phototrophically in an artificial seawater based medium as described by Janssen et al. (2018). The medium was modified by substituting elements containing sulphate forms (SO_4^{2-}) to chloride (Cl^-) forms. The buffer was changed from HEPES to Tris-HCl. No vitamins were added to the medium. The full medium composition: NaCl 444.9 mM; KNO_3 33.6 mM; Na_2SO_4 6.48 mM; K_2HPO_4 2.47 mM; $Na_2EDTA \bullet 2H_2O$ 84.1 μM; $MnCl_2 \bullet 4H_2O$ 19.3 μM; $CoCl_2 \bullet 6H_2O$ 1.20 μM; $CuCl_2 \bullet 2H_2O$ 1.30 μM; $Na_2MoO4 \bullet H_2O$ 104.1 nM; $ZnCl_2$ 4.20 μM; NaFeEDTA 27.8 μM; $MgCl_2 \bullet 6H_2O$ 2.96 mM; $CaCl_2 \bullet 2H_2O$ 2.45 mM; $NaHCO_3$ 10.0 mM; Tris-HCl 20.0 mM (Table 1). Medium was filter sterilised using a 0.2 μm filter (Sartobran 300). Pre-cultures were maintained in 250 mL shake flasks with a liquid volume of 150 mL in an orbital shaker incubator (Multitron, Infors HT, Switzerland) at: 25 °C, continuous mixing at (100 rpm), relative humidity of 50%, air enriched with 2.5% CO_2 and incident light of 100 μmol m^{-2} s^{-1} on a 16:8 light:dark cycle. Experimental cultures were inoculated at a starting OD_{750} of 0.5 in triplicates in 500 mL shake flasks with a liquid volume of 300 mL under the same conditions.

Table 1
ICP-OES parameters, operating conditions and elements measured in the ICP-OES including concentration ranges and wavelengths. Argon is symbolised with a * since this is the element used for the plasma.

ICP- OES Parameters and Operation conditions	
Nebulizer	Seaspray
Spray chamber	Baffled glass cyclonic
Sample uptake rate (mL/min)	1
RF power (W)	1500
Injector (mm id)	2.0 Alumina
Nebulizer gas flow (L/min)	0.7
Auxiliary gas flow (L/min)	0.2
Plasma gas flow (L/min)	10
Sample uptake tubing (mm id)	Black/Black (0.76)
Drain tubing (mm id)	Red/Red (1.14)
Software	Syngistix™
Replicates	$N = 3$
Wavelengths (nm)	*Arg (420.069), Ca (317.933), Cu (324.752), Fe (259.939), K (766.49), Mg (285.213), Mn (257.61), Na (589.592), P (213.617), S (180.669) and Zn (202.548)
Concentration ranges (mg/L)	*Arg (*), Ca (0.2–1), Cu (0.02–0.1), Fe (0.2–1), K (2–10), Mg (2–10), Mn (0.02–0.1), Na (0.2–1), P (2–10), S (2–10) and Zn (0.02–0.1)

*Argon is used in the ICP-OES to create the plasma.

2.2. Washing as a biomass pre-treatment prior to elemental composition determination

N. oceanica cultures were harvested on day 12, once they reached a dry weight of 2.9 ± 0.3 g/L. Aliquots of 50 mL were centrifuged (2000 *g*, 15 min at 20 °C) and the cell pellets were washed by adding 20 mL of 0.5 M ammonium formate and vortexing until complete cell resuspension. The centrifugation and washing steps were repeated 4 times. The resulting supernatants were collected, and the cell pellets were lyophilised (Sublimator 2 × 3 × 3-5, Zirbus Technology, Germany).

2.3. Cell recovery and cell debris during washing steps

Cell number and dry weight was determined during each step of the washing procedure. For size analysis, a cut-off of 1.8 μm was used to discriminate between *N. oceanica* cells (> 1.8 μm) and other particles e.g. debris, salt precipitates, etc. (<1.8 μm). Cell number was assessed with a cell counter (Beckman Multisizer™ 3 Coulter Counter) with a 50 μm aperture tube. Dry weight was performed according to (Kliphuis et al., 2012) in triplicate with the difference that ammonium formate (0.5 M) was used instead of de-mineralized (DI) water. Cell recovery was calculated by dividing the final cell number or dry weight value over the initial cell number or dry weight value and multiplying by one hundred, to give a percentage.

2.4. Microwave-assisted acid digestion

10 mL of supernatant or 50 mg of microalgal lyophilised samples were acid digested in a 100 mL closed Teflon vessel with Aqua Regia (10 mL dH_2O was added only to biomass samples, followed by 7.5 mL of hydrochloric acid (37%) and 2.5 mL of nitric acid (65%) from Merck (Darmstadt, Germany)). The biomass was then digested in a microwave oven (milestone S.r.l. ETHOS 1) with a temperature program as the

following: from zero to five min a ramp to 100 °C, from 5 to 10 ramp to 130 °C, from 10 to 15 ramp to 175 °C, remain at 175 °C from 15 to 30 min, cool down for 10 min. The total time is 40 min and the maximum energy used is 1400 W. After digestion, all samples were cooled for an hour at room temperature. Once cooled, the samples were transferred from the Teflon vessels using DI water into 50 mL glass volumetric flasks.

2.5. Standards for elemental analysis and Matrix matching method

1000 mg/L single element standards were obtained from Merck (CertiPUR®). Standards were prepared using matrix matching, in the concentration ranges expected in the sample matrix. Two standard mixes were produced from single element combined preparation using a Hamilton diluter (Hamilton™ Microlab™ 600 Diluter) and 10% aqua regia solution. Standard mixes were prepared separately to avoid precipitation of incompatible single element standards. Combined mix 1: P, S. Combined mix 2: Ca, Cu, Fe, Mg, Mn, Na, K, Zn. Yttrium was added as an internal standard to supernatant samples.

2.6. Calibration of the ICP-OES and operation conditions

ICP-OES (PerkinElmer Avio® 500) operation conditions were as described in Table 1. All elements were measured by axial view. An argon humidifier helped alleviate salts in the nebulizer and injector creating a more stable plasma and less cross-contamination.

2.7. Statistical analysis of elemental data

SPSS (IBM SPSS Statistics 25) was used for one-way analysis of variance (ANOVA) and post-hoc test (Tukey) to detect statistical differences between the ammonium formate washes within each element of interest, using a 5% level of significance.

3. Results and discussion

3.1. Washing steps effects on cell recovery

N. oceanica biomass was pre-treated with a series of 5 washing steps of ammonium formate and cell recovery was monitored. After the first centrifugation (Fig. 1) a decrease in both cell and smaller particles was detected but cell disruption was not observed. Cell concentration decreased by 11.1% after the first centrifugation step and then remained stable in the subsequent washes suggesting some dead cells, already present in the microalgal culture, are lost during centrifugation. In line with this hypothesis, dry weight values decreased after the first centrifugation by 45% and then remained stable in the subsequent washes. Cell debris also decreased by 40.1% after the first centrifugation step and continued to decrease with the washing steps (Fig. 1). A more pronounced decrease in dry weight values compared to cell numbers values can be attributed to the removal of other particles, including salts, since their presence contributes to dry weight measurements. This stresses the importance of washing as sample pre-treatment for accurate analysis. In line with our observation, Zhu and Lee (1997) investigated the effect of washing microalgae biomass on the assessment of dry weight and found that unwashed samples had a higher dry weight value due to the presence of salts.

3.2. Washing steps effects on elemental analysis

The washing steps were assessed by three factors: oversaturation of the equipment, matrix interference, and elemental composition of supernatant and biomass (Table 2, Table 3).

3.2.1. Oversaturation of the equipment

In our study, both supernatant and unwashed biomass samples led to an oversaturation of the ICP-OES detector (approximately ≥20 mg/L of Na). Similar to our findings, Napan et al. (2015) observed that salt has affects the accuracy of elemental results by either leading to unstable readings or to an overestimation of elements such as selenium (Se), tin (Sn), Cu and Zn.

3.2.2. Matrix interference on elemental analysis of supernatant samples

Matrix interference was investigated by adding yttrium to the calibration mixes and supernatant samples. After the first centrifugation step there was an yttrium recovery of 69–73% in supernatant samples, which indicates matrix interference. To overcome the matrix interference, samples were diluted up to 400 times, reaching an yttrium recovery of 98–100%. The downside of the dilution of supernatant samples is that elements of interest may become undetectable. Therefore, internal standards should be added to supernatant sample analysis.

3.2.3. Washing steps effects on supernatant and washes elemental determination

Elemental analysis was performed on supernatant samples from each of the 5 washing steps (Table 2). 'Unwashed' represents the concentration of elements remaining in the culture medium. The subsequent washes 1–5 represent the elements removed from the biomass samples

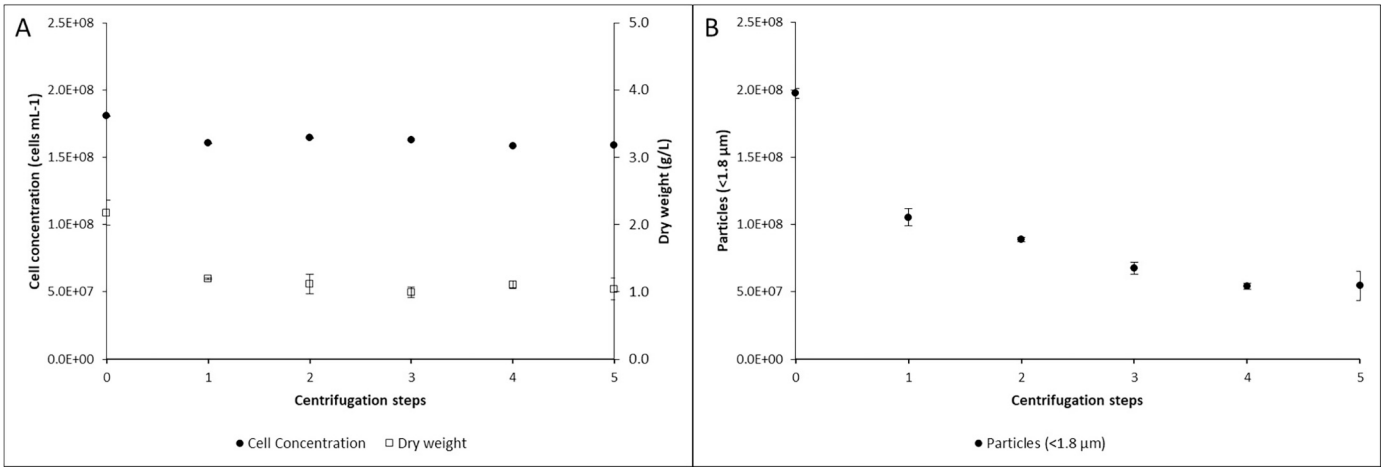

Fig. 1. Effect of washing steps on the recovery of microalgae biomass samples. A) cell concentration during washing steps (black circles) and dry weight (squares); B) particles in the sample smaller than 1.8 µm. All graphs represented in this figure are the average of four biological replicates. A total of 3 outliers were removed from the dry weight.

Table 2
Concentration of elements in supernatant collected during washing steps. Unwashed represents the supernatant of the first centrifugation and the subsquent washes are mostly ammonium formate which was used during the procedure. Each value represents an average of three technical replicates ($n = 3$).

	Supernatant concentrations									
	mg/L) (mg element /L supernatant)									
Treatment	Na	K	S	Ca	Mg	P	Mn	Zn	Fe	Cu
unwashed	12,237.6 ± 688.2 a	1598.4 ± 41.8 a	239.8 ± 5.4 a	104.3 ± 3.4 a	72.0 ± 1.8 a	52.9 ± 1.8 a	1.01 ± 0.04 a	0.43513 ± 0.01094 a	<0.1	<0.01
wash 1	236.0 ± 35.6 b	70.8 ± 12.8 b	6.6 ± 1.3 b	6.5 ± 0.5 b	2.8 ± 0.5 b	5.9 ± 2.1 b	0.04 ± 0.01 b	<0.01	<0.1	<0.01
wash 2	8.4 ± 1.4 b	36.9 ± 6.6 bc	3.7 ± 0.5 b	5.0 ± 1.0 b	2.6 ± 0.4 b	18.2 ± 2.6 c	0.12 ± 0.02 c	<0.01	<0.1	<0.01
wash 3	<1 b	13.6 ± 0.7 c	<1	3.8 ± 0.8 b	<1	7.4 ± 0.7 b	0.08 ± 0.01 bc	<0.01	<0.1	<0.01
wash 4	<1 b	10.0 ± 0.1 c	<1	3.5 ± 0.7 b	<1	<1	<0.01	<0.01	<0.1	<0.01
wash 5	<1	10.0 0.6 c	<1	<1	<1	<1	<0.01	<0.01	<0.1	<0.01

LOQ = limit of quantification.
Mo, Co were not detected in any of the supernatant digested samples.
a, b and c represent the statistical differences between the treatments ($p < 0.0.05$)

Table 3
Elemental composition of *N. oceanica* after centrifugation (unwashed biomass) and ammonium formate washed *N. oceanica* biomass (technical replicate for wash 1 $n = 9$, wash 2 n = 9, wash 3 n = 9, wash 4 $n = 6$ and wash 5 $n = 4$). Differences in technical replicates is due to the amount of sample available to perform the analysis,

	Biomass concentrations									
	(mg/g) (mg element /g microalgal biomass)									
Treatment	Na	Ca	K	S	Mg	P	Mn	Zn	Fe	Cu
unwashed	60.98 ± 0.68 a	19.53 ± 2.74 a	18.47 ± 0.37 a	5.61 ± 3.42 a	1.67 ± 0.04 a	8.32 ± 0.51 a	0.05 ± 0.00 a	0.07 ± 0.00 a	0.28 ± 0.03 a	0.02 ± 0.00 a
wash 1	3.79 ± 0.69 b	16.00 ± 0.74 a	5.66 ± 0.08 b	5.04 ± 1.89 b	1.46 ± 0.05 b	8.82 ± 0.20 a	0.06 ± 0.00 b	0.09 ± 0.01 b	0.33 ± 0.02 b	0.02 ± 0.00 b
wash 2	0.23 ± 0.06 b	14.01 ± 0.36 b	1.66 ± 0.33 c	5.66 ± 4.66 a	1.48 ± 0.08 b	7.69 ± 0.37 b	0.05 ± 0.01 c	0.10 ± 0.03 b	0.35 ± 0.04 c	0.02 ± 0.00 b
wash 3	0.14 ± 0.03 b	11.28 ± 0.19 b	1.05 ± 0.42 c	5.24 ± 1.28 b	1.39 ± 0.09 bc	6.63 ± 0.46 c	0.04 ± 0.00 d	0.08 ± 0.01 c	0.29 ± 0.05 d	0.02 ± 0.00 b
wash 4	0.13 ± 0.92 b	8.12 ± 0.06 b	0.70 ± 0.21 c	5.68 ± 2.50 b	1.57 ± 0.07 ab	6.61 ± 0.25 c	0.02 ± 0.00 e	0.06 ± 0.00 c	0.26 ± 0.03 a,d	0.02 ± 0.00 b
wash 5	0.12 ± 0.05 b	9.37 ± 0.14 b	0.68 ± 0.49 c	5.82 ± 1.21 a	1.63 ± 0.15 ab	6.90 ± 0.70 c	0.02 ± 0.00 f	0.06 ± 0.01 c	0.28 ± 0.04 a,b,d	0.02 ± 0.00 b

ND = not detected, or value is below detection limit.
Mo, Co were not detected in any of the biomass digested samples.

during each washing step (Table 2). Results suggest the remaining major salts of the media (Na, K, Ca) are removed from the biomass samples after two washes. The same can be observed for the remaining minor and trace elements analysed.

3.2.4. Washing steps effects on biomass elemental determination

Biomass samples, subjected to up to five washing steps, were also analysed. In line with the supernatants analysis, results show that the major seawater and medium components were completely removed from *N. oceanica* biomass samples after the second wash (Table 3). After the first washing step, a drastic reduction of 93.8% and 69.4% in Na and K concentrations was observed, respectively, compared to unwashed biomass samples (Table 3). After the second wash, Na and K concentrations were reduced further, up to 99.6% and 91%, respectively, in comparison to unwashed biomass samples. Thus, after wash 2 a total of 61 mg_{Na} was removed per kg of biomass, which represents salt not incorporated into the biomass. After wash 2 there is no statistical difference in Na concentration including washes 3, 4 or 5 ($p < 0.05$). Similarly, after the second wash a total of 17 mg_K was removed per kg of biomass and no more significant effect on K concentration was observed after additional washing steps. Cu, Zn, Mn concentrations show a decreasing trend during the washing. S, Mg and Fe concentrations varied slightly during the washing steps and overall they are considered to remain relatively constant during the experiment (Table 3). Two washes are required, to accurately measure the microalgal elements. The following order of element concentration of the elements in the biomass was found: Ca > P > S > K > Mg > Fe > Na > Zn > Mn > Cu > Co ≈ Mo, similar to what was found in other microalgae species (Ho et al., 2003).

Overall, the elemental analysis of *N. oceanica* biomass showed that there are clear residual amounts of major medium components, Na, K, and Ca, in the wet biomass pellet that do not represent the cellular elemental quota. Therefore, washing the biomass twice is recommended and sufficiently removes the excess of salts present in seawater media which, if left unwashed, lead to the risk of plasma interference and detector saturation. Further work should investigate the effect of salt removal on other biochemical analysis since there is little standardisation of microalgal sample pre-treatment and removing Na from marine microalgal biomass is essential for accurate elemental determination.

4. Conclusion

Our study is the first to emphasize the benefits of washing microalgal biomass twice prior to elemental analysis along with matrix matching. Thus, enabling the analysis of all elements in one single measurement. Washing the biomass does not rupture *N. oceanica* cells, removes cell culture debris and the excess of Na, K, Ca (which do not represent the cellular elemental quota), allowing for a more accurate elemental determination, saving time in processing and analysing samples. Elemental analysis of salt containing supernatant samples should also include an internal standard to counteract matrix interference.

CRediT authorship contribution statement

Bárbara O. Guimarães: Conceptualization, Methodology, Formal analysis, Visualization, Investigation, Writing - original draft, Writing - review & editing. **Pieter Gremmen**: Conceptualization, Methodology, Formal analysis, Visualization, Investigation, Writing - review & editing. **René H. Wijffels**: Conceptualization, Supervision, Writing - review & editing, Funding acquisiton. **Maria J. Barbosa**: Conceptualization, Supervision, Writing - review and editing. **Sarah D'Adamo**: Conceptualization, Formal analysis, Visualization, Writing - review & editing, Project administration.

Declaration of Competing Interest

The authors declare there are no conflicts of interest that could affect this work.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2021.736526.

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