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Using oil immersion to deliver a naturally-derived, stable bromoform product from the red seaweed *Asparagopsis taxiformis*

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ABSTRACT

Asparagopsis taxiformis (*Asparagopsis*) inhibits the production of enteric methane in ruminants. A next critical step in the implementation of this technology is the delivery of a naturally-derived product that maximises the concentration and longer-term retention of bromoform. This study (1) quantified the effects of solvent (water or oil), initial processing (intact or homogenised), and temperature (4 or 25 °C) on the stabilisation of bromoform over time, and (2) assessed the effects of increasing the biomass loading (g biomass mL^{-1} solvent) of *Asparagopsis* on the concentration of bromoform in a formulation. The most effective method was to homogenise freshly-collected *Asparagopsis* in oil, which resulted in the highest concentration of bromoform (19.2 ± 2.1 mg g^{-1} dw algae) in the homogeneous product in the shortest time (one day). In addition, the final product had a shelf life of at least 12 weeks, even when stored at room temperature (25 °C). Notably, there was an increase in the concentration of bromoform per mL of oil between each increment of biomass loading tested, with the highest concentration of bromoform of 4.04 ± 0.51 mg mL^{-1} in the maximum ratio of biomass to oil of 120 g 100 mL^{-1}. The method described here provides a viable processing alternative to freeze-drying, resulting in the stabilisation of the bromoform from *Asparagopsis*, which will be critical to the success of using *Asparagopsis* on a larger scale to mitigate the production of methane in ruminants.

1. Introduction

Halogenated compounds, which are defined by the presence of chlorine, iodine, fluorine, and bromine, are biologically active chemicals often with beneficial properties to humans and animals [1–4]. For example, bromoform ($CHBr_3$) and dibromoiodomethane ($CHBr_2I$) have antibacterial and antifungal properties mitigating the proliferation of *Pseudomonas aeruginosa*, *Escherichia coli*, *Candida albicans*, and *Staphylococcus aureus*, which occur in humans [4]. Halogenated methane analogues such as bromoform, dibromochloromethane ($CHBr_2Cl$), and bromochloromethane (CH_2BrCl) have anti-methanogenic properties and reduce the production of enteric methane in ruminants by inhibiting an essential enzymatic reaction required by methanogenic archaea [5], thus halting the formation of methane [3,6] and increasing productivity [7,8], with a concomitant decrease, but not an elimination, of methanogenic archaea in the rumen (*in vitro*) [9,10]. In these examples, the highest bioactivity was ascribed to bromoform. However, bromoform and chemically-related compounds, when purified and in high doses, have been identified as a probable carcinogenic and as ozone-depleting [11–13]. Therefore, there is a demand for natural resources with these compounds for human and animal applications [14,15].

Bromoform and chemically-related compounds are naturally produced in green, brown, and red marine macroalgae [1,2,16,17]. However, some of the highest concentrations of bromoform are produced in the red macroalgal genus *Asparagopsis* [3], where it is used as a chemical defence in the marine environment [18,19]. Recently, *Asparagopsis taxiformis* ([Delile] Trevisan de Saint-Léon 1845) and *A. armata* (Harvey 1855) have been successfully used to inhibit the production of enteric methane in ruminants. When included at a dosage of 2% of substrate organic matter (w/w) *in vitro*, methane was reduced by > 99% [20,21], and when included at 0.2–5% of substrate organic matter (w/w) *in vivo*, methane was reduced by 81–98% [6,8,10]. Importantly, these doses resulted in over 40% increases in weight gain and no negative effects on daily feed intake, feed conversion efficiencies, or rumen function, and no bromoform residues or changes in meat eating quality detected in beef cattle [8]. Dairy cows fed *Asparagopsis* at a 0.5% OM dose demonstrated no change in weight gain or milk productivity, while a higher dose of 1.0 OM inclusion led to both reduced weight gain and reduced milk production [22], indicating that the

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higher energy demands of lactating dairy cows require special consideration and management of dosing. More importantly than the % dosage used in those studies, is the concentration of bromoform in the biomass, which must remain above 1 mg g^{-1} organic matter for the biomass to maintain its efficacy and reduce methane by > 99%, at least *in vitro* [23]. However, the inherent difficulty with a halogenated compound such as bromoform, even as part of whole biomass, is that it can be lost to the environment through volatilisation [4,15,23]. Therefore, there is a need to develop innovative methods, with the fewest steps, for the processing of intact, fresh biomass, which maximise the concentration and longer-term retention of bromoform.

An effective processing method used to retain the content of antioxidants, phenols, vitamins, and other bioactives in natural products is freeze-drying [24–27]. To date, freeze-drying biomass of *A. taxiformis* (henceforth referred to as *Asparagopsis*) also yields the highest concentration of bromoform compared with other post-harvest processing methods [23]. However, freeze-drying is energy-intensive and can be difficult on a large scale, at least from a logistical perspective. An alternative is to use a food-grade solvent, such as vegetable oil, that captures, retains, and prevents the degradation of bioactive compounds. Phytosterols, tocopherols, and carotenoids can be preserved in vegetable oils when stored at room temperature [28,29] even with exposure to light and oxygen [30,31], due to their esterification and, therefore, lipophilicity [32,33]. Lipophilic compounds also have higher partition coefficients (log K_{OW}), a ratio measured by the difference in solubility in two immiscible liquids, which can range between −3 (extremely hydrophilic) and +10 (extremely lipophilic) [34]. Bromoform has a log K_{OW} of +2.38 [35] and partitioning from fresh algal biomass into oil may reduce the losses of bioactive compounds through volatilisation that occur during the processing and storage of harvested biomass.

The aims of this study were therefore to (1) quantify the effect of solvent (water or oil) and initial processing of *Asparagopsis* biomass (intact or homogenised) on the release of bromoform into the solvent; (2) quantify the effect of temperature on the loss of bromoform from these solutions over time; and (3) based on the most effective processing method, assess the effect of increasing the loading of biomass in the solvent on the concentration of bromoform.

2. Materials and methods

2.1. Biomass collection

Asparagopsis (gametophyte stage) was collected from Magnetic Island (QLD, Australia) in Austral winter (separate collections for each of the three experiments). In the first experiment, freshly-collected biomass was blotted dry, split into 30.0 g fresh weight (fw) portions, and immediately placed into individual 250 mL glass bottles (SCHOTT Australia Pty. Ltd.; Frenchs Forest, NSW, Australia) pre-filled with 100 mL of either blended vegetable oil (n = 6; canola oil 95%, sunflower oil 5%; Homebrand, Bella Vista, NSW, Australia) or milliQ water (n = 6). The bottles were capped, stored on ice, and transported to James Cook University (JCU, Townsville, QLD, Australia) where they were used to determine the effects of solvent and initial processing on the release of bromoform from the biomass into the solvent (see Section 2.2 '*Processing methods*'). The samples (bottles) were subsequently used to quantify the effects of storage time and temperature on the retention of bromoform in the solutions (see Section 2.3 '*Storage*'). In the second experiment, freshly-collected biomass was blotted dry and split into portions of 30, 60, 90, and 120 g fw (n = 3). According to the results from the processing methods, each portion was immediately placed into a 250 mL glass bottle prefilled with 100 mL of blended vegetable oil (as above). The bottles were capped, stored on ice, and transported to JCU to determine the effects of the loading of biomass on the concentration of bromoform in the solutions (see Section 2.4 '*Biomass loading*').

Additional biomass from each of these first two experiments was freeze dried (Virtis Benchtop 2 K, −55 °C, 120 μbar, VWR, Australia), and the content of bromoform in the biomass was quantified using the standard methanol extraction protocol [18] and used as a control for the comparative assessment of the effectiveness of each processing method. This biomass was split into 30 g fw portions (n = 3 per collection day), placed into separate resealable polyethylene bags, and stored on ice prior to the determination of dry weight (dw), the fw to dw ratio (fw:dw), and the concentration of bromoform in the biomass (see Section 2.5 '*Quantification of bromoform*'). In a third experiment, freshly-collected biomass was blotted dry, split into 30 g fw portions, placed into separate resealable polyethylene bags (n = 12), and stored on ice prior to being freeze-dried. Subsequently, 4 g dw samples were used to quantify the effects of storage time and temperature on the loss of bromoform from the freeze-dried *Asparagopsis* (see Section 2.3 '*Storage*').

2.2. Processing methods

Biomass samples were transported to the laboratory (less than 4 h from the time of collection), where biomass in three of the six replicate bottles from each solvent (vegetable oil or milliQ water) was homogenised (IKA ultra-turrax T-25, VWR Australia) for 60 s, while biomass in the remaining three replicates was left intact. All twelve bottles were then placed in the refrigerator (4 °C) and the concentration of bromoform was quantified after 1, 3, 5, 7, and 10 days to determine which method was most effective (*i.e.* which method released the most bromoform in the shortest amount of time). On each sampling day, 1.5 mL subsamples of solution (oil or water) were collected from each replicate bottle and centrifuged (12,000*g* for 1 min, Eppendorf 5425, VWR, Australia) to remove solids after which 1.0 mL subsamples of the clarified solutions were used for the quantification of bromoform (see Section 2.5 '*Quantification of bromoform*'). The concentrations of bromoform, reported as mg g^{-1} dw of algae, were subsequently used for statistical analysis.

2.3. Storage

After the initial 10 days of testing the effects of processing methods on the release of bromoform into the solvent (oil or water), the solvent from each bottle was separated from the biomass by pouring it through a 100 μm nylon mesh. The liquid was then centrifuged (3200*g* for 15 min, Eppendorf 5810R, VWR, Australia) to remove any remaining solids and the supernatant from each replicate sample was split into two 30 mL glass bottles (SCHOTT Australia Pty. Ltd.; Frenchs Forest, NSW, Australia). One of the bottles was stored at 4 °C and the other was stored at 25 °C. Importantly, the replicates (n = 3) used to determine any differences between the initial processing of the biomass (intact or homogenised) were combined for this experiment for statistical purposes based on the results from the processing methods (see results from Section 3.1 '*Processing methods*') and the fact that the biomass was removed from the solvent. Therefore, n = 6 per solvent (oil or water) per temperature (4°C or 25°C). Subsamples (1 mL) were collected from each bottle after 1, 4, 8, and 12 weeks of storage and used for the quantification of bromoform (see Section 2.5 '*Quantification of bromoform*') to determine which solvent and storage temperature resulted in the greatest retention of bromoform. The concentrations of bromoform, reported as the mg g^{-1} dw of algae originally included, were subsequently used for statistical analysis.

To determine the effects of storage time and temperature on the concentration of bromoform in the freeze-dried material, 4 g portions were placed into twelve 70 mL plastic containers (Sarstedt, Nümbrecht Germany) with one silica packet each. Three of these were used for the quantification of bromoform at time 0 (no storage). The remaining containers (n = 9) were then kept under three storage conditions – frozen (−20 °C), refrigerated (−4 °C), and room temperature (25 °C) (n = 3 for each storage condition). After twelve weeks under these

conditions, the concentration of bromoform in the biomass was quantified (see Section 2.5 '*Quantification of bromoform*') and the percent loss was calculated using the concentrations quantified at time 0 (no storage).

2.4. Biomass loading

Biomass samples were transported to the laboratory (less than 4 h from the time of collection), where the biomass (30, 60, 90, and 120 g fw; $n = 3$), which was mixed with vegetable oil (100 mL), was homogenised (IKA ultra-turrax T-25, VWR, Australia) and placed in the refrigerator (4 °C). The highest loading of biomass used in this study (120 g fw biomass per 100 mL oil) was based on the maximum quantity of biomass that could be physically packed into the bottle. Subsamples (1 mL) were collected after 24 h and used for the quantification of bromoform (see Section 2.5 '*Quantification of bromoform*') to determine which biomass loading yielded the highest concentration in the oil. The concentrations of bromoform, reported as mg g^{-1} dw of algae and mg mL^{-1} of oil, were subsequently used for statistical analysis.

2.5. Quantification of bromoform

For the aqueous subsamples (water as the solvent; Sections 2.2 and 2.3), bromoform was extracted from the 1 mL subsamples collected as described in Cancho et al. [36], with the modification of using naphthalene (10 μg mL^{-1} in methyl tert-buthyl ether [MTBE], Merck, Castle Hill, Australia) as the only internal standard. The MTBE phase was collected and analysed by gas chromatography–mass spectrometry (GC–MS) as described below.

For the oil subsamples (oil as the solvent; Sections 2.2, 2.3, and 2.4), bromoform was extracted by mixing the 1 mL subsample with 1 mL of high performance liquid chromatography-grade methanol, with naphthalene (10 μg mL^{-1}) as an internal standard followed by partitioning for 2 h at 4 °C. The partitioning conditions were selected based on previous experiments testing solvent (hexane, methanol, MTBE, and dichloromethane) and time (0.5–48 h). All organic solvents and naphthalene were from Merck, Castle Hill, Australia. The methanol phase was collected and analysed by GC–MS as described below.

The additional biomass from each of the two experiments was transported to the laboratory on ice (less than 4 h from the time of collection) and immediately frozen (−80 °C) and then freeze dried (Virtiz benchtop 2 K, −55 °C, 120 μbar, VWR, Australia) for 48 h. The freeze-dried biomass was weighed to calculate fw:dw, and then milled to 1 mm and stored at −80 °C in sealed jars prior to being used for the quantification of bromoform. The content of bromoform in the biomass was quantified within four weeks from collection for the control biomass, and after 12 weeks for the biomass used to quantify the effects of storage time and temperature. Briefly, a mixture of 10 mL of methanol and 50 mg of freeze-dried biomass was sonicated for 15 min, left for 72 h in a freezer, and then filtered through a syringe filter (0.2 μm; [18]). Naphthalene (10 μg mL^{-1}) was incorporated as the internal standard and the filtrate was collected and analysed by GC–MS as described below.

The GC–MS (Agilent 7890c [Agilent, Australia] equipped with a Zebron ZB-wax capillary column, 30 m × 0.25 mm × 0.25 μm, Phenomenex, Australia) analysis followed Paul et al. [18], with modifications as described in Machado et al. [3]. The analytical conditions were pulsed injections (1 μL, 35 psi) in splitless mode, with temperatures of the injection port (250 °C), GC–MS interface (300 °C), and oven (held at 40 °C for 1 min, ramped at 16 °C min^{-1} to 250 °C, then held at 250 °C for 2 min), with He as the carrier gas (2 mL min^{-1}). Separate standard curves were generated for each method (aqueous, oil, freeze-dried biomass) and the concentration of target compounds in each sample was calculated from the ratio of the peak areas of the target compound over the internal standard. Bromoform was identified by comparison with a commercial standard (Merck, Castle Hill Australia) and based on its characteristic ion fragments (molecular ion cluster at m/z 250, 252, 254, 256 [1:2:2:1]). The concentration of bromoform was normalised to the amount of dry biomass (calculated from the collection-specific fw:dw ratios) and reported as mean mg bromoform per g dw biomass.

2.6. Statistical analysis

A three-factor permutational analysis of variance (PERMANOVA) was used to determine the effects of solvent (oil or water; fixed factor) and initial processing (intact or homogenised; fixed factor) on the concentration of bromoform released from the biomass over time (1, 3, 5, 7, and 10 days; fixed factor). After 10 days, the algal biomass was removed from the media and a two-factor PERMANOVA was used to determine the effects of solvent (oil or water; fixed factor) and temperature (4 or 25 °C; fixed factor) on the concentration of bromoform that remained in the liquid over time (0 [*i.e.* the day 10 samples], 1, 4, 8, and 12 weeks; fixed factor). Importantly, the initial processing of the biomass (intact or homogenised) was not included in this statistical analysis and the replicates for each ($n = 3$) were combined, since initial processing had no effect on the concentration of bromoform at the end of the 10-day period, nor in the retention of bromoform over the storage time. One-factor PERMANOVAs were also used to determine whether biomass loading (30, 60, 90, and 120 g 100 mL^{-1}; fixed factor) had an effect on the concentration of bromoform as either mg g^{-1} dw of algae or mg mL^{-1} of oil.

The PERMANOVA analyses were performed using PRIMER 6 (v. 6.1.13 [37]) and PERMANOVA+ (v. 1.0.3. [38]). For PERMANOVA, Euclidean similarity matrices were produced using the untransformed raw data and p-values were calculated from 9999 random permutations. However, if there was a low permutation number, p-values were calculated using a Monte Carlo adjustment. Pairwise *a posteriori* comparisons were used to determine significant groupings, where applicable, and differences were only considered significant if $p < 0.05$. All data are presented as mean ± standard error.

3. Results

3.1. Processing methods

After one day, the highest concentration of bromoform was in the biomass homogenised in oil (19.2 ± 2.1 mg g^{-1} dw algae) and this did not increase by the end of the 10-day period, with similar amounts of bromoform measured on day 10 (19.8 ± 2.4 mg g^{-1} dw algae; Fig. 1A). Conversely, after one day, the concentration of bromoform in the biomass left intact in oil was similar to the water treatments, and 40.6% less than the biomass homogenised in oil. However, this concentration increased over the 10-day period and reached an equivalent concentration of bromoform (19.2 ± 1.5 mg g^{-1} dw algae) to the biomass homogenised in oil after seven days. Notably, there was no difference between treatments in the concentration of bromoform in the biomass in water (intact or homogenised) and, after ten days, the concentration of bromoform was 37.1–46.3% lower in water than in oil for both intact and homogenised biomass. This resulted in a significant interaction between solvent, initial processing, and time (PERMANOVA, pseudo-$f_{(4,40)} = 3.62$, $p = 0.014$). Therefore, the most effective method, resulting in the highest concentration of bromoform in the shortest amount of time, was to homogenise the *Asparagopsis* biomass in oil and let it steep for one day.

The concentration of bromoform of the control (freeze-dried biomass), quantified using the standard methanol extraction, was 16.7 ± 1.0 mg g^{-1} dw algae (dashed line in Fig. 1A).

3.2. Storage

After the samples were clarified and stored, the oil was more

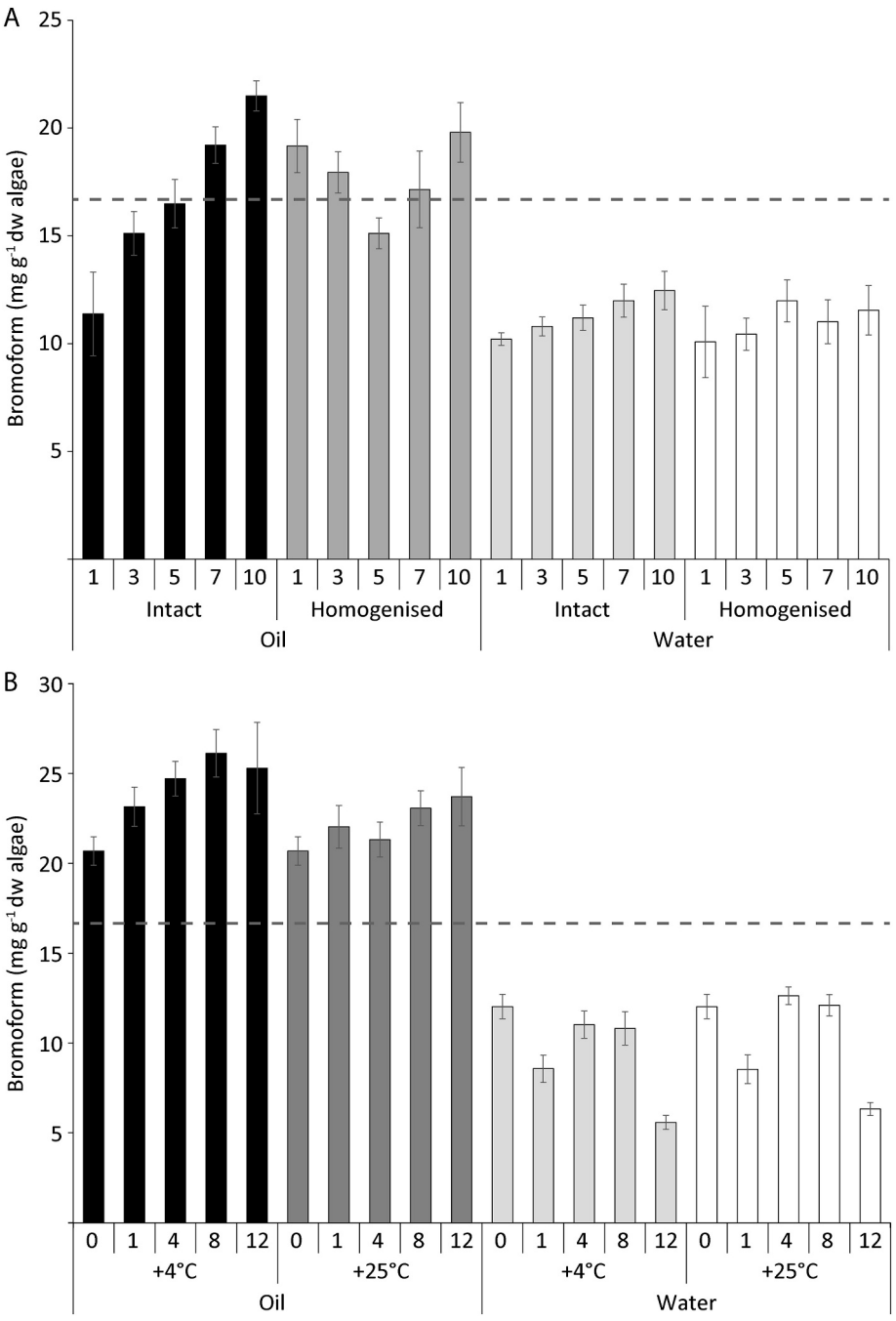

Fig. 1. The (A) concentration of bromoform released from intact or homogenised biomass of *Asparagopsis* into oil or water over 10 days and (B) the retention of bromoform in oil or water stored at 4 °C or 25 °C over 12 weeks. Data are presented as mean ± se; $n = 3$ for panel A and $n = 6$ for panel B. The dashed line represents the concentration of bromoform of freeze-dried biomass using the standard methanol extraction method.

effective at retaining bromoform than the water. More than 47% of the bromoform was lost after 12 weeks from water, regardless of temperature (Fig. 1B). In contrast, there was a statistically significant increase in the concentration of bromoform in oil stored at 4 °C over the 12-week storage period from 20.6 ± 1.9 mg g^{-1} dw algae (week 0) to a maximum of 26.1 ± 2.4 mg g^{-1} dw algae (week 8; Fig. 1B). This was not the case for the oil stored at 25 °C, where the concentration of bromoform was not statistically different over the 12 week storage period, although it did increase from 20.6 ± 1.9 to a maximum of 23.7 ± 4.0 mg g^{-1} dw algae. These increases were likely due to particulate biomass (< 100 μm) remaining in the clarified liquid that continued to release bromoform into the oil. Consequently, the oil stored at 25 °C had significantly lower concentrations of bromoform than the oil stored at 4 °C after 12 weeks, however, this difference was small (6.3%; Fig. 1B). These results caused a significant interaction between week and solvent (PERMANOVA, pseudo-$f_{(4,100)} = 12.27$, $p < 0.001$) as well as a significant interaction between temperature and solvent (pseudo-$f_{(1,100)} = 48.59$, $p = 0.006$).

The concentration of bromoform of the control (freeze-dried biomass), quantified using the standard methanol extraction, was 16.7 ± 1.0 mg g^{-1} dw algae (dashed line in Fig. 1B).

Finally, after freeze-drying and subsequent storage of *Asparagopsis* biomass for 12 weeks under room temperature (25 °C), refrigeration (4 °C), and freezing (−20 °C) conditions, the losses from the initial concentration of bromoform were 37.8 ± 6.1, 5.0 ± 4.1, and 1.3 ± 1.0%, respectively.

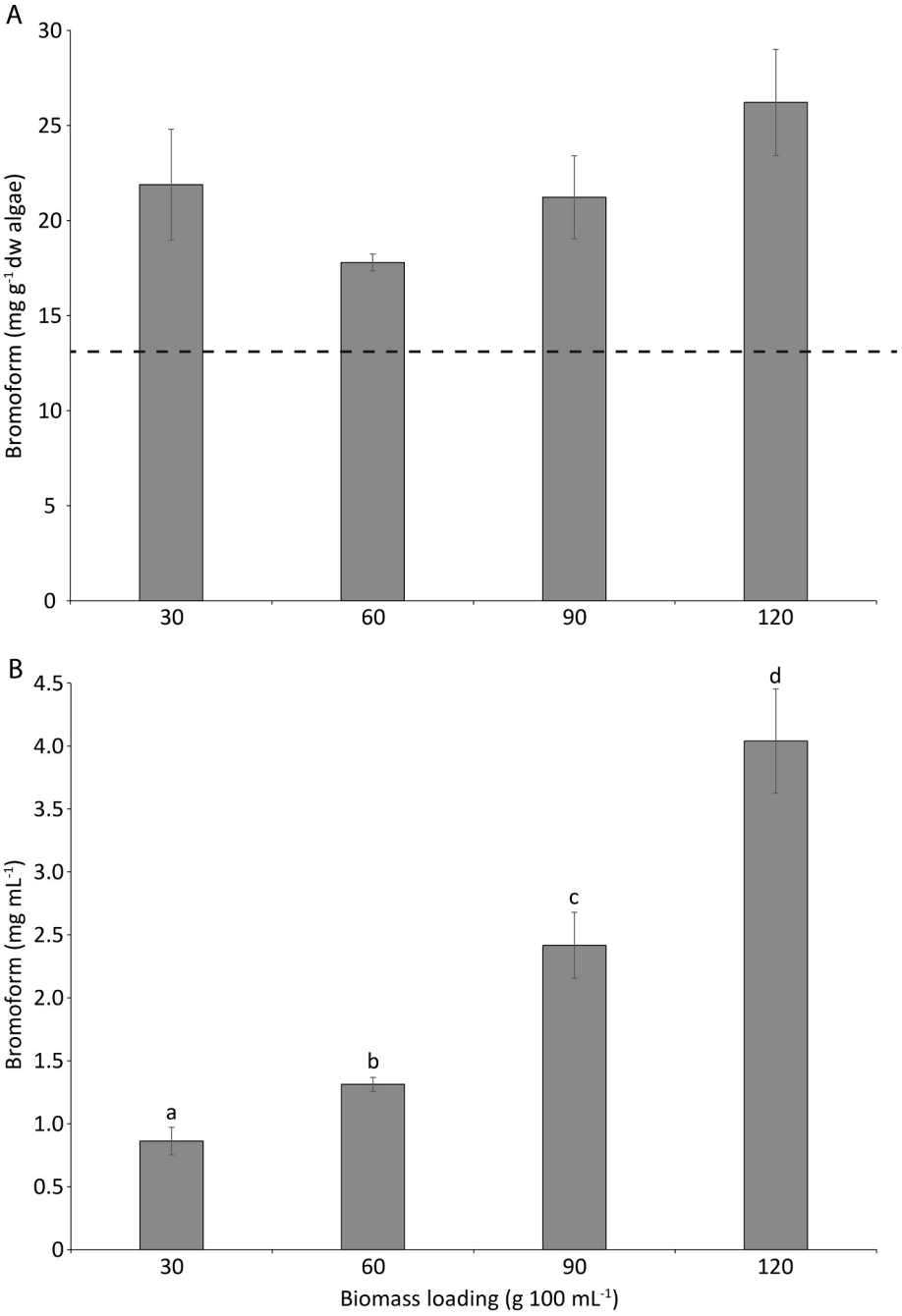

Fig. 2. The (A) concentration of bromoform (mg g^{-1} dw algal biomass) and the (B) concentration of bromoform (mg mL^{-1}) released into oil with increasing densities of homogenised biomass. Data are presented as mean ± se; $n = 3$. Dashed line represents the concentration of bromoform of freeze-dried biomass using the standard methanol extraction method. Superscript letters in Fig. 2B represent significant groupings.

3.3. Biomass loading

There was no difference in the concentration of bromoform in the oil per gram dw of algal biomass (17.79 ± 0.54 to 26.21 ± 3.41 mg g^{-1} dw algae; pseudo-$f_{(3,11)} = 2.25$, $p = 0.175$) and as the loading of the biomass increased, there was no increase or decrease in the effectiveness of the release of bromoform even though there was more or less oil per gram of algae, respectively (Fig. 2A). Consequently, there was a significant increase in the concentration of bromoform per mL of oil between each of the increments of biomass loading (PERMANOVA, pseudo-$f_{(3,11)} = 31.32$, $p < 0.001$; Fig. 2B), and there was 1.5, 1.8, and 1.7 times more bromoform released into oil per mL between 30 and 60, 60–90, and 90–120 g 100 mL^{-1}, respectively. Notably, although the highest biomass loading (1.2 g mL^{-1}) was four times that of the lowest biomass loading (0.3 g mL^{-1}), the concentration of bromoform was 4.7 times higher at 4.04 ± 0.51 mg mL^{-1} in the oil.

The concentration of bromoform of the control (freeze-dried biomass), quantified using the standard methanol extraction, was 13.11 ± 0.14 mg g^{-1} dw algae (dashed line in Fig. 2A).

4. Discussion

The immersion of *Asparagopsis* biomass into oil allowed for high concentrations of bromoform to be stabilised, with a shelf life of at least 12 weeks, even when stored at room temperature (25 °C). In contrast,

immersion in water resulted in ~40% less bromoform being extracted initially and a > 47% loss of bromoform when stored for 12 weeks. The most effective method to deliver a naturally-derived, stable bromoform product, was to homogenise *Asparagopsis* biomass in oil, which resulted in a homogeneous sample with the highest concentration of bromoform (19.2 ± 2.1 mg g^{-1} dw algae) in the shortest time (one day). The biomass left intact in oil resulted in a similar concentration of bromoform (19.2 ± 1.5 mg g^{-1} dw algae), but only after seven days, demonstrating a slow rate of release of bromoform to reach equilibrium between the solvent and the biomass.

From a practical perspective, the homogenisation method, where biomass is processed immediately upon immersion, results in a stabilised bromoform product that can be provided directly to animals. For example, ≥1 mg of bromoform per g dry weight of *Asparagopsis* at an inclusion rate of 2% of the organic matter fed to the animal is required to completely reduce methane production *in vitro* [23]. Therefore, beef cattle consuming 9–15 kg day^{-1} (dry matter intake) would require a daily supplement of 41–63 mL of oil, respectively. Furthermore, *in vivo*, 6.55 mg of bromoform per g dry weight of *Asparagopsis* at an inclusion rate of 0.2% of the organic matter fed to the animal reduces methane production by 98% [8]. Therefore, beef cattle would require a daily supplement of 27–45 mL of oil. This is based on an organic matter concentration in the feed of 92% ([39]) and a concentration of bromoform of 4.04 ± 0.51 mg mL^{-1} in the oil of the highest biomass loading tested here (120 g 100 mL^{-1}). Regardless of which processing method was used (homogenisation or intact biomass in oil), both resulted in higher concentrations of bromoform in the final product than the freeze-dried material which, up to now, has been the standard method to prepare *Asparagopsis* for use [3,6,8,20,40]. Similarly, the concentration of bromoform of the freeze-dried material used in the biomass loading trial was 13.11 ± 0.14 mg g^{-1} dw algae, while the bromoform content in the oil of the 120 g 100 mL^{-1} loading treatment was 26.21 ± 3.41 mg g^{-1} dw algae, or two times that of the freeze-dried material. This difference between the concentration of bromoform in the freeze dried material and the dry weight equivalent added as fresh material to oil may be due, in part, to the initial loss of bromoform from the *Asparagopsis* between harvesting and freeze-drying of the biomass and/or losses during the freeze-drying process due to the volatility of bromoform. Therefore, the results from this study show clear benefits to both the recovery and stability of bromoform through a naturally-derived oil immersion product from *Asparagopsis*. Importantly, high-lipid (oil) feed supplementation is already an adopted method for the reduction of methane in ruminants [41] where specific types of lipids – not protected from digestion – can decrease ruminal fermentation of organic matter or increase hydrogenation, both of which reduce the production of methane (reviewed in [42]; reviewed in [43]). High-lipid feed supplementation can also enhance dry matter intake, increase milk production, and improve energy balance in ruminants [44]. Therefore, using *Asparagopsis* in an oil formulation has the potential to provide additive effects on methane reduction above those achieved by dosing with freeze-dried *Asparagopsis* alone. As a caveat, feeds with higher concentrations of lipids generally have higher concentrations of protein and, therefore, nitrogen, resulting in higher rates of nitrous oxide production by the animal [45].

Notably, there was a difference between the concentration of bromoform in the freeze-dried biomass used for the processing and storage trials (16.7 ± 1.0 mg g^{-1} dw algae) and the freeze-dried biomass used for the biomass loading trial (13.11 ± 0.14 mg g^{-1} dw algae), which was collected at a later date. The differences in the concentration of bromoform can be due to within-plant variation, or seasonal and environmental conditions [18,46]. Understanding the genetic and environmental factors affecting the concentration of bromoform, and manipulating life-history phases to cultivate *Asparagopsis*, will play a critical role in future supply of quality biomass throughout the year. In addition, the form of the biomass and the time of storage affects quality. For example, freeze-dried biomass stored under refrigerated (4 °C) or room temperature (25 °C) conditions lost 5.0 ± 4.1% and 37.8 ± 6.1% of the initial concentration of bromoform after 12 weeks, respectively. In contrast, immersing the *Asparagopsis* into oil immediately after harvest, removing the biomass after 10 days, and storing the oil for 12 weeks resulted in no loss of bromoform at 25 °C and an increase in bromoform at 4 °C. Based on these results, it may be possible to increase the concentration of bromoform in the oil by letting it steep for the full 12 weeks, although this has yet to be demonstrated. In addition, minimising the volume of oil required to stabilise the bromoform from *Asparagopsis* (*i.e.*, maximising the biomass loading) will be critical to maximising dose efficacy and reducing the costs associated with this processing technique. Finally, the use of oil immersion will also facilitate seasonal harvesting of *Asparagopsis* depending on location and cultivation cycle. This is important as alternative methods for the storage of biomass to ensure year round availability such as ensilage [47,48] or thermal drying are less appropriate due to the volatile nature of bromoform with subsequent losses in temperature dependent processes [23].

There was no difference in the concentration of bromoform in the oil per gram dw of algal biomass, suggesting that the effectiveness of immersion in oil is constant regardless of the loading of biomass. In this study, the highest biomass loading presented was 120 g 100 mL^{-1}, since loadings > 120 g 100 mL^{-1} resulted in a homogenised solution that was too thick for subsequent processing. This is due to the presence of sulfated cell-wall polysaccharides in *Asparagopsis* that are typically water soluble [49], but form an emulsion with the oil and the residual external and internal water from the macroalgal biomass, which became denser as the biomass loading increased. Therefore, since the lowest ratio of biomass:oil is desired to minimise the quantity of oil required to stabilise the bromoform, homogenising 120 g of *Asparagopsis* in 100 mL of oil (ratio of 1.2:1) is optimal.

In conclusion, the method described here provides a viable processing alternative to stabilise the bromoform from *Asparagopsis* with the added advantage of minimising the logistically-difficult and energy-intensive requirements of sub-zero degree storage and freeze drying. Furthermore, since no bromoform is lost after 12 weeks of storage, the use of the oil would not be limited to regions proximate to areas of cultivation, but could be implemented within a transportation range where suitable feeding systems are in place. This is critical to the success of using *Asparagopsis* on a larger scale to mitigate the production of methane in ruminants.

Statement of informed consent

All the authors declare that informed consent is not applicable and there are no conflicts.

Declaration of author's agreement to authorship and submission

All authors have approved the final manuscript and agree with its submission to *Algal Research* for peer review.

CRediT authorship contribution statement

Conception and design of the study: MM, RdN; provision of study materials: MM, RdN; acquisition of data: LTN; analysis and interpretation of the data: MM, MJV, LTN; drafting the manuscript: MM, MJV, RdN; critical review of the manuscript; MM, MJV, RdN; final approval of the manuscript: MM, RdN.

Declaration of competing interest

This research is part of the Pacific Biotechnologies Research and Development program for the integrated production of macroalgae. De Nys and Magnusson are co-inventors on patents relating to the use of

Asparagopsis for methane mitigation (AU2015208661, AU2016/050689, WO2018/018062). De Nys and Magnusson are co-inventors on a patent application relating to novel processing options for the seaweed *Asparagopsis* for retention of bromoform (AU2018904642).

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