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Review article

Benefits and risks of including the bromoform containing seaweed *Asparagopsis* in feed for the reduction of methane production from ruminants

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ABSTRACT

The agricultural production of ruminants is responsible for 24% of global methane emissions, contributing 39% of emissions of this greenhouse gas from the agricultural sector. Strategies to mitigate ruminant methanogenesis include the use of methanogen inhibitors. For example, the seaweeds *Asparagopsis taxiformis* and *Asparagopsis armata* included at low levels in the feed of cattle and sheep inhibit methanogenesis by up to 98%, with evidence of improvements in feed utilisation efficiency. This has resulted in an increasing interest in and demand for these seaweeds globally. In response, research is progressing rapidly to facilitate *Asparagopsis* cultivation at large scale, and to develop aquaculture production systems to enable a high quality and consistent supply chain. In addition to developing robust strategies for sustainable production, it is important to consider and evaluate the benefits and risks associated with its production and subsequent use as an antimethanogenic feed ingredient for ruminant livestock. This review focuses on the relevant ruminal biochemical pathways, degradation, and toxicological risks associated with bromoform ($CHBr_3$), the major active ingredient for inhibition of methanogenesis in *Asparagopsis,* and the effects that production of *Asparagopsis* and its use as a ruminant feed ingredient might have on atmospheric chemistry.

1. Background

Methane (CH_4) is important in carbon cycling in natural environments and the predominant source is production by methanogenic archaea [1]. However, many anthropogenic practices have led to the disturbance of the natural cycle, leading to a net increase in the partial pressure of atmospheric methane [2]. From a climate perspective, the importance of methane emissions are second only to carbon dioxide (CO_2) emissions, having a global warming potential (GWP) 28–34 times that of CO_2 [3]. Methane is a key target for emissions reduction due to its GWP, and due to its short lifespan (8.4 years) in the atmosphere, the effects of new management strategies will be measurable in the short term. Importantly, strategies for the management of anthropogenic methane emissions from key sources such as fossil fuels, and municipal waste are being implemented (e.g. phasing out use of fossil resources, (catalytic) combustion, waste minimization, aeration) [4,5], while those for emissions from agriculture are lagging. Emissions of methane from agriculture are dominated by the farming of ruminants (39% [2], Fig. 1) and therefore the development of management tools and strategies to reduce the carbon footprint of this socially and economically important industry are essential.

There are a number of management strategies for ruminant methane mitigation under investigation with varying capacity to reduce enteric methane emissions [6–8]. These include selective breeding, vaccines, methanogenesis inhibitors, and dietary measures. While the effective management of enteric methane emissions is likely to be integrated

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across strategies, methanogenesis inhibitors in the form of feed ingredients are the best performers to date with the highly bioactive seaweeds of the genus *Asparagopsis* having the highest activity [9–13].

In the context of dietary intervention, seaweeds are a traditional part of livestock diets in coastal regions and have been used in stock feeds since the recording of agricultural practices began [14]. Brown seaweed (kelp and fucoids) has traditionally been the main macroalgal group used to supplement animal feeds, however, select species from all seaweed phyla (Rhodophyta, Chlorophyta, and Ochrophyta) have been investigated for their capacity to reduce enteric methane emissions in ruminants [15–19]. Of these seaweeds, the species of the genus *Asparagopsis (A. taxiformis* and *A. armata*) stand out for their efficacy, inhibiting methane production (methanogenesis) through specific effects on rumen methanogenic archaea (methanogens) [9–13]. These species consistently and significantly reduce methane emissions from sheep [10] and cattle [9,11–13] consuming *Asparagopsis* at inclusion levels of less than 1% of the feed organic matter (OM) intake. Notably, the effective level of inclusion is dependent on the bioactive content of the seaweed and the formulation of the basal feed [9,12]. The effect is linked to the halogenated methane analogue (HMA) components (Fig. 2) of *Asparagopsis,* which inhibit key steps involved in methanogenesis [20–22]. Although *Asparagopsis* contains a wide range of organobromine compounds [23–26] with potential bioactive and environmental effects, bromoform is the most abundant of the bioactive constituents by an order of magnitude over the next most abundant compound, dibromochloromethane (DBCM) [27], with an equivalent or higher anti-methanogenic activity compared to other HMAs [20,28].

Although HMAs are widely used in a biosecurity context as fumigants and in the chemical industry as solvents, there are documented health and environmental concerns over the mitigation of agricultural methane production, particularly enteric methane, with products that contain HMAs and related compounds [6,30]. Consequently, the use of *Asparagopsis* as a livestock feed ingredient containing a naturally produced HMA bioactive, bromoform, for the mitigation of methane production in ruminants has raised similar concerns regarding potential carcinogenic and ozone-depleting effects [31–33]. However, the mechanistic details associated with the mode of action of bromoform and its degradation by ruminant microbes have not previously been explicitly described, and this is central to understanding any risks. Consequently, the mode of action of bromoform and its degradation as it inhibits methanogenesis is reviewed, and the benefits and risks of this methane mitigation strategy assessed and discussed. Bromoform is considered the most important contributor to the anti-methanogenic activity of *Asparagopsis* and therefore the discussion has a focus on 1) the significant biochemical pathways in the rumen, 2) toxicology, and 3) atmospheric chemistry of bromoform.

2. Significant biochemical pathways

Nutrition in ruminants is predominantly dependent on the microbial fermentation of feed, producing volatile fatty acids (VFAs) from carbohydrate fermentation, and further microbial metabolism resulting in proteins from bacterial biomass. Nutrition is, to a lesser extent, also obtained from feed escaping fermentation in the rumen to be utilized by the animal in the lower digestive tract in the same way as their monogastric counterparts. Rumen fermentation is concomitant with gaseous waste by-products (e.g. CO_2, CH_4, and H_2) (Fig. 3) and methanogens are responsible for the capture of H_2 and CO_2 in a reductive process resulting in the formation of CH_4. In this regard, methanogenesis is responsible for energy losses of between 2 and 12% of the total feed energy in ruminant livestock [34,35]. Methane mitigation strategies, including supplementation with *Asparagopsis* [9,11–13] redirect energy lost as CH_4 into beneficial metabolism resulting in improved feed utilisation and improved animal productivity [35].

At low feed inclusion levels, *Asparagopsis* (containing bromoform) and synthetic HMAs inhibit the production of methane in ruminants with a consequent reduction in the abundance of methanogenic archaea [37–45]. The latter is accompanied by a net increase in levels of eructed H_2 [9,13,37,41,44] and changes in volatile fatty acid profiles [9–11,13,37,39–42,45–47]. Depending on inclusion level and the test animal, variable changes in weight gain, dry matter intake, feed conversion efficiency, and milk production and quality (for dairy) have been measured (Table 1). Importantly, when *Asparagopsis* at the minimum effective inclusion level has been used as the methanogenesis inhibitor, elevated levels of bromoform in animal products have not been detected [9–13]. The following sections discuss the significant biochemical pathways affected by bromoform including methanogenesis, volatile fatty acid production, and reductive dehalogenation as the degradation pathway of bromoform.

2.1. Methanogenesis

The relative importance of methanogenic archaea in the rumen ecosystem as utilizers of metabolic hydrogen [H] produced during feed fermentation is unclear between studies [9,11,41,50–53]. However, recent studies suggest that a dramatic reduction in CH_4 production can have a beneficial effect on feed use efficiency [11,12] and ruminant animal performance [9,11]. Methanogenesis is the key mechanism for energy production in methanogens, driving metabolic and biosynthetic pathways and therefore the growth and abundance of methanogens [50]. Therefore, the inhibition of methanogenesis is expected to indirectly result in reduction of methanogen abundance [38]. In vitro trials with *Asparagopsis* [28,44] and synthetic HMA methanogenesis inhibitors [38,40,41] have also demonstrated the same correlation in ruminant animals. Inhibition of methanogenesis by halogenated alkanes (e.g. bromoform) occurs by blocking the action of key metalloenzymes [21,22,54–58] of the Wolfe cycle (Fig. 4) [59,60]. The Wolfe cycle details the stepwise reduction of CO_2 to CH_4 in the general reaction $CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$ (Table 2) by rumen hydrogenotrophic methanogenic archaea [6]. Steps *vi* and *vii* in the Wolfe cycle are catalysed by

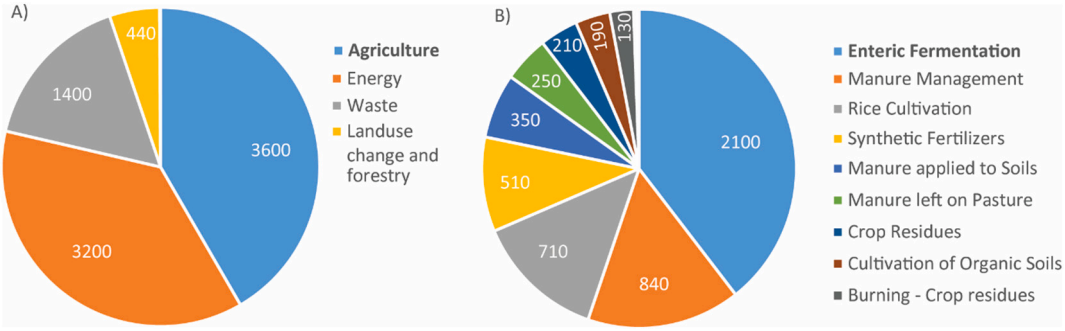

Fig. 1. Global annual anthropogenic methane (CH_4) emissions (million metric tonnes of carbon dioxide equivalents, $MtCO_2eq$) in 2016 by A) sector, and B) subsectors within the agricultural sector, data sourced from [2].

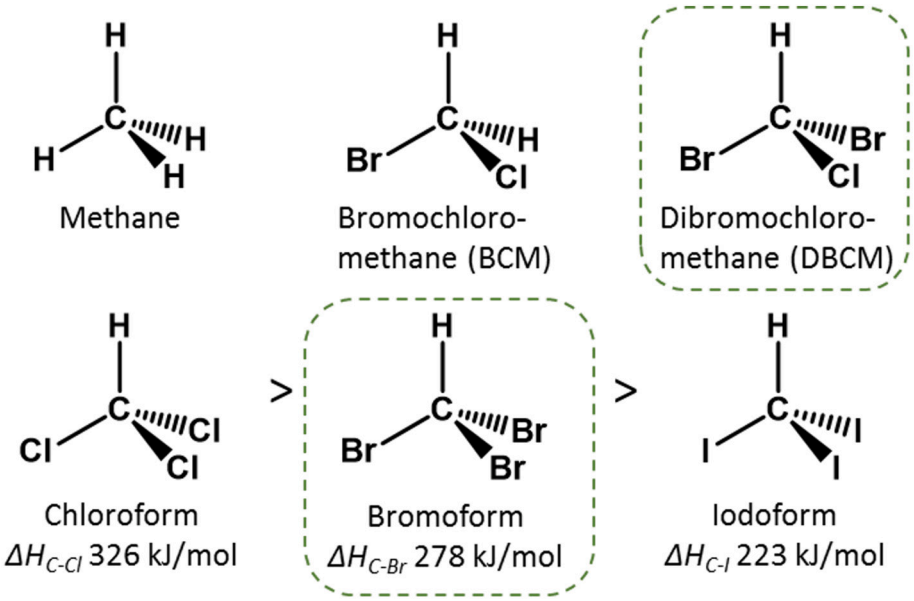

Fig. 2. The structure of methane and halogenated methane analogues (methane, bromochloromethane, dibromochloromethane, chloroform, bromoform, and iodoform) that block methanogenesis. Of these, bromoform and dibromochloromethane (dashed frames) are present in *Asparagopsis*. Carbon-halogen bond energies decrease from chloroform to iodoform with concomitant increase in reactivity [29].

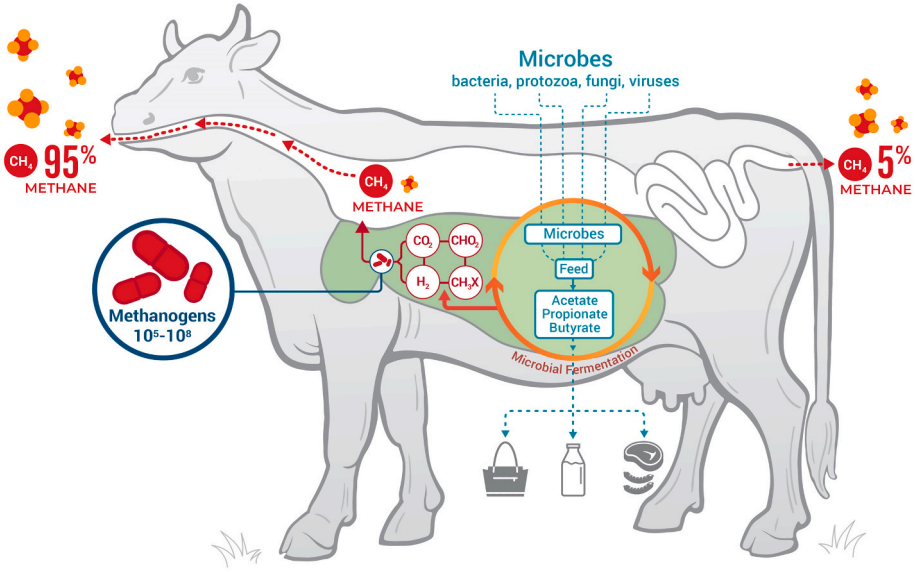

Fig. 3. Ruminant fermentation processes and products, and microbial contributors. *Notes:* Microbial numbers are listed per mL or gram of ruminal contents. Methane emissions from eruction (95%) far outweigh flatulence (5%). Abbreviations: CO_2 = carbon dioxide; H_2 = hydrogen, CHO_2^- = formate; CH_3X = methoxy compounds or methylamines; CH_4 = methane.
Adapted from [36].

coenzyme M methyltransferase (with a cobalamin prosthetic group) and methyl coenzyme M reductase (with nickel tetrapyrrole as a prosthetic group; syn. cofactor F_{430}), respectively, and are susceptible to competitive and/or oxidative inhibition [21,22,55,56,61–63]. Importantly, halogenated alkanes react competitively with the substrates of coenzyme M transferase and methyl coenzyme M reductase, inhibiting methyl transfer from methyl-H4MPT to CoM-SH (Fig. 4 *vi.* cobalamin) and the reductive release of methane from methyl-coenzyme M (Fig. 4 *vii.* Cofactor F_{430}), respectively [21,54,57,58,62].

The most widely cited mechanism for the inhibitory activity of HMAs in ruminants is competitive binding with coenzyme M methyltransferase (Fig. 4. step *vi.* Cobalamin) [21], thus inhibiting methyl transfer in methanogenesis. However, as discussed above halogenated alkanes also block the activity of methyl coenzyme M reductase (Fig. 4. step *vii.* Cofactor F_{430}), that catalyses the final and rate limiting step of methane production [57]. It is likely that HMAs inhibit both coenzyme M methyltransferase and methyl coenzyme M reductase in vivo, however the relative influence of these inhibition pathways on methane production has not been quantified.

2.2. Volatile fatty acid production and other products

A consequence of the inhibition of methanogenesis is the increase in levels of [H] and subsequently H_2 in the rumen. Metabolic hydrogen [H]

Table 1
Summary of relevant ruminant animal studies using synthetic halogenated methane analogues (HMAs) or *Asparagopsis* for methane reduction purposes, including inclusion level, measured results, and citation (organised by bioactive).

HMA	Animal	Treatment size (n)	Treatment duration (days)	Inclusion level* (mg·kg^{-1} LW day^{-1}	CH_4[a] (Δ%)	H_2 (Δ%)	Change in VFAs (Δ%)	Acetate: Propionate (Δ%)	Productivity (Δ%)	Ref.
BCM	Cattle[b]	4 × 4 LS	28	5.5[c]	–	–	↓	–	–	[42]
BCM	Cattle	4 × 4 LS	28	10.9	↓100[d]	–	n.s.	↓33[d]	↑31[e].	[48]
BCM	Cattle	6	28	0.66	↓29	–	n.s.	↓5	n.s.[e]	[37]
BCM	Cattle	5	28	0.66	↓93	–	–		n.s.[e]	[30]
BCM	Cattle	5	90	0.66	↓38	–	–	–	–	[30]
BCM	Cattle	12	85	0.66	–	–	–	–	n.s.[e]	[30]
BCM	Goats[f]	9	70	0.66	↓32	–	n.s.	↓31	↑36[g]	[47]
BCM	Cows	4 × 4 LS	14[h]	9.0	↓85	–	–	–	–	[49]
BCM	Goats	3	33[i]	4.0	↓91	↑ > 650	n.s.	↓33	–	[39]
$CHCl_3$	Sheep	3	98	3.3	↓89[j]	↑ > 250[k]	↓26	↓31	–	[46]
$CHCl_3$	Cows	3	42	3.71	↓38	–	–	↓30	n.s.	[45]
$CHCl_3$	Cattle[b,l]	4	30[m]	1.69	↓58	↑3.16g[n]	↓10	↓32	–	[41]
$CHCl_3$	Cattle[b,o]	4	30[m]	1.69	↓55	↑1.73g[n]	↓16	↓32	–	[41]
$CHCl_3$	Cattle[b]	8	21[p]	1.04	↓40	↑1.2g[n]	n.s.	↓35	n.s.	[40]
A. taxiformis	Sheep	5	72	0.07[q]	n.s.	–	↓6	↓34	–	[10]
A. taxiformis	Sheep	5	72	0.14[q]	↓52	–	↓19	↓45	–	[10]
A. taxiformis	Sheep	5	72	0.16[q]	↓62	–	↓25	↓42	–	[10]
A. taxiformis	Sheep	5	72	0.18[q]	↓81	–	↓29	↓45	–	[10]
A. taxiformis	Cattle	5	60	0.10[q]	↓9	n.s.	n.s.	↓14	n.s.	[9]
A. taxiformis	Cattle	5	60	0.25[q]	↓38	↑380[r]	n.s.	↓29	↑51	[9]
A. taxiformis	Cattle[b]	5	60	0.45[q]	↓98	↑1700[r]	n.s.	↓35	↑42[s]	[9]
A. taxiformis	Cattle	6	147	0.87[q]	↓50.6	↑318[r]	–	–	n.s.	[12]
A. taxiformis	Cattle	6	147	1.58[q]	↓74.9	↑497[r]	–	–	n.s.	[12]
A. armata	Cows	3 × 3 LS	21	0.44[q]	↓26.4	↑163[r]	–	–	n.s.	[11]
A. armata	Cows[f]	3 × 3 LS	21	0.62[q]	↓67.2	↑236[r]	–	–	↓11.6[g]	[11]
A. taxiformis	Cows[f]	4 × 4 LS	28	–[t]	n.s.	↑234[r]	n.s.	n.s.	n.s.	[13]
A. taxiformis	Cows[f]	4 × 4 LS	28	–[t]	↓34.4	↑627[r]	n.s.	↓11.7	↓6.5[g]	[13]

Abbreviations: dash (–) = not quantified; n.s. = not significant; LS = latin square experimental design; LW = live weight; BCM = Bromochloromethane; VFAs = Volatile Fatty Acids, $CHCl_3$ = chloroform.

[*] Calculations are based on average dry matter intakes (DMI) and animal weights.
[a] Percent reduction in eructed CH_4 over control animals.
[b] Meat.
[c] Units of g day^{-1}.
[d] 6 h post-dose.
[e] Live weight gain.
[f] Dairy.
[g] Milk yield (kg animal^{-1} day^{-1}).
[h] Plus 14-day recovery period.
[i] Test animals exposed to each of 3 doses (0.4, 1.6, and 4.0 mg kg^{-1} day^{-1}) in succession with each treatment lasting 11 days.
[j] Measurements from harvested rumen fluid.
[k] Based on the lowest measure value of H_2 in Fig. 1 of [46].
[l] Hay:concentrate diet.
[m] Test animals exposed to each of 3 doses (0.65, 1.04, and 1.69 mg kg^{-1} day^{-1}) in succession with each treatment lasting 11 days.
[n] g kg^{-1} DMI.
[o] Hay diet.
[p] Test animals exposed to each of 3 inclusion levels (0.4, 1.6, and 4.0 mg kg^{-1} day^{-1}) in succession with each treatment lasting 11 days.
[q] Bromoform, except in [10] which is total HMAs.
[r] Expressed as % of control.
[s] Average daily weight gain (ADWG, kg day^{-1}).
[t] *A. taxiformis* added at 0.25 and 0.5% dry weight (dw), respectively, bromoform was not quantified in fed *Asparagopsis*.

is released during the fermentation of feed by ruminant microbes, contributing to the production of VFAs, microbial biomass, and reduced electron acceptors, as well as H_2 in reactions catalysed by hydrogenases (Table 2). It has been reported that high partial pressure of H_2 inhibits microbial dehydrogenases leading to a reduction in fermentation, dry matter intake, and feed digestibility [50,64]. The primary VFAs utilized in ruminant production are acetate, propionate, and butyrate, which represent >95% of total VFAs [50,52]. The concentration of H_2 in the rumen influences fermentation pathways in different ways (described below), and consequently influences ruminant nutrition [50]. The free energy change of fermentation pathways influences the relative production of VFAs, with higher levels of H_2 in the rumen favouring substrate transformations that are accompanied by the consumption of H_2, and lower levels favouring pathways accompanied by the production of H_2 (Table 2) [50,53]. Thus, higher concentrations of H_2 in the rumen favours the production of propionate over acetate (and to a lesser extent butyrate) with a consequent decrease in the acetate:propionate ratio. This relationship has been demonstrated in studies quantifying the effects of HMA methanogenesis inhibitors on methane production in both in vitro fermentation studies [16,20,40,44,65] and in in vivo studies with goats [38,39], sheep [10,46], and cattle [9,13,37,40,41,47,48]. However, other [H] sinks also play a role in utilising the excess [H] in the methanogenesis inhibited rumen and in animal nutrition [53,64], discussed below.

The production of one CH_4 molecule from CO_2 requires 8[H] (Table 2) and measured changes in VFAs (in vitro and in vivo) do not account for the differences between the theoretical increase in [H] and the measured partial pressure of H_2 in the methanogenesis inhibited

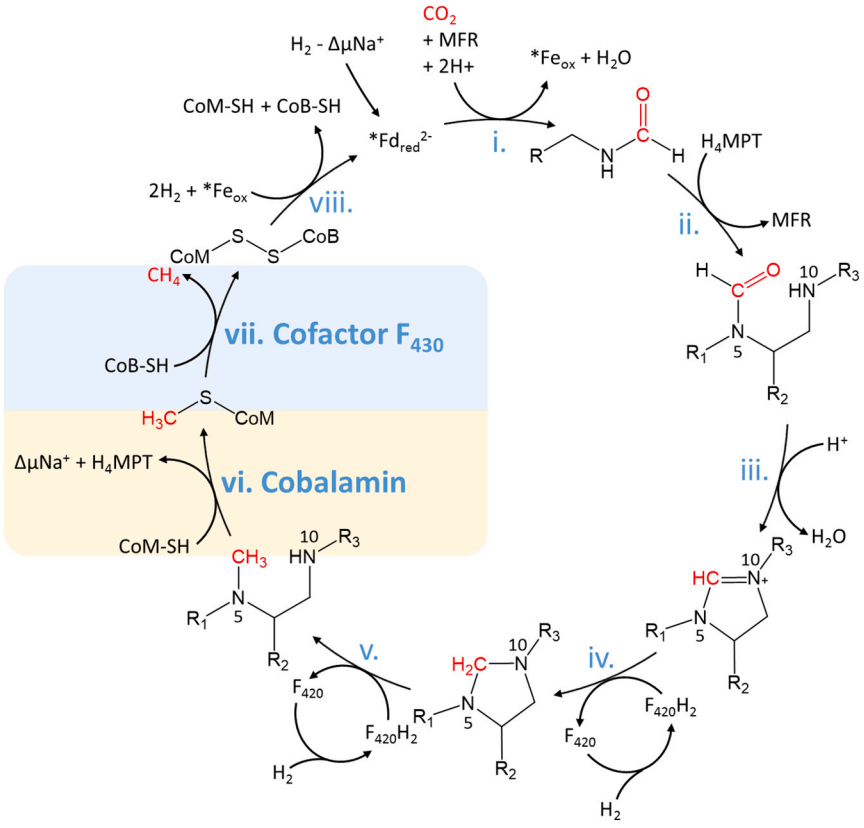

Fig. 4. The Wolfe cycle for the reduction of CO_2 to CH_4 in hydrogenotrophic methanogenic archaea. Steps: *i.* CO_2 reacts with methanofuran (MFR) to produce formyl-MFR; *ii.* The formyl group is transferred to tetrahydromethanopterin (H_4MPT); *iii.-v.* Intramolecular imine formation and successive reductions; *vi.* Methyl transfer from methyl-H_4MPT to CoM-SH catalysed by coenzyme M (CoM) methyltransferase (cobalamin); *vii.* Methyl group reduced to methane catalysed by methyl-CoM reductase (cofactor F_{430}); *viii.* Ferredoxin mediated regeneration of CoM. Adapted from [60].

Table 2
Stoichiometry of glucose ($C_6H_{12}O_6$) fermentation reactions of the rumen and effects on methane production.

VFA	Reaction equation	CH_4/glucose (mol/mol)
Acetate	$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 4[2H] + 2CO_2$	+1.0
Propionate	$C_6H_{12}O_6 + 2[2H] \rightarrow 2CH_3CH_2COOH + 2H_2O$	−0.5
Butyrate	$C_6H_{12}O_6 \rightarrow CH_3(CH_2)_2COOH + 2[2H] + 2CO_2$	+0.5
Valerate	$C_6H_{12}O_6 + [2H] \rightarrow CH_3(CH_2)_3COOH + CO_2 + 2H_2O$	−0.25
Methane	$CO_2 + 4[2H] \rightarrow CH_4 + 2H_2O$	NA

rumen [9,11,12,40,41,53,66]. In this regard, a recent meta-analysis of in vitro results reports the mean [H] incorporated into H_2 for both batch and continuous fermentations with 100% methanogenesis inhibition as only 10% and 6%, respectively [53]. This has been verified in in vivo studies in lactating dairy cows [11,66] and beef cattle [9,12,40,41]. For example, Kinley et al. reported that at a 98% inhibition of methanogenesis with *A. taxiformis* there was a 17-fold increase in H_2 production [9]. However, based on the reduction of methane emissions (↓10.8 g $kg^{-}1$ DMI), and the increase in H_2 (↑1.7 g kg^{-1} DMI), 69% of the [H] that would otherwise be directed into CH_4 production is redirected elsewhere and is not lost as emissions of H_2. This is in agreement with a study of lactating dairy cows supplemented with *A. armata* where only 5.6% of [H] went into the production of H_2 when methanogensis was inhibited by 67.2% [11]. Furthermore, in a long-term study (147 days) where *A. taxiformis* was included in high forage, medium forage, and low forage diets, typical of the life stage specific total mixed rations for growing beef steers, 90.3%, 89.3%, and 88.6% of the theoretical [H] produced from methanogenesis inhibition was redirected to other [H] sinks [12]. In this regard, a number of alternative nutritionally beneficial [H] sinks have been identified including reductive acetogenesis, formate formation, and increased microbial biomass production [53,64]. Indeed, the production of H_2 in the methanogenesis inhibited rumen can be managed using diet [41] and/or supplementation [40], and animal productivity gains can be achieved.

To convert the feed energy saved by the inhibition of methanogenesis into increased animal productivity, [H] needs to be redirected into nutritionally beneficial [H] sinks. However, inhibiting methanogenesis does not lead to consistent improvements in animal production metrics [67]. For example, dairy cows fed *A. armata* (1.32 mg bromoform g^{-1} biomass) included in feed at 0.5% OM inclusion had a similar weight change (Δwt = 32.7 kg) and milk production (milk = 37.2 kg per animal) compared to control fed cows (Δwt = 31.0 kg; milk = 36.2 kg per animal), while *A. armata* fed at 1.0% OM inclusion resulted in significant reductions in both weight change (Δwt = 21.3 kg) and milk production (milk = 32.0 kg per animal) [11]. In this study, while there was an 11.6% drop in milk production per animal there was also a 74% increase in feed conversion efficiency (i.e. kg milk kg^{-1} DMI). This is a critical point, and depending on the cost of feed, stock, and infrastructure, economics and animal health will dictate business decisions. In another study, Brahman-Angus cross steers receiving *A. armata* (6.55 mg bromoform g^{-1} biomass) supplemented feeds at 0.10% and 0.20% OM inclusion had a reduction in eructed methane by 40% and 98%, and weight gain improvements over control animals (Δwt = 53 kg) of 53% (Δwt = 81 kg) and 42% (Δwt = 75 kg), respectively [9]. Furthermore, there was no significant change in DMI, indicative of a higher feed conversion efficiency. Higher feed conversion efficiencies were also recorded in a study where Angus-Hereford beef steers received *A. taxiformis* (7.8 mg bromoform g^{-1} biomass) supplemented feeds at 0.25% and 0.50% OM inclusion leading to reductions in eructed methane by 50.6% and 74.9%, respectively [12]. However, in this study no change in weight gain was detected. The above examples are indicative of the variability associated with differing ruminant feeding systems, feed formulations, animal species and condition (health and stress), and *Asparagopsis* quality (bromoform concentration). There is

not yet enough published work available to clearly interpret the relationship of these factors, however, it is apparent that the relationship between bromoform concentration of the as-fed seaweed and enteric CH_4 mitigation is not linear in vivo.

The development of new feed formulations and supplements that promote the redirection of [H] to beneficial sinks in the methanogenesis inhibited rumen is an important research focus. In this regard, two key studies have demonstrated the effect of feed formulation [41] and supplementation [40] on the production of H_2 in the methanogenesis inhibited rumen. In terms of feed formulation, Brahman steers fed a roughage hay diet (Rhode grass hay) supplement with a high dose of a HMA formulation produced 1.72 g of H_2 kg^{-1} DMI; while steers fed a mixed roughage hay:concentrate diet (60:40) produced 3.16 g of H_2 kg^{-1} DMI. In this study, animals fed roughage diet redirected a greater proportion (67%) of [H] to alternative sinks than those animals fed a mixed diet (55%). In a second study, Brahman steers with inhibited methanogenesis receiving the same mixed hay concentrated diet were supplemented with a [H] sink (phloroglucinol) and this significantly reduced the eruction of H_2 by 51% over control animals [40]. Furthermore, this redirection in [H] translated to significant improvements in average daily weight gain, noting a caution due to small sample size ($n = 4$ per treatment). Importantly, [H] can be productively redirected through feed formulation and supplementation, although genetics also plays a role [68]. To better understand the effect of the inhibition of methanogenesis with *Asparagopsis* on hydrogen metabolism in different ruminant production systems, further long-term in vivo studies are required to investigate the beneficial effects of diet and supplementation.

To date there are three major findings: 1. Methanogenesis inhibition can be achieved in conjunction with increased feed energy conservation; 2. Matching of inclusion level based on seaweed quality with diet formulation is critical to ensure the effective inhibition of methanogenesis; 3. Further long-term animal feed trials are required to determine the effects of inclusion levels and diet formulations (supplementation) on different animal production systems.

2.3. Reductive dehalogenation as the degradation pathway of bromoform

Animal studies investigating the use of *Asparagopsis* for the inhibition of methanogenesis have not detected elevated bromoform levels in animal tissues or products above background levels when administered over 21 days [11], 28 days [13], 60 days [9], 72 days [10], or 147 days [12] (see also Section 3), showing it is either metabolised or excreted. Notably, methanogens are effective metabolisers of HMAs in nature and have the capacity to dehalogenate HMAs [69–71]. Studies with methanogens have demonstrated that coenzyme M methyltransferase [58] and methyl-coenzyme M reductase [57] reductively dehalogenate a range of HMAs (including bromoform) [21] to methane and other less halogenated intermediates, with methyl-coenzyme M reductase 50 times more active than coenzyme M methyltransferase [57]. The efficiency of HMA dehalogenation also increases according to expected carbon-halogen bond dissociation energies which decrease in the order $F > Cl > Br > I$. Therefore, bromoform would be more efficiently dehalogenated than chloroform. Furthermore, while methanogens make up only 0.3–3.3% of the microbial consortia in the rumen [72], their contents of coenzyme M methyltransferase (100–1400 nmol g^{-1} dry weight [dw] archaea) is 5–6 orders of magnitude higher than other bacteria including *Escherichia coli* (<0.01 nmol g^{-1} dw bacteria) [73], and with concomitant high levels of methyl-coenzyme M reductase (227–800 nmol g^{-1} dw archaea) [74], methanogens demonstrate considerable capacity for reductive dehalogenation. Importantly, dehalogenation of HMAs by methanogens combined with the design of ruminant digestive tract (i.e. first stop: rumen) may limit transfer of HMAs, including bromoform from *Asparagopsis*, into ruminant derived food products. This is evidenced by no detection of elevated bromoform in meat, edible offal, fat, milk, or faecal matter of ruminants receiving *Asparagopsis* included in their feed when administered at minimum effective inclusion levels [9–13]. This collectively increases the utility of *Asparagopsis* as a tool for the mitigation of ruminant methane production but further long-term studies are required to verify the fate of bromoform and its degradation products in different ruminant production systems.

From a management perspective, dehalogenation of bromoform has several implications. To achieve a balance between methane mitigation and animal and human health, the population of methanogens in the rumen may need to be reduced, not eradicated. To achieve ongoing methane mitigation continuous inclusion of *Asparagopsis* is required, although a specific minimum feeding interval is yet to be determined. This has two further important implications for the use of *Asparagopsis* as a methane mitigation strategy in ruminants. Firstly, due to the volatility of the bioactive, innovative approaches and technologies to supplement grass fed stock, and managing logistics around the processing, transport and supply chain, are required. This includes stabilisation of the bromoform (e.g. [75]) and standardised methods for analysis and provisions of certificates of analysis of *Asparagopsis* biomass or formulated feeds [76]. Secondly, ongoing inclusion supports an industry focused on seaweed biomass production. The large-scale cultivation of *Asparagopsis* is required for this approach to have a meaningful effect on the mitigation of ruminant methane production, with common considerations for responsible farming considered in [77].

2.4. Summary of biochemical pathways and their implications

- *Asparagopsis* (bromoform) inhibits methanogenesis by up to 98% leading to increased levels of rumen H_2 and concurrent changes in volatile fatty acids profile.
- Methanogens in the rumen metabolise bromoform through dehalogenation, which in conjunction with adopting minimum effective inclusion levels of *Asparagopsis* limits transfer of bromoform into animal tissues and food products.
- Determination of minimum effective inclusion levels of *Asparagopsis* in different ruminant production systems with specific feed formulations is critical to maintain animal health, food quality, and improved animal productivity. To achieve this, further dose-response animal studies specific to diet formulation (including potential for supplementation with alternative [H] sinks) are required.
- Accurate inclusion levels will therefore require that each batch of *Asparagopsis* biomass or formulation be accompanied by a certificate of analysis for content of bromoform. Ultimately, a product of consistent bromoform content would facilitate greater confidence and wider acceptance by producers and consumers.

3. Toxicological risk assessment for ruminants of bromoform present in fed *Asparagopsis*

3.1. Absorption, distribution, metabolism, and excretion (ADME)

To provide context, the key principles of toxicology relevant to this risk assessment are summarised. The first principle of toxicology is that "all things are poisonous, and it is the dose that distinguishes between a drug (or something else of value) and a poison" (Paracelsus $C15^{th}$). The second principle is that "exposure of experimental animals to toxic agents in high doses is a valid method of discovering possible hazards in humans or other animals" [78]. A third critical component is an understanding of absorption, distribution, metabolism, and excretion (ADME) (Fig. 5) of compounds of interest, both from a toxicological perspective [79] and to ascertain and address residue concerns [80].

While acknowledging that toxicological risks need to be carefully considered if seaweed is to be used for ruminant methane reduction, it is also important to acknowledge that the vehicle for the active ingredient (i.e. biomass) will affect ADME and bioavailability of the active ingredients. Bioavailability in this context is the proportion of a drug or

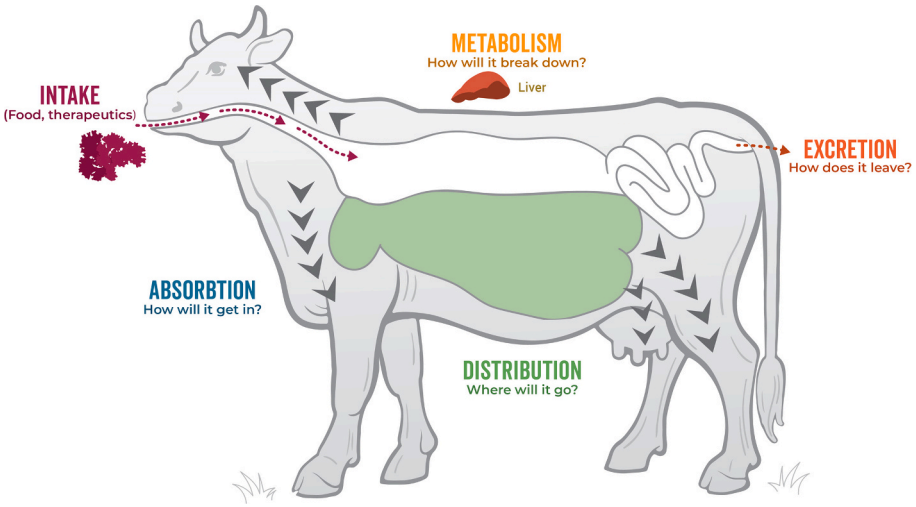

Fig. 5. The pharmacokinetic principle of absorption, distribution, metabolism, and excretion (ADME) for compounds of interest. Oral delivery of *Asparagopsis* in feed is primarily absorbed and metabolised by rumen microbiota, and waste products from this process are absorbed or excreted via the animal's digestive system.

other substance that enters the circulation when introduced into the body and will be dose dependent. The ADME of bromoform as delivered in seaweed is important in the context of use of seaweed from the genus *Asparagopsis* for the reduction of ruminant methane production. For xenobiotics or natural compounds to produce systemic pharmacological or toxicological effects, i.e. affecting the whole body or an area distant from its entry point rather than a specific (local) area, absorption into the blood from the gut and distribution to target organs is a prerequisite. As previously described, when *Asparagopsis* is included at low levels in feed formulations, a corresponding low concentration of bromoform is released into the rumen where it efficiently inhibits methanogenesis and undergoes dehalogenation in the process. This scenario differs from animal toxicology studies where maximising bioavailability is achieved by the administration of large doses of the active ingredient in solution to rats and other test species by oral gavage (stomach tubes), rather than ingested at low concentrations in feed. Regardless, these animal toxicology studies put the risk of toxicity from bromoform into perspective when different ruminant production systems are fed feed formulation with minimum effective inclusion levels of *Asparagopsis*.

Studies in ruminants fed *Asparagopsis* demonstrate no quantifiable uptake or accumulation of bromoform in milk, tissue, edible offal, fat, or faecal matter when administered for the purpose of quantifying methane mitigation and minimum effective inclusion levels (Table 1) [9–13], supporting the conclusion that bromoform in *Asparagopsis* has negligible bioavailability to the animal in treatments that significantly reduce the emission of methane. The lack of bioavailability in ruminants at the minimum effective inclusion level is consistent with the seaweed exerting its effect in the rumen and its microbiota, not systemically. Furthermore, in laboratory animal studies where large bolus doses of bromoform of 100 to 150 mg kg^{-1} were administered by oral gavage to rats and mice, the absorbed bromoform was quickly excreted with a half-life of 0.8 h in rats and 8 h in mice [81]. Hence, in scenarios where there was absorption of small amounts of bromoform in ruminants, excretion is likely to be very rapid, though this requires confirmation. Recently, an assessment of the toxicology of *Asparagopsis taxiformis* in dairy cows was made with concurrent monitoring of the transfer of the seaweed derived bromoform to milk, urine, faeces, and animal tissue [82]. The study administered *Asparagopsis* (1.26 mg bromoform g^{-1} dw biomass, 51% salts as ash) as a seaweed-mix containing *Asparagopsis* at 5.9% (Low, n = 8), 10.5% (Medium, n = 2), or 20.0% (High, n = 2) of the seaweed-mix dry matter (as fed). This seaweed-mix was fed twice daily as a bolus 1–2 h prior to offering the basal diet, and milking occurred 1–3 h following the morning feeding. Muezilaar et al. [82] reported poor (Low and Medium treatments) to no (High treatment) acceptance of the seaweed-mix and low overall feed intake indicating difficulties with the experimental design that subsequently led to the study's early termination. It is worth noting that inclusion levels of the seaweed-rich bolus (5.9–20% dw) in the Muezilaar study were in the upper range or exceeded inclusion levels in studies that focused on determining the minimum effective inclusion level in cattle and sheep (0.2–3% of OM [9–11,83]), and that at high levels of inclusion animals self-regulate intake. While the study was terminated early due to poor feed intake and subsequent animal health issues [82] there were results indicative that high inclusion levels of *Asparagopsis* biomass as a bolus can result in elevated bromoform levels in animal products and may impact on animal health. For example, on day 1, bromoform was detected in some milk samples in the Low (mean 9.1 μg L^{-1}, n = 5) and Medium (11 μg L^{-1}, n = 2) treatments, and on day 9 bromoform was detected in milk from one animal in the high treatment (35 μg L^{-1}). Notably, in the latter example this animal only consumed 16–20% of a healthy DMI (3.1–3.6 kg vs. 15–19 kg DMI day^{-1} prior to commencing treatment) in the days preceding sample collection. Thus, when dairy cows are fed high levels of *Asparagopsis* under feed deprivation it is possible to detect elevated levels of bromoform in milk, but at levels well below the World Health Organisation standard for bromoform in drinking water of 100 μg L^{-1} [84]. Additionally, on completion of the study, two low treatment animals showed localised abnormalities of the rumen mucosa with invasion of inflammatory cells [82]. While the authors could not conclude that the abnormalities were due to the fed *Asparagopsis*, an earlier study where sheep were fed *Asparagopsis* (0.5–3% OM) also detected similar rumen abnormalities in five out of ten sheep examined that were fed *Asparagopsis* [10]. Given the chronicity of the lesions, the cause could not be determined. All sheep, including controls (no *Asparagopsis*) showed changes in rumen mucosa consistent with mild acidosis [10]. We suggest that where feasible, the quantification of residues and excretion of bromoform, and histology of organs in treated animals, are included in future long-term feed studies where *Asparagopsis* is implemented as a feed ingredient for methane mitigation at minimum effective inclusion levels and delivery scenarios.

3.2. Toxicology

Toxicological assessments of bromoform have been performed on rodents, using doses many orders of magnitude higher than seaweed inclusion levels required to inhibit methane production in ruminants. For example, Condi et al. (1983) investigated the toxic effects of the HMAs bromodichloromethane, bromoform, chloroform, dibromochloromethane, and methylene chloride in mice [85]. In this study, mice

were administered bromoform by oral gavage over the dose range of 72–289 mg kg^{-1} day^{-1} and ranked bromoform as one of the least toxic HMAs tested [85]. There was evidence of renal and hepatotoxicity at the highest dose (289 mg kg^{-1} day^{-1}). This amount represents 183–2890 times higher amounts than the inclusion levels applied to achieve *Asparagopsis*-based methane inhibition (0.10–1.58 mg kg^{-1} day^{-1}; Table 1) in cattle [9,11,12] and sheep [10], and is therefore not comparable to dietary administration of seaweed for the purposes of methane inhibition. Similarly, Chu et al. (1982) reported no changes in rats at the highest dose of bromoform in drinking water (500 mg kg^{-1} day^{-1}) [86], which is 316–5000 times higher per kg per day than feedlot cattle might receive. The LD_{50} (the amount of an ingested substance that kills 50% of a test sample) for bromoform for female and male rats is 1147 and 1388 mg kg^{-1}, respectively [87]. Conservatively, livestock fed 0.50 mg kg^{-1} day^{-1} for a three-year production lifespan would consume 548 mg kg^{-1} in total, suggesting that cattle would not consume enough bromoform in a lifetime to reach half the LD_{50} for rats. Furthermore, in contrast to rats, as previously described bromoform from consumed seaweed is assimilated and metabolised by methanogenic bacteria in the rumen in the process that inhibits methanogenesis. Based on the short half-life of bromoform [81] it is expected that if small amounts were absorbed systemically it would not persist in tissues but be rapidly metabolised and excreted. This hypothesis is consistent with the lack of elevated bromoform in milk, meat, edible offal, fat, or faeces from cattle and sheep fed *Asparagopsis* at or near the minimum effective inclusion level as compared to their counterparts that did not receive *Asparagopsis*.

3.3. Summary of toxicological risk assessment

- The following factors contribute to the low or negligible bioavailability of bromoform in cattle in *Asparagopsis* treatments that significantly reduce the emission of methane:
  - o Vehicle, i.e. the complex structure of whole seaweed versus oral gavage or bolus dosing of pure bromoform.
  - o Low inclusion levels of *Asparagopsis* in stock feed compared with toxicology studies on rodents using large bolus doses.
  - o The robust biology (physiology and microbiology) of the rumen gastrointestinal tract and its ability to consume and degrade HMAs.
- After feeding, bromoform was not detectable above background levels in products (e.g. milk, meat, and organs) or waste products (faeces) when fed as part of the basal feed offering at or near minimum inclusion levels.
- The lack of uptake of bromoform is consistent with its decomposition by the methanogens where its binding competitively interferes with methanogenesis.
- Systemic toxicology associated with bromoform in animals requires absorption of bromoform into the blood stream and the exposure of target organs to significant concentrations of bromoform. However, supplementation of *Asparagopsis* at minimum effective doses results in minimal or no absorption of bromoform in animal products or tissues. Thus, the systemic toxic effects detected in small animals used in the toxicology assessment of bromoform are not detected.

4. Effect on atmospheric chemistry

4.1. Atmospheric fate of bromoform

While the cultivation and development of feed formulations with *Asparagopsis* will target the retention and metabolism of the bioactive bromoform, the production process will have a minimal effect on atmospheric levels of ozone depleting inorganic bromine (Br_y). Bromoform is naturally released to the atmosphere and although there is a large amount of uncertainty in the estimates, these natural contributions to atmospheric bromoform are mainly of oceanic origin with the turnover of seaweed biomass (all species) estimated to produce 70% of the total global flux of bromoform [88]. During transport of volatilised bromoform through the troposphere and stratosphere, bromoform reacts with hydroxyl or chlorine radicals, or undergoes photolysis resulting in the production of water-soluble reactive product gases and inorganic bromine (Br_y) (Fig. 6) [89–91]. These reactive products can contribute to catalytic decomposition of tropospheric and stratospheric ozone, or be removed from the troposphere via wet deposition, limiting transport into the stratosphere [91,92]. However, transport and removal processes are temporally and spatially highly variable [92]. Additionally, bromoform is classified as a very short-lived substance (VSLS) with a lifetime of 24 days and therefore has a relatively low ozone depletion potential overall [93,94].

4.2. Global emissions

To place the contribution of atmospheric bromoform originating from the potential large scale aquaculture of *Asparagopsis* into context; brominated marine VSLSs contribute 10% - 40% of the total atmospheric bromine [95], with brominated VSLS contributions from anthropogenic (e.g. bromomethane and halons) or other natural (e.g. methylene bromide) sources [94] making up the remainder. The total production of bromine from brominated VSLSs is estimated at 204–980 Gg Br yr^{-1}, with bromoform contributing 120–820 Gg Br yr^{-1} [93]. In the open ocean biological production of bromoform is linked to phytoplankton (microalgae), while coastal production is linked mainly to seaweed (macroalgae) [96]. Bromoform is released slowly during the natural life cycle of algae and during their senescence and decay. Estimates of total annual global seaweed biomass production ranges between 244 and 1072 million tonnes dry weight [97], and is estimated to contribute 70% (84–574 Gg Br yr^{-1}) of the world's total flux of bromoform [88]. Total cultivated seaweed biomass was 31.2 million tonnes fresh weight (i.e. ~3.12 million tonnes dry weight) in 2016 [98] and contributes 0.3–1.2% of the total global seaweed standing stock and thus 0.2–0.8% of the total global bromoform flux. These data have several important implications for the cultivation and application of *Asparagopsis* for ruminant methane mitigation. Primarily, the total production of *Asparagopsis* (i.e. bromoform) will be very small (estimated in Section 4.3) compared with both the total production of all cultivated seaweeds, and the total global standing stock of seaweed (natural + cultivated). Secondly, the influence over atmospheric bromine will be minimised through aquaculture and processing aimed at the retention of the

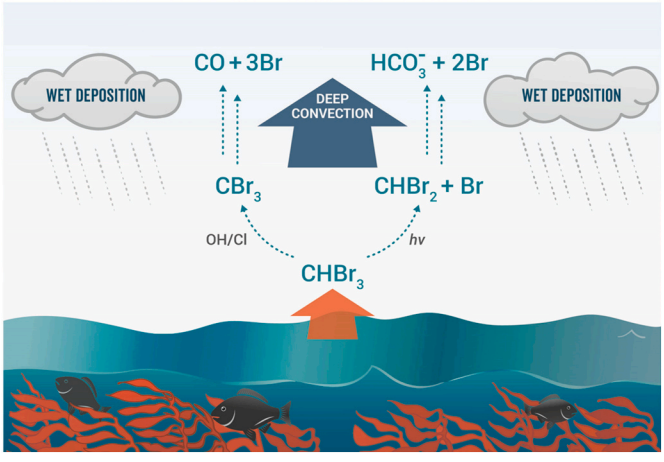

Fig. 6. Primary and secondary processes in the photo-dissociation of bromoform, yielding reactive bromine species that are either washed-out via wet deposition, or participate in ozone depletion. In a simplified model atmospheric bromoform ($CHBr_3$) reacts with OH/Cl radicals producing a tribromomethyl radical (CBr_3), or undergoes photolysis generating a dibromomethine radical ($CHBr_2$) and a bromine radical (Br). CBr_3 and $CHBr_2$ are further degraded to carbon monoxide (CO) and Br, and bicarbonate (HCO_3^-) and Br, respectively.

bioactive bromoform [13,99,100]. Furthermore, the metabolism of bromoform in the target animals will limit atmospheric emissions of bromoform (Section 2). Consequently, measured effects will be contextual (e.g. aquaculture production site and methods, processing, storage and transport chain, target animal, and feed supplementation regime) and require a case-by-case assessment in the first instance to provide baseline data for modelling.

4.3. Biomass requirements

Recent data from the use of high quality *Asparagopsis* biomass to reduce methane production in vivo can be used to assess the cultivation demands and the potential atmospheric impact of *Asparagopsis* used in this way by considering market penetration, the target herd (e.g. dairy cows, feedlot beef cattle, sheep, or goats), feed inclusion of *Asparagopsis*, and dry matter intake. For example, a market penetration of 20% targeting 1.26 million dairy cattle in New Zealand (total NZ dairy herd = 6.3 million in 2018 [101]), *Asparagopsis* (i.e. 6.55 mg g^{-1} dw bromoform) supplemented feed at 0.20% OM inclusion (i.e. ~0.4% dw seaweed inclusion) with a daily and yearly dry matter intake of 13.5 kg day^{-1} and 4.93 t per animal (average LW = 450 kg [102]), respectively (sum 6.2 million tonnes dry matter intake) [103], equals a total of ~25,000 t dry weight *Asparagopsis* biomass (~0.8% of the annual total amount of cultivated seaweeds) with a total of ~164 t of bromoform (0.156 Gg Br yr^{-1}). The latter represents ~0.016–0.076% of the estimated total global bromine flux from brominated marine VSLSs (see Section 4.2). Given this inclusion rate results in a 98% reduction of methane emission in cattle compared to control animals (11 g CH_4 kg^{-1} DMI), the potential reduction in methane emissions is estimated at ~68,000 t per year. A second scenario is based on an average animal in the Australian feedlot beef cattle industry (i.e. 520 kg live weight) dry matter intake of 15.6 kg day^{-1} with a finishing period of 105 days [104]. Here, less *Asparagopsis* biomass (20,573 t dw; ~0.7% of the annual total cultivated seaweeds) is required to supplement feed (0.2% OM inclusion) for the annual throughput of cattle in Australian feedlots (3.14 million head in 2018–2019, www.mla.com.au). Projected methane reduction in this scenario is 56,577 t per year. This modelling allows management programs to be developed that optimise animal and human health parameters, limit potential atmospheric emissions of bromoform, and enhance feed conversion efficiencies, that incidentally are likely to further reduce the greenhouse gas footprint of the ruminant industry. However, scaled up production of *Asparagopsis* and large-scale animal feed trials are required to better understand and optimise the technology, as also discussed in [77].

4.4. Summary of the effects on atmospheric chemistry

- While evidence suggests bromoform is decomposed on ingestion in target animals, further work is required to quantify volatilised bromoform from end-to-end of the process (i.e. volatilisation during cultivation and processing, through to excretion).
- A small quantity of high quality *Asparagopsis* has a large influence on ruminant methane production.
- Bromoform from cultivated *Asparagopsis* biomass will have a very low effect on the total natural and anthropogenic atmospheric loading of bromine.
- Scaled up aquaculture cultivation of *Asparagopsis* and further large-scale animal feed trials are required to better understand and optimise the technology for potential commercial implementation and roll-out.

5. Concluding remarks

Mitigation of methane production in ruminants is critical for the sustainability of red meat, dairy, and wool industries. Greenhouse gas emission management strategies including methanogenesis inhibitors provide an effective measure, with *Asparagopsis* (bromoform) the most promising performer to date. Importantly, evidence to date demonstrates that animal health and product quality is not compromised at the minimum effective feed inclusion levels of *Asparagopsis* targeting inhibition of ruminant methanogenesis. At or near the minimum effective feed inclusion levels of *Asparagopsis*, the ruminant digestive system combined with the decomposition of bromoform by ruminal methanogenic archaea to degrade bromoform reduces risk to animal health and product quality. However, methane production utilises excess [H] generated during rumen fermentation and subsequent H_2 production, and, similar to methane emissions but to a lesser degree, energy lost as H_2 emissions represent a feed utilisation inefficiency, and excessive H_2 has potential to reduce the efficiency of rumen fermentation. Alternative [H] sinks have been identified but more research is required to verify and optimise feed formulations to increase productivity across different production systems. Our modelling analyses in this review demonstrate that the risk of *Asparagopsis* contributing to ozone depletion is very small relative to the collective total of all natural and anthropogenic sources of bromine. However, research to develop effective production and management protocols, and strategies for the aquaculture and processing of *Asparagopsis* targeting maximum bioactivity (e.g. by minimising bromoform losses) is needed, as part of a portfolio of integrated management strategies for enteric methane emissions.

In conclusion, this synthesis of the literature suggests that large-scale aquaculture of *Asparagopsis,* and its application in methane mitigation strategies for ruminants at or near minimum effective inclusion levels, may not negatively impact animal health, food quality, and ozone depletion.

Statement of Informed Consent

No conflicts, informed consent, or human or animal rights are applicable to this study.

Authorship contribution statement

Glasson: Conceptualization, Methodology, Investigation, Visualization, Formal analysis, Writing - Original Draft, Writing - Review & Editing.

Magnusson: Conceptualization, Methodology, Visualization; Investigation, Writing - Original Draft, Writing - Review & Editing, Funding.

Kinley: Conceptualization, Investigation, Writing - Original Draft, Writing - Review & Editing.

de Nys: Conceptualization, Writing - Review & Editing.

King: Conceptualization, Writing - Review & Editing.

Adams: Conceptualization, Writing - Original Draft, Writing - Review & Editing, Funding acquisition.

Romanazzi: Conceptualization, Writing - Review & Editing.

Packer: Conceptualization, Writing - Original Draft, Writing - Review & Editing, Visualization, Funding acquisition.

Svenson: Conceptualization, Writing - Review & Editing, Visualization.

Eason: Conceptualization, Writing - Original Draft, Writing - Review & Editing, Funding acquisition.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Magnusson, de Nys, and Kinley 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* spp. for retention of bromoform (AU2018904642).

Magnusson and de Nys are on the advisory board of SeaForest,

however, Seaforest did not fund this research, and had no involvement in the conduct of the research; preparation of the article; study design; the collection, analysis and interpretation of data; in the writing of the report; or in the decision to submit the article for publication.

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