INVESTIGATING THE METHANE MITIGATION POTENTIAL OF LOCALLY ABUNDANT SEAWEEDS IN CATTLE FEED SUPPLEMENTS THROUGH CHEMICAL ANALYSES

by

NATALIE ATHERTON

B.Sc. Royal Roads University, 2021

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE ENVIRONMENTAL SCIENCE

in the Department of Physical Sciences

Thesis examining committee:

Dipesh Prema (PhD), Supervisor and Associate Professor, Physical Sciences, Thompson Rivers University

John Church (PhD), Co-Supervisor and Professor, Natural Resource Science, Thompson Rivers University

Kingsley Donkor (PhD), Committee Member and Professor, Physical Sciences, Thompson Rivers University

Stefanie Zaklan Duff (PhD), External Examiner and Professor, Fisheries and Aquaculture, Vancouver Island University

July 2025

Thompson Rivers University

ABSTRACT

Methane emissions from enteric fermentation in cattle represent a significant portion of Canada's agricultural greenhouse gas output. As part of targeted mitigation strategies, seaweed-based feed additives have attracted growing interest due to their potential to alter rumen fermentation and reduce methane production. However, most research has focused on tropical red seaweeds like *Asparagopsis taxiformis*, which are not ecologically available or sustainable in the northern Pacific of western Canada. This thesis investigates three seaweed species native or abundant in British Columbia: *Macrocystis pyrifera* (giant kelp), *Saccharina latissima* (sugar kelp), and *Mazzaella japonica* (*M. japonica*), to evaluate their potential as methane supressing additives in cattle feed through chemical profiling of omega-6s and omega-3s, phlorotannins, and heavy metal content.

Fatty acid analysis using low-solvent and rapid proton nuclear magnetic resonance (^{1}H NMR) spectroscopy revealed that both sugar kelp and giant kelp contain favourable omega-6 to omega-3 (n-6/n-3) ratios, supporting their nutritional value and possible methane-reducing potential. Dehydration was identified as the preferable drying method for seaweed samples, yielding clearer and more consistent spectra compared to oven-dried samples. In contrast, *M. japonica* showed minimal fatty acid content and was excluded from quantitative analysis due to spectral resolution limitations.

Phlorotannins, polyphenolic compounds plentiful in brown seaweeds, were quantified using Folin-Ciocalteu (F-C) assay, identified using ^{1}H NMR, and trialled using capillary electrophoresis (CE). Sugar kelp showed slightly higher mean concentrations of polyphenolic content than giant kelp, however, method variability limited the reliability of the results. Additionally, phloroglucinol aromatic H (hydrogen) peaks were successfully identified by ^{1}H NMR near 8.4 ppm. Although CE trials showed inconsistencies, the phlorotannin analysis results highlighted the promise and current limitations of minimal solvent techniques for phlorotannin detection and quantification.

The concentration of trace elements, including heavy metals, in seaweeds was analysed to assess potential toxicity risks these additives may pose to livestock. Through inductively coupled plasma mass spectrometry (ICP-MS), concentrations of key elements of interest including aluminum, arsenic, cadmium, lead, and mercury were determined and compared to

Governmental Action Levels for livestock feed. All species had concentrations of key elements well below acceptable levels, with *M. japonica* containing the lowest concentrations.

These findings underscore the importance of site- and species-specific monitoring when considering harvesting for livestock feed. Altogether, this study demonstrates that two locally abundant brown seaweeds, giant kelp and sugar kelp, exhibit promise as cattle feed additives for improved ruminant nutrition and potential methane mitigation. Favourable fatty acid ratios and phlorotannin content support their potential efficacy, while trace elements including heavy metals levels suggest they are safe for animal consumption under current guidelines. This research also highlights the utility of minimal solvent analytical techniques including ^{1}H NMR and CE for more environmentally conscious seaweed analysis.

TABLE OF CONTENTS

ACKNOWLEGEMENTS

As a student at Thompson Rivers University, most of my work took place on the traditional and unceded territory of the Tk'emlúps te Secwépmec people, within Secwépmec. I also want to acknowledge that I live, work, and play on the traditional, ancestral, and unceded lands of the Coast Salish peoples, including the xʷməθkʷəy̓əm (Musqueam), Skwxwú7mesh (Squamish), Sḵwx̱wú7mesh (Tsleil-Waututh), and kʷikʷəƛ̓əm (Kwikwetlem) Nations.

I am forever grateful to have completed this work under my wonderful supervisory committee consisting of Dr. Dipesh Prema, Dr. John Church, and Dr. Kingsley Donkor.

Kingsley, you took a chance on me and were the first person to encourage me to pursue a master's degree at TRU. I wouldn't have committed to this project if it wasn't for your kindness and guidance in the early days. Your knowledge and passion for analytical chemistry was contagious and I am beyond thankful for the opportunity to have worked with you.

John, thank you for your continual encouragement and enthusiasm throughout this process. I have learned so much about how research can transcend academia and be applied for real-world solutions from you. Your generosity, guidance, and leadership of this committee was paramount to my success.

Finally, to my supervisor Dipesh, I am immensely grateful and thankful to have been your first master's student. Your patience, kindness, and expertise made this a positive experience I will look back fondly on. Not only are you an outstanding teacher, but your willingness to learn for me and with me was inspiring and eye opening. I thank you from the bottom of my heart for taking this project on with me.

Moving to Kamloops alone to pursue this degree was intimidating, but Jacqueline Schoen, Sydney Miller, and Shae Turner made it feel like my home away from home. I feel so incredibly lucky to have met you three, and to have gone through this program alongside you. Whether it be a midday coffee run to vent about the problems our projects were facing, or weekend get togethers to celebrate our wins, you ladies encouraged and inspired me every step of the way.

To my family, I seriously could not have done this without your support. My lovely parents Beena, Jerome, and Laura, my brother James and sister-in-law Lindsay and family, thank you for being my biggest cheerleaders and always checking in on me. Your advice, love, and support carried me through this program even from a distance.

Finally, to my partner Alex, holy cow (no pun intended) what a journey this has been. Thank you for every facetime pep talk, all the words of encouragement, and for always lending an ear. You have been my rock and my shoulder to cry on, and your unwavering support has meant more than you know. Thank you.

I have received funding for this research from a Ken Lepin Research Graduate Student Award, a British Columbia Graduate Scholarship, and a conference travel grant from the TRU Students Union. This research was mainly supported by Dr. John Church's Genome B.C. Grant. Thank you to all who have supported this research.

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1: GENERAL INTRODUCTION

Greenhouse Gases and Climate Change

Climate change is a major global threat with far-reaching consequences, including global warming, more frequent and severe extreme weather events, and sea level rise (Nikzad et al., 2017; Ahmed et al., 2023; IPCC, 2022; Nicholls & Cazenave, 2010). These events not only devastate ecosystems but pose significant threats to human societies (IPCC, 2021 summary; Bayram et al., 2017; Russco et al., 2019). Extensive research has demonstrated that the frequency and severity of such climatic events are heavily influenced by the emission of greenhouse gases (GHGs) (Stips et al., 2016; Hegerl et al., 2007; Bindoff et al., 2013; Hansen et al., 2000).

GHGs are defined by their ability to absorb and emit infrared radiation through a process defined as radiative forcing, which contributes to warming at the earth's surface (Fouli et al., 2021; Johnson et al., 2007). Common GHGs include carbon dioxide (CO_2), methane (CH_4), nitrous oxides (NO_x), and fluorinated gases, with CO_2 accounting for approximately 76 % of global GHG emissions (Centre for Climate Change and Energy Solutions, 2025). The impact of each GHG on the atmosphere is typically assessed using global warming potential, a metric based on radiative forcing, mean atmospheric lifetime, and emissions relative CO_2 (Johnson et al., 2007; Government of Canada, 2025).

GHGs are essential for planetary function and as such, are released and absorbed through various biogeochemical processes (Tian et al., 2016). Biogenic sources include volcanic activity, forest fires, and organic matter decomposition, which release significant amounts of CO_2 and CH_4 into the atmosphere (Climate Atlas of Canada, 2025). However, natural sinks such as forests, grasslands, and oceans typically absorb slightly more GHGs than the natural sources emit, maintaining a near-equilibrium (IPCC, 2013). Human-induced disruptions to these natural sinks through industrialization, combined with anthropogenic sources, have contributed to a pronounced GHG imbalance, accelerating climate change and prompting a need for urgent mitigation strategies (IPCC, 2013).

In response, many countries have initiated comprehensive climate action plans. Canada has pledged to achieve net-zero GHG emissions by 2050 and, as part of this goal, launched the

2030 Emissions Reduction Plan. This plan targets a 40% reduction in GHG emissions below 2005 levels by 2030 through a sector-by-sector approach (Government of Canada, 2023). Despite this commitment, emissions have steadily increased since 2005, with a temporary decline between 2019 and 2021 due to the COVID-19 global pandemic (Government of Canada, 2023). As economic activity and stability resumes, integrating low-emission frameworks and technologies into industrial practices while preserving economic growth has become increasingly important.

Within the agricultural sector, specific initiatives have been outlined to support emission reductions. These include substantial federal investments in programs such as the Agricultural Climate Solutions: On-Farm Climate Action Fund, the Agricultural Clean Technology Program, and research initiatives aimed at developing a sustainable, climate-resilient agricultural sector aligned with Canada's 2050 net-zero target (Government of Canada, 2025). Within this goal, it is likely that strategies will target GHG emissions associated with the Canadian agricultural industry through technological advancement and green solutions.

Western Canadian Cattle Agriculture and GHG Emissions

Raising cattle is a key economic driver in Canada, with beef cattle production alone contributing an average of $22 billion annually to the national gross domestic product (GDP) between 2019 and 2021 (Statistics Canada, 2022). The industry is the second-largest single source of farm cash receipts nationwide (Canadian Cattle Association, 2024). However, the 2024 Canadian Census of Agriculture reported that the national cattle herd size has declined to their lowest levels since January 1989, largely due to ongoing stressors such as increased drought conditions and limited feed availability (Animal Production, 2024).

In addition to its economic contributions, the cattle industry is a major source of national methane emissions. Agriculture in Canada accounts for approximately 10% of the country's total GHG emissions, with enteric fermentation from cattle alone responsible for 33.1% and manure management for an additional 4.9% (Fouli et al., 2021) (Figure 1.1). Methane is a potent GHG with a global warming potential approximately 28 times greater than CO_2 over a 100-year period, although it has a much shorter atmospheric lifespan of about 12 years

(World Energy Outlook, 2022). This relatively short lifespan suggests that targeted methane reduction strategies could lead to measurable climate benefits within decades.

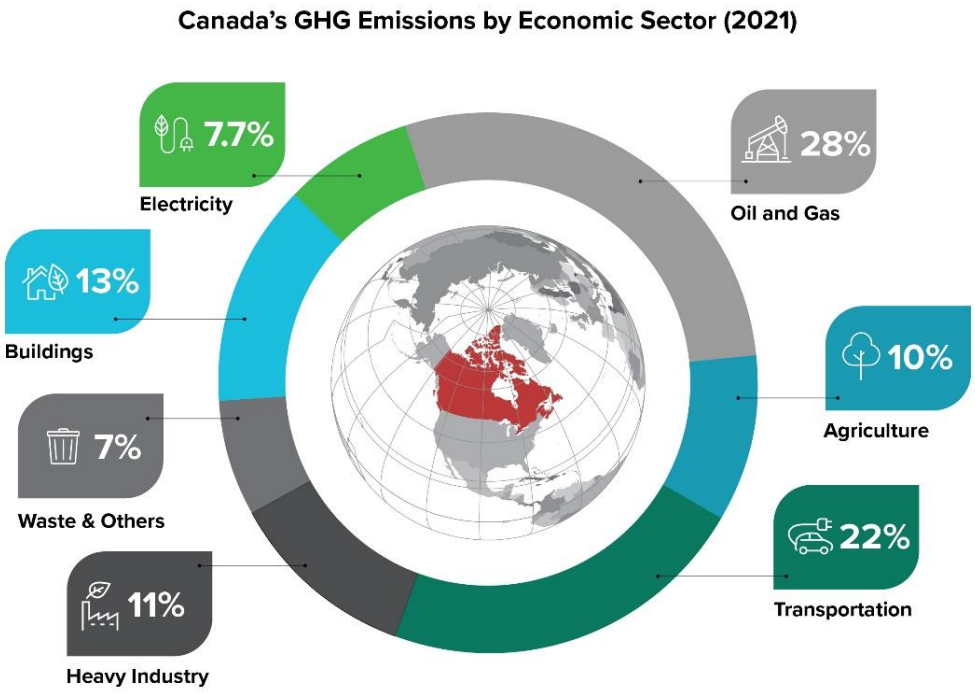


Figure 1.1 Canada's GHG Emission by Sector: 2021 Average (Government of Canada, 2023)

Enteric fermentation is the primary source of methane emissions in cattle. This is a microbial process that enables ruminants to digest cellulose, generating volatile fatty acids (VFAs) for energy, but also producing hydrogen (H) and CO_2 as substrates for methanogenesis (Vijn et al., 2020). Methanogenic archaea convert these substrates into CH_4, which is expelled by the animal (Hook et al., 2010; Morgavi et al., 2010). Methane loss represents a net reduction in feed efficiency; hence strategies such as dietary modifications, genetic selection, and improved livestock management are being explored for mitigation (Hristov et al., 2015).

A practical and emerging approach to combatting enteric methane emissions is by improving animal nutrition. Dietary interventions can influence rumen fermentation by altering the microbial presence and processes to reduce hydrogen availability for methanogens. Supplementing ruminant diets with certain fats, tannins, or essential oils has demonstrated

methane-reducing effects by either direct suppression of methanogenic archaea or through the modification of fermentation end-products (Beauchemin et al., 2008; Patra & Saxena, 2010). In general, high-quality forages with lower fiber content and greater digestibility have shown to reduce methane expulsion intensity per unit of animal product (Beauchemin et al., 2008; Hristov et al., 2013). Among emerging approaches, the use of seaweed-based feed additives has gained particular interest as they may offer a promising and potentially scalable solution for reducing enteric methane production in cattle.

Seaweed Feed for Enteric Methane Mitigation

Seaweeds, also known as marine macroalgae, are non-flowering photosynthetic macrophytes that are known to promote numerous health benefits in ruminants largely due to their synthesis of bioactive secondary metabolites, as well as their fatty acid profiles (Min et al., 2021). They are of particular interest for incorporation into cattle feed systems because unlike typical food sources, seaweeds do not compete for arable land or freshwater resources and are generally simple to grow and harvest (Leyton et al., 2020). In addition, they provide CO_2 sequestration, heavy metals sequestration, contribute to ocean cooling, and reduce ocean acidification – all of which would increase should renewable aquaculture and harvesting expand (Duarte et al., 2017; Chung et al., 2017). These considerations pose them as an attractive renewable biomass source for a range of applications in the livestock industry and beyond (Buschmann et al., 2017).

There are three distinct types of seaweeds: Rhodophyta (red), Phaeophyceae (brown), and Chlorophyta (green) varieties. All three types have been investigated to an extent for their enteric methane reduction abilities when used as cattle feed additive, of which the results have been highly variable (Vijn et al., 2020). For the purposes of this research and based on local prevalence and abundance, three macroalgal varieties were investigated, two brown and one red.

Red Macroalgae

The potential for red macroalga to mitigate enteric methane emissions from ruminants have been extensively evaluated using both *in vitro* and *in vivo* approaches and have produced substantial and promising results. Notably, *Asparagopsis taxiformis*, a tropical/subtropical red seaweed species, has been appointed the most impactful for enteric methane mitigation

with reduction ranges seen between 40 – 98% in steers (Nord University, 2021). The relative success of enteric methane suppression of *A. taxiformis* is due to the abundance of halogenated compounds, specifically bromoform ($CHBr_3$), in the species. Unlike brown macroalgae, red species often possess large quantities of bromoform, with *A. taxiformis* typically containing anywhere from 0.86 – 6.55 mg of bromoform/g dry weight (Machado et al., 2016; Roque et al., 2019; Kinley et al., 2020).

The anti-methanogenic properties of halogenated compounds come from their ability to inhibit methanogenesis by enzyme disruption (McGurrin et al., 2023). More specifically, bromoform acts as a structural analog for methyl-CoM and competitively inhibits the methyl-CoM reductase (Mcr) enzyme in the methanogenesis pathway that carries that cobamide-dependent methyl group (De Bhowmick & Hayes, 2023). An approximation of methane reduction potential of seaweed has been successfully investigated by the quantification of bromoform and other halogenated compounds in various seaweed species, and some research suggests that the relationship between bromoform content and methane reduction potential can be used as a starting point to standardise seaweed inclusion levels in ruminant feed (McGurrin et al., 2023).

Although a positive relationship between bromoform content and the ability to suppress methanogenesis in ruminants has been established, these halogenated compounds in certain concentrations pose both negative environmental and health effects. As bromoform and many other trihalomethanes are considered possible carcinogens, a maximum inclusion of 0.100 mg/L in drinking water has been set as a standard in Canada (Health Canada, 2024). Research by Muizelaar et al. (2021) found that when *A. taxiformis* was introduced into cattle feed of lactating cows, bromoform residue was present in their milk and urine within one day of ingestion. Although the study did not find bromoform content in animal tissue, it did find rumen abnormalities and inflammation during a histological analysis (Muizelaar et al., 2021).

Additionally, it was reported that the cows regularly refused the feed or specifically selected against the *A. taxiformis* component of the feed (Muizelaar et al., 2021). On top of its possible health impacts, bromoform and the act of harvesting bromoform-rich seaweeds poses environmental threats. As bromoform is a volatile compound that influences atmospheric and ozone chemistry, there is a potential for the volatilization of bromoform

from the harvesting and processing of *A. taxiformis* (De Bhowmick & Hayes, 2023). The contribution of bromoform to the atmosphere must be carefully considered when planning such aquaculture harvesting events.

Regardless of the successful enteric methane suppression and associated toxicological risk of red seaweeds like *A. taxiformis,* these halogen-rich species are typically only found in tropical and sub-tropical climates within temperate marine ecosystems (Min et al., 2021). Consequently, if the inclusion of seaweed in cattle feeds is to be utilised in western Canadian agriculture systems, the source of such seaweed should ideally originate from the local marine ecosystem for increased sustainability and reliance. As such, a new tropical red seaweed species may not be suitable for cultivation in B.C. and would involve bringing in non-native and potentially invasive species, potentially disrupting local ecosystems. Instead, an already established and abundant invasive red species, *Mazzaella japonica*, may be an interesting cattle-feeding alternative for investigation of secondary metabolite content.

M. japonica is native to the northwest Pacific (Japan, Korea, and China) and was likely introduced to B.C. through oyster aquaculture activities in the 1970s (Hansen et al, 2022; Saunders, 2008). Since its introduction, *M. japonica* has established populations along the coast of Vancouver Island and other intertidal regions of B.C. (Hansen et al., 2022). Similar to other red macroalga, *M. japonica* typically contains a suite of bioactive secondary metabolites, that may have anti-methanogenic properties in the context of animal feed (Wells et al., 2017). Due to its abundance as an introduced species off the coast of B.C., as well as its potential to reduce methane emissions in ruminants, *M. japonica* should be investigated further for viability as cattle feed additive on a local scale.

Brown Macroalgae

Brown macroalgae (Order Laminariales [kelps]) are prevalent in B.C.'s marine ecosystems, and like red seaweeds, have been explored as potential feed additives to reduce methane emissions in ruminants. These seaweeds constitute most of the biomass found in the intertidal and upper subtidal zones along B.C.'s coast, and as such, play a critical ecological and economic roles (Bates, 2025). Kelp beds provide habitat for marine life, supply oxygen to nearshore food webs, and protect shorelines from erosion by dampening wave and storm

energy (Bates, 2025). B.C. is home to a diverse and unparalleled diversity of kelps with over 30 species accounted for (eFlora BC, 2025).

Among the most abundant species are *Macrocystis pyrifera* (giant kelp) and *Saccharina latissima* (sugar kelp), both of which are important in aquaculture and have multiple industry applications. Giant kelp is the largest of all seaweeds and can form extensive canopies that result in ocean modification and habitat opportunities that foster coastal biodiversity (Druehl & Clarkston, 2016). Giant kelp has additional applications in food, feed, pharmaceuticals, and bioenergy due to its high nutrient and secondary metabolite content (Biparva et al., 2023; Purcell-Meyenrink et al., 2021). Sugar kelp is widely consumed by humans and livestock and is considered one of the most promising species for commercial cultivation in B.C. because of its fast growth, cold tolerance, and compatibility with integrated aquaculture systems (Grebe et al., 2019; Kim et al., 2017).

In addition to their nutritional value, brown macroalgae produce bioactive secondary metabolites including phlorotannins, fucoidans, laminarins, and other sulfated polysaccharides with antioxidant, antimicrobial, and anti-inflammatory properties (Hermund et al., 2018; Holdt & Kraan, 2011). While they lack halogenated compounds like bromoform, known for strong methane suppression ability in some red seaweeds, the presence of phlorotannins in brown species has led to growing interest in their potential to mitigate enteric methane through alternative biochemical pathways and improved nutrition (Abbot et al., 2023; Machado et al., 2016). Most studies investigating the methane mitigation potential of brown seaweeds have been limited to *in vitro* trials with variable results that depend on species, inclusion rates, and growing conditions (Nord University, 2021). The variability highlights the need for further research on species-specific metabolite profiles and their potential functional outcomes (Kunzel et al., 2023; Rosa et al., 2019).

The growing interest in seaweed-based solutions for enteric methane mitigation highlights the worldwide shift and interest in sustainable agricultural strategies and solutions. However, most of the research to date has focused on tropical red species such as *A. taxiformis* with high bromoform content, a known methane inhibitor. Recent efforts have focused on determining the chemical composition and bioactive potential of macroalgae found along the B.C. coast, offering a more local approach. Given the increasing demand for sustainable and

regionally available feed additives, there is a clear need to evaluate local seaweed species that could offer both environmental and economic benefits to Western Canadian cattle producers.

Research Objective

To address this knowledge gap, this thesis investigates plentiful red and brown seaweed species, *M. japonica*, sugar kelp and giant kelp, found along the B.C. coastline and investigates their fatty acid, phlorotannin, and heavy metals content, to assess their suitability for use as livestock feed additive. In addition to the assessment of these key compounds, this research aims to develop and optimize rapid, low-solvent analysis techniques to accurately quantify these chemical components. The findings will contribute to the growing research supporting seaweed as a promising methane mitigation feed that improves cattle health and offers a localized perspective for integrating marine resources into sustainable agriculture.

LITERATURE CITED

Abbott, J., Min, B. R., Renaud, D. L., & McAllister, T. A. (2023). Anti-methanogenic potential of seaweeds and seaweed-derived compounds in ruminant feed: Current perspectives, risks and future prospects. *Journal of Animal Science and Biotechnology, 14*(1), 47. https://doi.org/10.1186/s40104-023-00946-w

Ahmed, S., Ahmad, M., Rafique, M., & Khan, S. (2023). A review on climate change impacts, models, and its consequences on different sectors: A systematic approach. *Journal of Water and Climate Change, 15*(1), 104–122. https://doi.org/10.2166/wcc.2023.164

Animal Production. (2024). *Census of Agriculture: 2024 summary findings*. Statistics Canada

Bates, A. (2025). *Ecological functions of intertidal kelp systems in British Columbia*. BC Marine Studies.

Bayram, H., Bauer, A. K., Abdalati, W., Carlsten, C., Pinkerton, K. E., Thurston, G. D., et al. (2017). Environment, global climate change, and cardiopulmonary health. *American Journal of Respiratory and Critical Care Medicine*, 195(6), 718–724.

Beauchemin, K. A., Kreuzer, M., O'Mara, F., & McAllister, T. A. (2008). Nutritional management for enteric methane abatement: A review. *Australian Journal of Experimental Agriculture, 48*(2), 21–27. https://doi.org/10.1071/EA07199

Bindoff, N. L., Stott, P. A., AchutaRao, K. M., Allen, M. R., Gillett, N., Gutzler, D., … & Zhang, X. (2013). Detection and attribution of climate change: From global to regional. In *Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change* (pp. 867–952). Cambridge University Press.

Biparva, P., Ashrafi, O., & Balasubramanian, V. N. (2023). Potential industrial uses of Canadian kelp species in bioenergy and biomaterials. *Canadian Journal of Chemical Engineering, 101*(2), 312–321.

Buschmann, A. H., Camus, C., Infante, J., Neori, A., Israel, Á., Hernández-González, M. C., ... & Golberg, A. (2017). Seaweed production: Overview of the global state of exploitation, farming and emerging research activity. *European Journal of Phycology, 52*(4), 391–406.

Canadian Cattle Association. (2024). *Cattle industry statistics*. https://www.cattle.ca

Centre for Climate and Energy Solutions. (2025). *Greenhouse gas emissions*. https://www.c2es.org/content/greenhouse-gas-emissions/

Chung, I. K., Oak, J. H., Lee, J. A., Shin, J. A., Kim, J. G., & Park, K. S. (2017). Installing kelp forests/seaweed beds for mitigation and adaptation against global warming: Korean project overview. *ICRI Proceedings*, 22–28.

Climate Atlas of Canada. (2025). *Greenhouse gas sources*. https://climateatlas.ca

De Bhowmick, G., & Hayes, M. (2023). Halogenated compounds in seaweeds and their environmental interactions. *Marine Environmental Research, 191*, 106052. https://doi.org/10.1016/j.marenvres.2023.106052

Druehl, L. D., & Clarkston, B. E. (2016). *Pacific seaweeds: A guide to common seaweeds of the West Coast* (Rev. ed.). Harbour Publishing.

Duarte, C. M., Wu, J., Xiao, X., Bruhn, A., & Krause-Jensen, D. (2017). Can seaweed farming play a role in climate change mitigation and adaptation? *Frontiers in Marine Science, 4*, 100. https://doi.org/10.3389/fmars.2017.00100

eFlora BC. (2025). *Kelp and seaweed flora of British Columbia*. https://linnet.geog.ubc.ca

Fouli, Y., Duval, P., & Hall, K. (2021). Livestock methane emissions in Canada: Trends and mitigation strategies. *Canadian Journal of Animal Science, 101*(4), 613–625.

Government of Canada. (2023). *Canada's national greenhouse gas inventory: 1990–2021*. Environment and Climate Change Canada. https://www.canada.ca/en/environment-climate-change/services/climate-change/greenhouse-gas-emissions/inventory.html

Government of Canada. (2025). *2030 Emissions Reduction Plan*. Environment and Climate Change Canada. https://www.canada.ca/en/services/environment/weather/climatechange/climate-plan.html

Grebe, G. S., Byron, C. J., St. Gelais, A., Kotowicz, D. M., & Olson, T. K. (2019). An ecosystem approach to kelp aquaculture in the Americas and Europe. *Aquaculture Reports, 15*, 100215. https://doi.org/10.1016/j.aqrep.2019.100215

Hansen, J., Sato, M., Ruedy, R., Lacis, A., & Oinas, V. (2000). Global warming in the twenty-first century: An alternative scenario. *Proceedings of the National Academy of Sciences, 97*(18), 9875–9880. https://doi.org/10.1073/pnas.170278997

Health Canada. (2024). *Guidelines for Canadian drinking water quality*. https://www.canada.ca/en/health-canada/services/environmental-workplace-health/water-quality/drinking-water.html

Hegerl, G. C., Zwiers, F. W., Braconnot, P., Gillett, N. P., Luo, Y., Marengo Orsini, J. A., … & Stott, P. A. (2007). Understanding and attributing climate change. In *Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change* (pp. 663–745). Cambridge University Press.

Hermund, D. B., Plaza, M., Turner, C., & Jónsdóttir, R. (2018). Structure–activity relationship of radical scavenging and anti-inflammatory sulfated polysaccharides from Icelandic seaweeds. *Algal Research, 29*, 34–40. https://doi.org/10.1016/j.algal.2017.11.009

Holdt, S. L., & Kraan, S. (2011). Bioactive compounds in seaweed: Functional food applications and legislation. *Journal of Applied Phycology, 23*(3), 543–597. https://doi.org/10.1007/s10811-010-9632-5

Hook, S. E., Wright, A. D. G., & McBride, B. W. (2010). Methanogens: Methane producers of the rumen and mitigation strategies. *Archaea, 2010*, 945785. https://doi.org/10.1155/2010/945785

Hristov, A. N., Oh, J., Firkins, J. L., Dijkstra, J., Kebreab, E., Waghorn, G., ... & Tricarico, J. M. (2015). Special topics — Mitigation of methane and nitrous oxide emissions from animal operations: II. A review of manure management mitigation options. *Journal of Animal Science, 91*(11), 5070–5094.

Hristov, A. N., Oh, J., Giallongo, F., Frederick, T. W., Harper, M. T., Weeks, H. L., ... & Bravo, D. M. (2013). Mitigation of methane and nitrous oxide emissions from animal operations: I. A review of enteric methane mitigation options. *Journal of Animal Science, 91*(11), 5045–5069. https://doi.org/10.2527/jas.2013-6583

Imbs, T. I., & Zvyagintseva, T. N. (2018). Phlorotannins are polyphenols of brown algae. *Russian Journal of Marine Biology, 44*(4), 263–273.

Intergovernmental Panel on Climate Change (IPCC). (2013). *Climate Change 2013: The physical science basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change* (T. F. Stocker et al., Eds.). Cambridge University Press. https://doi.org/10.1017/CBO9781107415324

IPCC. (2022). *Climate change 2022: Impacts, adaptation, and vulnerability. Contribution of Working Group II to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change* (H.-O. Pörtner, D. C. Roberts, M. Tignor, E. S. Poloczanska, K. Mintenbeck, A. Alegría, … & B. Rama, Eds.). Cambridge University Press. https://www.ipcc.ch/report/ar6/wg2/chapter/chapter-4/

IPCC. (2021). High confidence in human emissions driving extreme weather. *Time* (summarizing IPCC).

Johnson, D. E., Ward, G. M., & Ramsey, C. B. (2007). Livestock methane: Current emissions and mitigation potential. *Environmental Science & Technology, 41*(2), 405–409.

Kim, J. K., Kraemer, G. P., & Yarish, C. (2017). Development of a simple seedstring for improving the efficiency of sugar kelp (*Saccharina latissima*) aquaculture. *Journal of Applied Phycology, 29*, 411–420. https://doi.org/10.1007/s10811-016-0959-z

Kinley, R. D., de Nys, R., Vucko, M. J., Machado, L., & Tomkins, N. W. (2020). The red macroalgae *Asparagopsis taxiformis* is a potent natural antimethanogenic that reduces methane production during in vitro fermentation with rumen fluid. *Animal Production Science, 56*(3), 282–289.

Kunzel, S., Abbas, W., Hasan, R. H., & Reza, M. S. (2022). Brown seaweeds and methane mitigation: A review of nutritional potential and constraints. *Marine Pollution Bulletin, 183*, 114010.

Leyton, C., Vásquez, J. A., & Piñones, A. (2020). Perspectives on seaweed cultivation in Chile for bioenergy and climate change mitigation. *Journal of Applied Phycology, 32*, 3451–3466.

Machado, L., Magnusson, M., Paul, N. A., Kinley, R., de Nys, R., & Tomkins, N. (2016). Dose–response effects of *Asparagopsis taxiformis* and *Oedogonium* sp. on in vitro fermentation and methane production. *Journal of Applied Phycology, 28*, 1443–1452. https://doi.org/10.1007/s10811-015-0639-9

McGurrin, H., Keogh, D., Murray, P., & Walsh, P. J. (2023). Bromoform and related halogenated compounds in seaweed-based ruminant feed. *Frontiers in Veterinary Science, 10*, 1156748. https://doi.org/10.3389/fvets.2023.1156748

Min, B., Solaiman, S., & Gurung, N. (2021). Seaweed supplementation for methane mitigation in ruminants: A review. *Agronomy, 11*(3), 494. https://doi.org/10.3390/agronomy11030494

Morgavi, D. P., Forano, E., Martin, C., & Newbold, C. J. (2010). Microbial ecosystem and methanogenesis in ruminants. *Animal, 4*(7), 1024–1036. https://doi.org/10.1017/S1751731110000546

Muizelaar, W., Smits, M. C. J., van Duinkerken, G., Mol, J. G. J., & de Boer, I. J. M. (2021). Safety and transfer study of bromoform from red seaweed to milk and urine in lactating dairy cows. *Journal of Dairy Science, 104*(6), 6723–6734.

Nicholls, R. J., & Cazenave, A. (2010). Sea-level rise and its impact on coastal zones. *Science, 328*(5985), 1517–1520. https://doi.org/10.1126/science.1185782

Nikzad, R., Gholami, V., & Darand, M. (2017). Climate change: Global impacts and mitigation strategies. *Atmospheric and Climate Sciences, 7*(3), 361–375.

Nord University. (2021). *Seaweed and methane mitigation in ruminant livestock*. Norwegian Institute of Bioeconomy Research.

Patra, A. K., & Saxena, J. (2010). A new perspective on the use of plant secondary metabolites to inhibit methanogenesis in the rumen. *Phytochemistry, 71*(11–12), 1198–1222.

Purcell-Meyenrink, M., Walker, T. R., & Scheinberg, J. (2021). Seaweed aquaculture in British Columbia: A case study for sustainable coastal development. *Marine Policy, 134*, 104792.

Roque, B. M., Brooke, C. G., Ladau, J., Polley, T., Marsh, L. J., Najafi, N., ... & Kebreab, E. (2019). Effect of the macroalgae *Asparagopsis taxiformis* on methane production and the rumen microbiome across diets and production systems in vivo. *Frontiers in Microbiology, 10*, 2051.

Russco, H., et al. (2019). Combined impacts of climate and air pollution on human health and agricultural productivity. *Environmental Research Letters*, 14(11), 113001.

Saunders, G. W. (2008). Using DNA barcoding to reveal cryptic diversity and the identity of introduced species in Canadian marine algae. *Botany, 86*(8), 802–810.

Statistics Canada. (2022). *Cattle statistics*. https://www.statcan.gc.ca

Stips, A., Macias, D., Coughlan, C., García-Gorriz, E., & Liang, X. S. (2016). On the causal structure between CO_2 and global temperature. *Scientific Reports, 6*, 21691. https://doi.org/10.1038/srep21691

Tian, H., Xu, R., Canadell, J. G., Thompson, R. L., Winiwarter, W., Suntharalingam, P., ... & Saikawa, E. (2016). Global methane and nitrous oxide emissions from terrestrial ecosystems due to multiple environmental changes. *Environmental Research Letters, 11*(3), 034012.

Vijn, S., Compart, D., Dutta, N., Foukis, A., Hess, M., Hristov, A. N., ... & Tricarico, J. (2020). Key considerations for the use of seaweed to reduce enteric methane emissions from cattle. *Frontiers in Veterinary Science, 7*, 597430. https://doi.org/10.3389/fvets.2020.597430

World Energy Outlook. (2022). *Methane report*. International Energy Agency. https://www.iea.org/reports/global-methane-tracker-2022

Wells, M. L., Potin, P., Craigie, J. S., Raven, J. A., Merchant, S. S., Helliwell, K. E., ... & Dupont, C. L. (2017). Algae as nutritional and functional food sources: Revisiting our understanding. *Journal of Applied Phycology, 29*, 949–982.

CHAPTER 2: FATTY ACID ANALYSIS

ABSTRACT

This research determined omega-6 (n-6) to omega-3 (n-3) fatty acid ratios in three locally abundant seaweed species, *Macrocystis pyrifera* (giant kelp), *Saccharina latissima* (sugar kelp), and *Mazzaella japonica* (*M. japonica*), by proton nuclear magnetic resonance (^{1}H NMR); and assessed the applicability of a developed dietary fatty acid analysis methodology using capillary electrophoresis (CE). Results indicated that giant kelp had the highest n-6/n-3 ratio, while still being well within a favourable range, followed by sugar kelp, whereas *M. japonica* yielded variable and unquantifiable spectra likely due to the low fatty acid content of the species. Drying techniques, either oven-dried or dehydrated seaweed samples, were compared; and it was found that dehydrated samples showed better ^{1}H NMR spectral clarity and therefore improved peak integration and n-6/n-3 quantification. Statistical analyses confirmed that drying methods produced significantly different ratios; and the method was validated for n-6/n-3 ratio quantification in sugar kelp and giant kelp through repeatability assessments. Although pilot CE analysis of fatty acids proved variable and overall unsuccessful, the results of the trial provide background information for further optimization investigations. Despite these challenges, the favourable fatty acid ratios in giant kelp and sugar kelp samples suggest their potential to be used as sustainable cattle feed-additive to improve animal nutrition and support methane mitigation strategies.

INTRODUCTION

Lipids are essential energy-providing macronutrients that are necessary for the survival of animals including humans (Martínez-Pinilla et al., 2016; McDonald et al., 2011). The composition of dietary lipids can significantly influence overall health (Sacks et al., 2017). While both animal-based and plant-based foods are important sources of lipids, those rich in unsaturated fats, specifically monounsaturated (MUFAs) and polyunsaturated (PUFAs) fatty acids, are generally considered the most beneficial for health (National Research Council, 1989; Bradbury & Appleby, 2019; "Types of Fats", 2014).

Unsaturated fats are defined by the presence of one or more double bonds in their hydrocarbon chains (Nelson & Cox, 2017; Gurr et al., 2016). They are classified as either MUFAs, containing one single double bond, or PUFAs, containing two or more double bonds (Nelson & Cox, 2017; Gurr et al., 2016). PUFAs are further categorized as omega-3 (n-3), omega-6 (n-6), or omega 9 (n-9) fatty acids based on the position of the first double bond from the methyl (omega) end of the fatty acid chain (Liu et al., 2023). Specifically, the first double bond appears between the third and fourth carbon atoms in omega-3s, the sixth and seventh in omega-6s, and ninth and tenth in omega-9s (Silva et al., 2020). The term "omega" refers to the final letter of the Greek alphabet and in this context, denotes the location of the double bond in relation to the methyl group (Gunstone, 2001). Common PUFAs and the structural distinctions among omega-3, omega-6, and omega-9 fatty acids are presented in Figure 2.1**,** below.

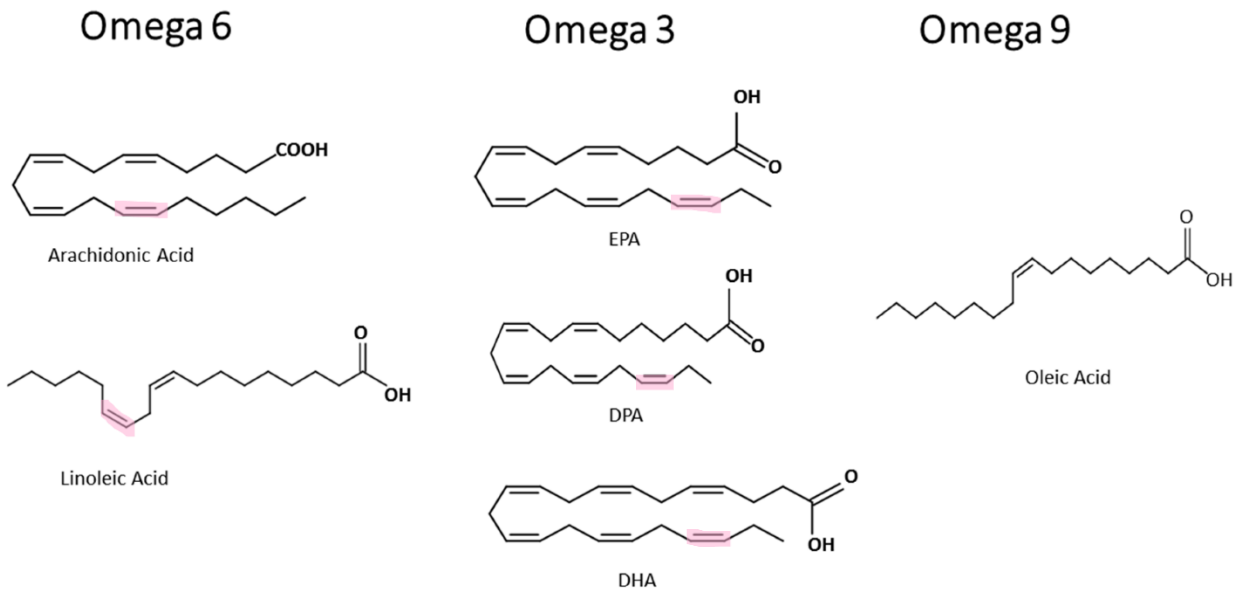


Figure 2.1 Common Omega-3's, Omega-6's, and Omega-9 and their Structural Differences Highlighting the First Double Bond of which the Fatty Acid is Grouped By (Adapted from Silva et al., 2020)

Although MUFAs are synthesized endogenously in humans and animals and are therefore not considered essential dietary fats, they still comprise approximately 15% of total dietary fat intake (Lunn, 2007). MUFAs are found in substantial quantities in animal products such as meat, eggs, and dairy, as well as in plant-based oils like olive and sunflower oil. In contrast, PUFAs, namely omega-3 and omega-6 fatty acids, are essential because they cannot be synthesized by the body and must be obtained through diet. Common dietary PUFA sources include plant seeds and marine fish oils for omega-3s, and vegetable oils for omega-6s (Kapoor et al., 2021; "Types of Fat", 2014). Seaweeds are also known to be rich in bioactive compounds, including long-chain (LC) PUFAs (Schmid et al., 2014).

LC omega-3 PUFAs, such as eicosapentaenoic acid (EPA; 20:5 n-3) and docosahexaenoic acid (DHA; 22:6 n-3), have been associated with numerous health benefits, including the prevention of cardiovascular disease and certain cancers, as well as supporting nervous system development (Schmid et al., 2014). The dietary ratio of omega-6 to omega-3 fatty acids has been shown to greatly influence health outcomes. For instance, Rocha et al. (2021) cited studies indicating n-6/n-3 ratios between 3:1 and 5:1 were linked to reduced risks of breast, prostate, colon, and renal cancers. Ratios between 2:1 and 3:1 were associated with

reduced inflammation in patients with rheumatoid arthritis (Rocha et al., 2021). Conversely, higher ratios such as 10:1 have been associated with negative health outcomes (Rocha et al., 2021). These findings suggest that a lower n-6/n-3 ratio is optimal for health benefits.

In cattle, dietary LC-PUFAs, particularly omega-3s, are important for improving reproductive performance, supporting immune function, and reducing inflammation (Fabjanowska et al., 2023). As such, the inclusion of these fatty acids in livestock feed can alter the fatty acid profile of resulting meat and dairy products, thereby enhancing their nutritional value for human consumption. For example, Daley et al. (2010) found that grass-fed cattle produced meat with higher concentrations of omega-3 PUFAs, resulting in more favourable n-6/n-3 ratio. Supporting this, Prema et al. (2016) used proton nuclear magnetic resonance spectroscopy (^{1}H NMR) to analyse the n-6/n-3 ratios of cereal grains and forages (including hydroponic sprouts and haylage). It was found that forages yielded more favourable (lower) n-6/n-3 ratios. These findings emphasize the importance of incorporating LC-PUFAs into cattle diets, not only to support animal health but also to improve the quality of animal-derived food products.

In addition to their health benefits, fatty acids, particularly LC-PUFAs, have demonstrated potential to supress methane emissions when used as cattle feed-additives. Patra (2013) conducted a meta-analysis on fat supplementation in cattle diets, focusing on enteric methane production, digestibility, and rumen fermentation. Analysing 105 treatment means from 1,339 observations across 29 experiments, it was found that increasing dietary fat content to a maximum of 6% dry matter (DM) reduced enteric methane emissions by up to 15%, compared to typical diets that contained about 2% fat (Patra, 2013). Among the fatty acids studied, lauric acid (C12:0), α-linolenic acid (C18:3 n-3), and various PUFAs demonstrated the most significant methane-reducing effects. A similar meta-analysis by Grainger et al. (2011) confirmed the prolonged methane-suppressing effect of dietary fatty acids in ruminants. These findings highlight the value of feed management strategies that include LC-PUFAs for mitigating methane emissions from livestock.

Typically, LC-PUFAs are supplied to diets through marine fish oils, either via direct fish consumption or dietary supplementation. However, increasing concerns about the overexploitation of marine resources and demand for sustainable, plant-based alternatives

have highlighted seaweeds are a promising substitute (Rocha et al., 2021; Schmid et al., 2013). Seaweeds are a highly renewable resource that can be cultivated without competing for arable land or freshwater. Additionally, seaweed cultivation contributes to carbon sequestration, as macroalgae absorb atmospheric CO_2 during photosynthesis (Duarte et al., 2017). These attributes position marine macroalgae as an abundant and potentially sustainable source of fatty acids for integration in livestock feed systems.

Identifying and quantifying fatty acid profiles, including SFAs, PUFAs, LC-PUFAs, and MUFAs, in potential feedstocks is crucial for predicting methane mitigation potential and optimizing animal nutrition. Therefore, the analysis of fatty acid profiles in local seaweed species such as *Macrocystis pyrifera* (giant kelp), *Saccharina latissima* (sugar kelp), and *Mazzaella japonica* (*M. japonica*) is central to evaluating their application as feed in the local cattle industry for both nutritional and environmental benefits.

The objective of this research is to develop and apply low-solvent analytical methodologies to identify and analyse the fatty acid profiles of giant kelp, sugar kelp, and *M. japonica*. Specifically, this includes quantifying SFAs, PUFAs, and determining omega-6 to omega-3 (n-6/n-3) ratios. ^{1}H NMR is employed as a rapid, technique for characterizing n-6/n-3 ratios, while the viability of using capillary electrophoresis (CE) for investigating/separating SFAs and PUFAs is analysed. This research contributes to the development of efficient analytical methods for fatty acid characterization and to evaluate the potential of these seaweeds as more sustainable sources of beneficial fatty acids for improving cattle health, enhancing the nutritional quality of animal-derived products, and supporting enteric methane mitigation strategies.

MATERIALS AND METHODS

Biological Materials Sampling

The study species for this analysis included two brown seaweed species, giant kelp and sugar kelp, and one red seaweed species, *M. japonica*. Fresh brown seaweed samples were collected by Cascadia Seaweed Inc. (Cascadia) from their farm site in Clayoquot sound, B.C. (approximate location shown in Figure 2.2). Harvesting took place at approximately 17:00 on June 5, 2024. According to Cascadia's documentation, live specimens were cut from the stalk with a knife, and visible biofouling was removed. The cleaned samples were wrapped in damp towels and transported in coolers containing seawater. They were shipped via air transport to Kamloops, B.C., and were received by TRU on June 7, 2024. The *M. Japonica* was hand collected by TRU as storm-cast from the beaches of Deep Bay, Vancouver Island, B.C. on August 26th, 2024 (Figure 2.2). The fresh red seaweed samples were packed in coolers with ice and transported to the TRU laboratory over the subsequent days.

Figure 2.2 Approximate Locations of Sugar Kelp, Giant Kelp Harvest and *M. japonica* Harvest in Clayoquot Sound, B.C., and Deep Bay, B.C (image created via iMap BC).

Seaweed Sample Storage and Preparation

Upon arrival at the TRU laboratory, all macroalgal samples were sorted by species, rinsed with deionized water to remove impurities, and stored in Ziploc bags at -80 ºC until further processing. Prior to analysis, the samples were removed from the freezer for drying. Two drying methods were employed: (1) mechanical convection oven drying using an Economy Model 18EM set at approximately 105 ºC for one hour (or until fully dry), and (2) low-temperature dehydration using a Hamilton Beach household dehydrator set at 40 ºC for approximately three hours. Following drying, all samples were ground to a fine powder using an electric mill grinder. The resulting powders were stored in Ziploc bags, categorized by species and drying method, and kept at room temperature until analysis. Dried samples were prepared in small ~100 g batches at a time to ensure freshness.

Chemical Supplies

Methanol (HPLC grade, ≥99.9%) was used in both ^{1}H NMR and CE protocols and was obtained from Millipore Sigma Canada Ltd. (Oakville, Ontario, Canada). High-purity water (18 MΩ·cm), used throughout sample preparation and analysis, was produced using a Milli-Q purification system (Millipore, Billerica, MA, USA). For ^{1}H NMR analysis, deuterated chloroform ($CDCl_3$, 99.8 atom % D) was sourced from Millipore Sigma Canada Ltd. This solvent was used for fatty acid extraction and spectral acquisition. Fatty acid standards for CE analysis included lauric acid, myristic acid, palmitic acid, stearic acid, behenic acid, α-linolenic acid, γ-linolenic acid, stearidonic acid, arachidonic acid (≥95.0%), eicosatrienoic acid (IS), eicosapentaenoic acid, and docosahexaenoic acid, all purchased from Millipore Sigma Canada Ltd., and linoleic acid (99%) was obtained from Fisher Scientific Canada, a division of Thermo Fisher Scientific Inc. (Ottawa, Ontario, Canada). Other reagents used in CE, including buffer components β-cyclodextrin, urea, and sodium dodecyl sulfate (SDS) were acquired from Millipore Sigma, and 0.1 M and 1.0 M sodium hydroxide (NaOH) conditioning solutions were prepared in volumetric flasks using 18 MΩ water.

Fatty Acid Detection and Quantification by ^{1}H NMR

^{1}H NMR Instrumentation

^{1}H NMR spectra were acquired using a Bruker Ultrashield 500 MHz spectrometer (Bruker BioSpin, Rheinstetten, Germany) equipped with a proton-optimized inverse detection (PULPROG: zg30) probe. The instrument was operated at 298 K. TopSpin software platform (version 3.5 pl 7, Bruker Biospin) was used for spectral processing and integration. IconNMR (component of Topspin) automation software was further utilized for automated acquisitions of sample sets. Each spectrum was collected using 256 scans with a sweep width of 20 ppm and relaxation delay (d1) of 1 second.

Sample Preparation for Rapid n-6/n-3 Ratio Determination by ^{1}H NMR

For the rapid determination of n-6/n-3 fatty acid ratios, protocols were adapted from Prema et al. (2016). Dried samples of the three seaweed species, sugar kelp, giant kelp, and *M. japonica*, prepared by both drying methods (oven drying [OV] and dehydrator drying [DH]), were weighed to approximately 200 mg using an analytical balance (see **Appendix A.1, Table A.1.1** for exact weights). Each sample was transferred into a clean glass vial, and 2.5 mL of $CDCl_3$ was added. The vials were manually agitated and then placed into a Bransonic Ultrasonic Cleaner (50/60 Hz) for one hour of sonication to facilitate extraction. Following sonication, samples were allowed to settle for an additional hour at room temperature. The resulting solutions were then filtered through Kimwipe (Kimberly-Clark, Mississauga, Canada) directly into NMR tubes for analysis. Each sample condition (species x drying method) was prepared in triplicate, resulting in six samples per species and a total of 18 samples, with the intention of determining which drying method resulted in a better spectral resolution.

Spectra resulting from dehydrated samples were clearer and more interpretable and therefore lead to a more accurate quantification of n-6/n-3 ratios. As such, optimized dehydrated samples of each species were re-prepared and re-analysed in triplicate over three separate days to assess inter- vs. intra-day variation as part of a method validation process **(Appendix A.1, Table A.1.2).**

In an attempt to attain improved omega-3 and omega-6 fatty acid extraction for ^{1}H NMR analysis, a secondary extraction method was investigated on a trial basis following a fatty acid extraction approach outlined by Soliman et al. (2013). For this trial, giant kelp was weighed via analytical balance (actual mass: 1.0024 g) and added to a glass vial with 10 mL of 50:50 v/v methanol/water. The mixture was sonicated for two hours, and the methanol/water was rotary evaporated off at 50 °C, spun at 120 rpm. The crude product was re-dissolved in $CDCl_3$ and filtered directly into an NMR tube. The sample was analysed under the same parameters as previous samples. As the process did not improve fatty acid extraction or the ^{1}H NMR spectral clarity, coupled with the considerably longer preparation time, further trials were not explored.

n-6/n-3 Determination from ^{1}H NMR Spectra

For the quantification of n-6/n-3 ratios (or LA/ALA ratio), an approach adapted from Prema et al. (2016) was applied. This approach was originally used for the determination of n-6/n-3 ratios in cereal grains and cattle forages and involved the integration of two peaks located at approximately 2.77 ppm (LA region) and 2.81 ppm (ALA region) when analysed in $CDCl_3$. For the consistency of this analyses, integration between 2.74 – 2.79 ppm corresponded to the LA region while integration between 2.79 – 2.84 ppm corresponded to the ALA region. An example of this integration from a ^{1}H NMR spectra obtained for forage samples from Prema et al. (2016) is shown in Figure 2.3. n-6/n-3 ratios were determined by the following calculation:

$$\text{n-6/n-3} = \frac{I_{LA}}{\frac{I_{ALA}}{2}}$$

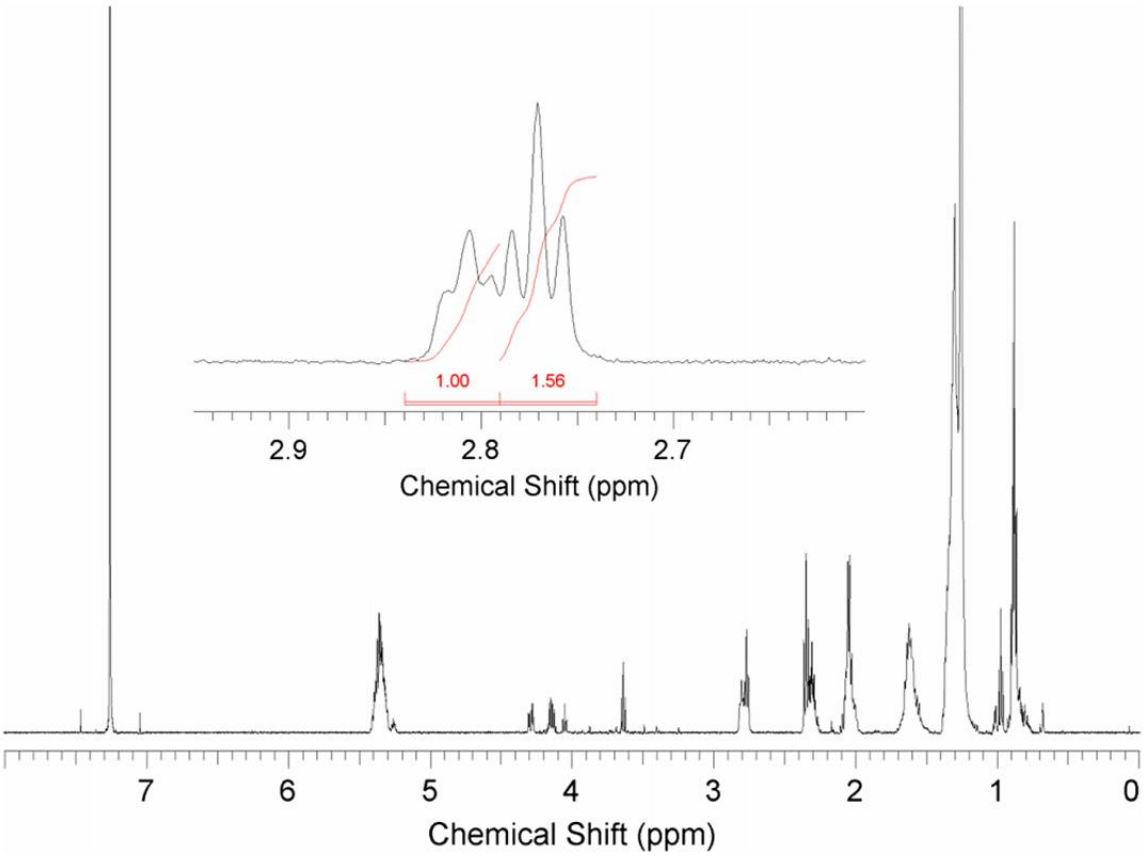


Figure 2.3 Example of the Integration of 18:2n-6 (LA) at 2.77 ppm and 18:3n-3 (ALA) at 2.81 ppm From a Forage Sample in $CDCl_3$ (Prema et al., 2016)

With the goal of quantifying oleic acid and total unsaturated fatty acid content using the same ^{1}H NMR spectra obtained through this analysis, a methodology developed by Siudem et al. (2022) used for the determination of LA, ALA, oleic acid, and total unsaturated fatty acids in vegetable oils was investigated. LA, ALA and oleic quantities were determined using the integrated values of specific fatty acid peaks denoted by "*I"* (Siudem et al., 2022). The chemical shifts of relevant fatty acid compounds from Siudem et al. (2022) are detailed in **Appendix A.1, Table A.1.3**, and the formulas for fatty acid determination are detailed below.

$$ALA = \frac{I_{(2)}}{I_{(2)} + I_{(1)}}$$

$$LA = \frac{1}{2}\left(I_{(7)} - 4ALA\right)$$

$$OlEIC = \frac{I_{(9)} - 4LA - 6ALA}{2}$$

$$TOT_{unsat} = OLEIC + LA + ALA$$

Pilot Investigation for Fatty Acid Detection and Determination by CE

CE Instrumentation

CE analysis was conducted utilising a SCIEX P/ACE MDQ Plus Capillary Electrophoresis system (Fullerton, California). Analytes were detected with an ultraviolet (UV) detector at 214 nm wavelength using direct absorbance, and data was acquired and processed using 32Karat software. For fatty acid separation, uncoated, fused-silica capillaries (Polymicro Technologies, Phoenix, AZ) of an inner diameter of 50 μm were used. The length of the capillary was 50.0 cm, with an effective length of 40.0 cm. Prior to CE analysis, ultraviolet-visible spectroscopy was utilized to confirm 214 nm was an appropriate wavelength to be used for the analysis.

For the analysis of SFAs, the capillary was rinsed for 4 min with 0.1 M NaOH, 1 min with 18 MΩ water, and 5 min with buffer solution all at 20 psi prior to analysis. Standards and samples were injected at 1.0 psi for 5 s and separated at 20.0 kV for 25.00 min each. Four sets of standards and samples (one blank, six standards, and three samples – resulting in 10 vials per set) were analysed at one time.

Prior to analysis of PUFAs, initial capillary rinsing followed the same process as stated above. However, in addition to the initial rinse, short interim rinses were introduced between each sample set (every 10 vials) in attempt to mitigate suspected contamination carryover. Short rinses consisted of 4 min of 1.0 M NaOH, 5 min of 0.1 M NaOH, and 5 min of 18 MΩ water, all at 20 psi. PUFAs were injected at 1.0 psi for 5.0 s and separated at 25.0 kV for 35.0 min each. Voltage and duration of separation were adjusted in attempt to optimize results based on the variable results produced by the SFA analysis.

Fatty Acid Profile Determination by Capillary Electrophoresis

A pilot analysis was conducted to determine the ability for CE instrumentation to be utilised for fatty acid separation and determination to support and supplement the n-6/n-3 ratio analysis obtained via ^{1}H NMR. The methodology used was developed, optimized, and validated by Soliman et al., (2013) for the separation of dietary omega-3s and omega-6s in

food materials. The same methodology was applied to SFAs in addition to PUFAs for the analysis of seaweed species.

First, fatty acid standard stock solutions of either 150 ppm or 250 ppm were created in 50:50 v/v methanol/ 18 MΩ water. Six concentration levels of working standard solutions and one blank (50:50 v/v methanol/ 18 MΩ water) were created from each FA stock solution and used to curate calibration curves for subsequent FA analyses. The exact concentrations of working standards for each FA are presented in Table 2.1 below, and the actual concentrations of standard stock solutions can be found in **Appendix A.2**, **Table A.2.1**. As determined to be the optimal buffer by Soliman et al. (2013), a 40 mM tetraborate buffer at pH 9.5 containing 50 mM SDS, 6 M urea, 10 mM β-cyclodextrin (β-CD), and 10% acetonitrile (ACN) was used for the analyses of both SFAs and PUFAs (Soliman et al., 2013).

Table 2.1 *List of 11 FAs, Including Internal Standard (20:3 n-3), and the Exact Concentrations of Standard Mixtures used for CE Analysis*

Fatty Acid	**Symbol**	**Standard levels (mg/L or ppm)**					
		1	2	3	4	5	6
Lauric acid	12:0	6.53	19.5	39.0	65.3	97.5	129.7
Myristic acid	14:0	5.19	15.5	31.0	51.9	77.5	103.1
Palmitic acid	16:0	5.36	16.0	32.0	53.6	80.0	106.4
Stearic acid	18:0	5.86	17.5	35.0	58.6	87.5	116.4
Linoleic acid	18:2 n-6	9.98	49.9	79.9	114.8	159.7	199.9
α-Linolenic acid	18:3 n-3	9.94	49.7	79.6	114.4	159.1	198.9
ɣ-Linolenic acid	18:3 n-6	10.05	50.3	80.4	115.6	160.8	201.0
Arachidonic acid	20:4 n-6	5.02	15.0	30.0	50.2	75.0	99.7
Stearidonic acid	18:4 n-3	5.25	15.7	31.4	52.5	78.4	104.3
Eicosapentaenoic acid (EPA)	20:5 n-3	5.03	15.0	30.0	50.3	75.1	99.8
Docosahexaenoic acid (DHA)	22:6 n-3	5.02	15.0	30.0	50.2	75.0	99.7
Eicosatrienoic acid (IS)	20:3 n-3	-	-	-	-	-	-

Sample preparation started with measuring approximately 1 g of powdered seaweed in 10 mL of 50:50 v/v methanol/18 MΩ water. The solution went through three rounds of 30-min sonication, centrifuging, and supernatant collection. The collective supernatant was made up to 25 mL with 50:50 v/v methanol/18 MΩ water, resulting in a 40,000-ppm sample stock solution of each species. All solutions, including the samples, standards, and buffer were filtered through 0.45 µm Nylon syringe filters (Canadian Life Science Inc., ON, Canada) and stored at 4°C.

Statistical Analysis

Rapid ^{1}H NMR n-6/n-3 Ratio Statistical Analysis - Drying Technique Comparison

Statistics Canada considers a coefficient of variation (CV) between 0% and 16.5% acceptable, between 16.6% and 33.3% marginal and greater than 33.3% unacceptable (Statistics Canada, 2025). These acceptable and unacceptable value ranges were used when evaluating the validity of results obtained from the n-6/n-3 fatty acid ratio analyses conducted via ^{1}H NMR. First, the reproducibility and accuracy of n-6/n-3 fatty acid ratios in seaweed samples that underwent different drying methods was determined by assessing the standard deviation (SD) of fatty acid ratios from the mean and the resulting CV for each species run in triplicate. These analyses were conducted via Microsoft Excel, and are presented in **Appendix A.1**, **Table A.1.4.**

Based on the Statistics Canada guidelines, the variation of n-6/n-3 fatty acids in *M. japonica* for both drying methods was too high to be accepted during the initial analyses. Additionally, the variation for OV sugar kelp at ~85.5% is over the accepted range, while the CV for DH sugar kelp was considered marginal. Due to the CV results, the *M. japonica* n-6/n-3 fatty acid ratios will not be considered in subsequent analyses. Additionally, as the OV sugar kelp results had a variation over the accepted range, a subsequent analysis was conducted looking only at dehydrated samples. Giant kelp, which contained the overall highest ratios of n-6/n-3 fatty acids produced low-variability results within the accepted range and therefore can be retained. The lower variability may be attributed to overall higher quantity of fatty acids in the species and therefore the production of a more stable ^{1}H NMR spectra baseline allowing for accurate integration.

A two-tailed paired t-test was applied to the giant kelp data to determine if drying methods caused significantly different n-6/n-3 ratio results within a species**.** As the CV was not accepted for *M. japonica* and was found either unacceptable or marginal for sugar kelp, a t-test was not applied to this data. The p-value for giant kelp generated by the t-test was 0.03 ($p<0.05$). Based on this result, the null hypothesis stating that drying methods cause no significant difference in n-6/n-3 fatty acid ratios within a seaweed species was rejected. As such, the n-6/n-3 ratios for oven dried giant kelp samples compared to dehydrated giant kelp samples were found to be significantly different.

Rapid ^{1}H NMR n-6/n-3 Ratio Statistical Analysis – Dehydrated Samples & Method Validation

For secondary n-6/n-3 fatty acid ratio results obtained from optimized dehydrated samples analysed in triplicate on three separate occasions, an intra-day and inter-day statistical analysis was conducted to assess the repeatability and validity of the ^{1}H NMR method. The intra-day analysis showed that all triplicate samples within each individual trial, aside from sugar kelp samples run on trial day 1, had a CV of < 16.5% and therefore were deemed acceptable. The set of sugar kelp samples from the first trial day produced a CV of 21.0% and was considered a marginable result. Inter-day analysis assessed method validity by looking at the CV across all nine replicates (3 samples per species x 3 days). Both sugar kelp and giant kelp n-6/n-3 fatty acid ratios were shown to have acceptable variation and therefore support the validity and repeatability of ^{1}H NMR methodology. Both the intra-day and inter-day analyses results are provided in **Appendix B.1**, **Table B.1.5**, and **Table B.1.6**, respectively.

Fatty Acid Determination by CE – Statistical Analysis

The pilot CE trials were conducted to support and expand on ^{1}H NMR omega-6 and omega-3 findings, however, the results of the trials produced considerably variable results. For results from a CE fatty acid analysis to be manipulated statistically, a thorough method optimization and method validation would be necessary. As such, the raw results of this pilot trial will be presented directly in **Section 3.0** in hopes to support future method optimization and investigation.

RESULTS

Rapid Determination of n-6/n-3 Ratio by ^{1}H NMR

A total of 18 samples using two different drying techniques were analysed for n-6/n-3 ratios using the aforementioned methodology developed by Prema et el. (2016). Then, using the optimal drying method that resulted clearer spectra, a secondary analysis was performed using dehydrated samples of each species run in triplicate on three separate days for an inter- vs. intra-day comparison analysis. Prior to integration, all spectra were subjected to an automatic phase correction and referenced to 7.26 ppm for residual protons in $CDCl_3$. The results of the average n-6/n-3 ratio analysis categorized by species as well as drying technique (oven-dried (OV) and dehydrated (DH)) are found in Table 3.2, and the average results for the subsequently prepared optimized dehydrated samples are presented in Table 3.3. Figure 3.4 depicts how spectra were integrated following the method outlined by Prema et al. (2016), showing an example of LA and ALA regions for giant kelp (OV). Full results of all samples can be found in **Appendix B.1**, **Table B.1.7,** and **Table B.1.8.**

Table 3.2 *Average ^{1}H NMR n-6/n-3 Fatty Acid Ratios of Oven-Dried Seaweeds Compared to Dehydrated Seaweeds*

Drying Method	Sugar Kelp	Giant Kelp
DH mean	0.17	0.31
OV mean	0.13	0.36

Table 3.3 *Secondary Analysis of n-6/n-3 Fatty Acid Ratios Results for Optimized Dehydrated Samples per Trial Day via ^{1}H NMR*

Trial Day	Sugar Kelp	Giant Kelp
1	0.24	0.32
2	0.27	0.32
3	0.23	0.37

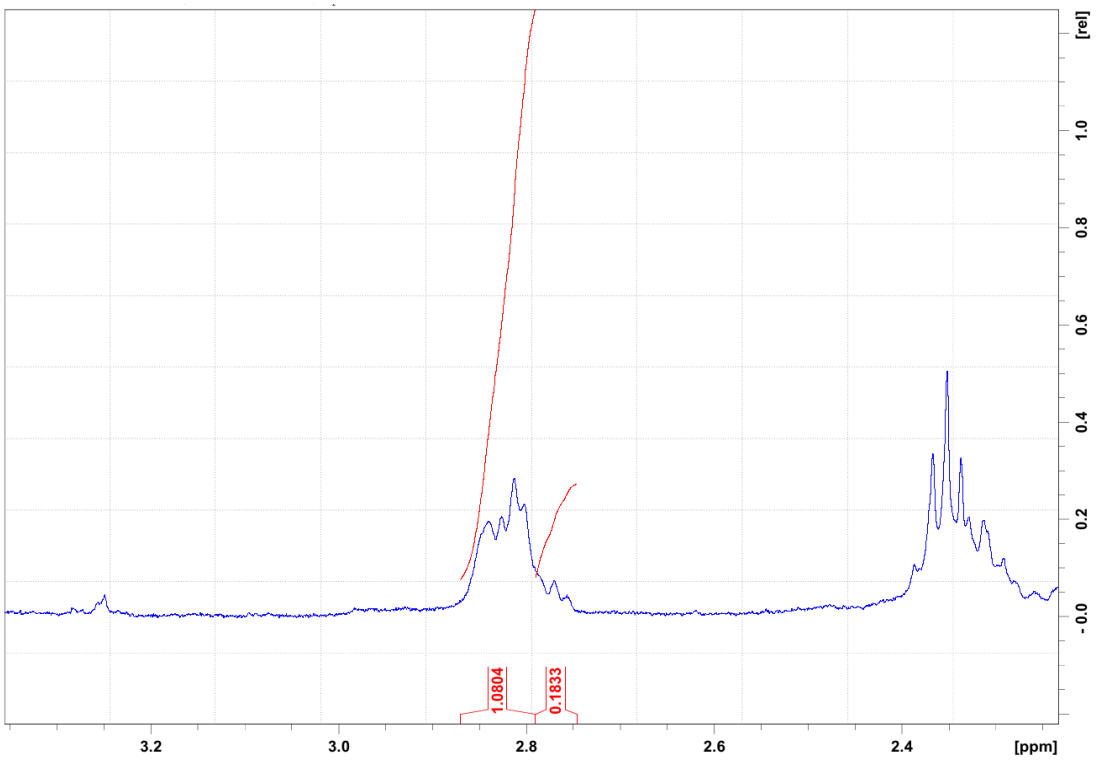


Figure 3.4 ^{1}H NMR spectra of LA and ALA extracted from giant kelp (Trial 1 - 500 MHz, 256 scans, $CDCl_3$; 18:2n-6 (LA) at 2.74 – 2.79ppm; 18:3n-3 (ALA) at 2.79 – 2.81 ppm).

The initial analysis comparing the two drying techniques as well as the species indicated that n-6/n-3 fatty acid ratios are lower in sugar kelp compared to giant kelp samples when dried by either method. In addition, results from the statistical analysis revealed that the difference in drying methods used, either dried in a mechanical oven at approximately 105 °C or dehydrated at approximately 40 °C, produced statistically different n-6/n-3 ratios within a species. As the dehydrated samples from the initial analysis resulted in a more optimal baseline and clearer spectra, dehydrated samples were utilized in a secondary "optimized" analysis to assess inter- vs. intra-day variation in results.

As Table 3.3 depicts, the average n-6/n-3 fatty acid ratios found during the optimized secondary analysis were lower in sugar kelp than in giant kelp per trial day. The variation of results per trial day were found to be acceptable for both giant kelp and sugar kelp, excluding the first trial day for sugar kelp which was found to be marginable. The *M. japonica* results of these analyses consistently produced unreliable results and as such, were deemed inaccurate and non-useable, and were not part of the statistical analysis. Overall, in both sets

of analyses, the *M. japonica* samples produced a noisier baseline that was difficult to integrate and therefore is likely indicative of very low omega-6 and omega-3 quantities.

Determination of Oleic and Total Unsaturated Fatty Acids via Analysis of ^{1}H NMR

Oleic acid and total unsaturated fatty acids were calculated using a methodology outlined by Siudem et al. (2022) for the initial ^{1}H NMR results that compared drying methods. As with the n-6/n-3 ratio analysis, the ^{1}H NMR spectra were subjected to an automatic phase correction and referenced to 7.26 ppm for residual protons in $CDCl_3$ prior to integration. Due to the low ratio of omega-6 to omega-3, the integration of corresponding peaks to determine oleic acid and total unsaturated fatty acid content in samples were rendered inaccurate and produced negative results. The full results from the FA profile calculations can be found in **Appendix A.1, Table A.1.9.**

Fatty Acid Determination by CE

The pilot trials for fatty acid analysis using the CE method outlined by Soliman et al. (2013) produced variable results for seaweed samples. Potentially useable results were produced only for SFAs lauric acid and myristic acid in which the calibration curve (produced with IS peak consideration) R^2 values indicated marginal fit (**Appendix A.2**, **Graph's A.2.1** and **A.2.2**, and **Table's A.2.2** and **A.2.3**). Based on the calibrations curve equations of the line, the concentration of lauric acid and myristic acid in the three seaweed species was calculated (Table 2.4, below).

Table 3.4 *Concentration (ppm) of Lauric Acid and Myristic Acid in Seaweeds Using CE*

SFA	**Sugar Kelp**	**Giant Kelp**	***M. japonica***
Lauric Acid	133.3 ppm	97.8 ppm	75.1 ppm
Myristic Acid	6.31 ppm	29.6 ppm	3.55 ppm

The results for this pilot trial analysis indicated that sugar kelp had the highest concentration of lauric acid compared to the other two species, while giant kelp had the highest concentration of myristic acid. In both cases, *M. japonica* had the lowest concentration of

lauric acid and myristic acid. Although quantifiable electropherograms were produced from this analysis, it should be noted that calibration curve R^2 values for lauric acid and myristic acid standards were 0.7150 and 0.7064, respectively. As such, the concentrations produced using the resulting equations of the line should be considered a rough estimate that would be better approximated with improved calibration.

The calibration electropherograms produced by the other SFAs and all PUFAs were unquantifiable and therefore could not be used to determine concentrations in seaweed samples. This result could be due to incompatible buffer solution, contamination of standard and sample vials from instrumentation carry-over, or lack of fatty acid extraction in the medium used. The present study did not divulge alternative buffers or extraction methodologies; however, these results are provided in hopes to support and supplement future instrumental optimization for fatty acid analysis.

DISCUSSION

This research aimed to evaluate the omega-6 to omega-3 (n-6/n-3) fatty acid ratios of three local seaweed species – giant kelp, sugar kelp, and *M. japonica* by ^{1}H NMR spectroscopy. In addition, pilot trials were conducted to assess the ability of CE to be used for fatty acid analysis using a pre-validated method developed by Soliman et al. (2013). The results provide insight into both the methodological suitability of ^{1}H NMR spectroscopy for seaweed fatty acid analysis, and the nutritional potential of these species for cattle feed applications.

Of the three seaweed species analysed, giant kelp consistently exhibited the highest n-6/n-3 ratios, followed by sugar kelp, while *M. japonica* produced unquantifiable and variable ratios due to overall low concentrations of omega-6s and omega-3s. The n-6/n-3 fatty acid ratios of giant kelp and sugar kelp were both within a favourable range for the support of animal health if incorporated into animal feed. Ratios present in sugar kelp and giant kelp aligned with previous research that suggested ratios below 5:1 are optimal for reducing inflammatory responses and in turn, improve the health of livestock (Rocha et al., 2021; Fabjanowska et al., 2023).

Although giant kelp was found to have higher n-6/n-3 ratios than sugar kelp for both dehydrated and oven-dried samples, ratios were still very low and considered favourable for health outcomes. For example, it was found that milk production was increased, and ovarian

function was improved in cattle fed “low” (2.5:1) to “moderate” (4.5:1) n-6/n-3 compared to cows fed a higher ratio of n-6/n-3 (Najafi et al., 2020). Similarly, Greco et al. (2015) compared diets with n-6/n-3 ratios of ~3:1, ~4:1, and ~5:1, in early lactation cows, and found that the ~3:1 ratio diet resulted in the highest milk output, the lowest inflammatory markers, and the highest dry-matter intake. As the n-6/n-3 ratio in both sugar kelp and giant kelp were found to be very low, both species would contribute to health benefits in cattle. This also suggests that both brown seaweed species, particularly giant kelp (due to its higher content of PUFAs in general), could serve as promising functional feed additives capable of contributing to beneficial fatty acid profiles to ruminant diets.

In contrast to the brown seaweeds, *M. japonica*, the one red seaweed species analysed, yielded low and sometimes negative n-6/n-3 values. These results are likely due to the combination of low overall fatty acid content in the species and limitations in spectral resolution. A noisier baseline was observed in *M. japonica* spectra compared to the spectra produced by the other species and further highlights the sensitivity limitations of the ^{1}H NMR method for fatty acid detection in seaweed matrices. Given the unreliability of the results, *M. japonica* was excluded from further statistical analysis.

The comparison between oven-dried and dehydrated giant kelp samples revealed that drying method significantly influenced omega-6 and omega-3 fatty acid detection. Dehydrated samples produced cleaner and more interpretable ^{1}H NMR spectra with less baseline noise, which enabled more accurate integration and quantification. This is consistent with the findings by Daley et al. (2010) that noted high temperature drying can degrade or alter lipid profiles in certain biological materials. Statistical analysis confirmed that drying method had a significant effect on the n-6/n-3 fatty acid ratios in giant kelp ($p = 0.03$), suggesting that methodological consistency is critical for reproducible fatty acid analysis. Based on this result, future lipid analyses of seaweeds may want to prioritize low-temperature drying methods like dehydration instead of high-temperature oven drying to preserve fatty acid integrity and improve spectral clarity.

Statistical evaluation of ^{1}H NMR detected n-6/n-3 ratios demonstrated acceptable reproducibility and precision for giant kelp and sugar kelp, with coefficients of variation (CVs) falling within or near acceptable ranges set by Statistics Canada (2025). Inter- vs.

intra-day variation in dehydrated samples was low, particularly in giant kelp, affirming the reliability of the NMR method for this purpose. Higher variability observed in sugar kelp and *M. japonica* emphasizes the limitations of this methodology if the sample material has overall low concentrations of fatty acids. The rapid analysis time and low solvent consumption associated with ^{1}H NMR positions the instrument as a valuable tool for screening n-6/n-3 ratios in seaweeds. However, the method's limitations in detecting very low fatty acid concentrations must be acknowledged, and extraction processes must be optimized for more favourable spectral outcomes.

In addition to ^{1}H NMR spectroscopy, this analyses briefly investigated the use of capillary electrophoresis (CE) as an alternative method for the separation and detection of saturated and polyunsaturated fatty acids (SFAs and PUFAs) in seaweed extracts. The method used was based on the work of Soliman et al. (2013), which demonstrated successful separation of dietary omega-3 and omega-6 fatty acids using a micellar electrokinetic chromatography (MEKC) buffer. Standard mixtures of 11 fatty acids were prepared and run under the CE conditions outlined by Soliman et al. (2013) to assess method performance and applicability to seaweed analysis.

Calibration curves were only successfully generated for two fatty acids, lauric acid and myristic acid, both SFAs, and even so, produced marginal R^2 values. The production of inconsistent electropherograms was likely due to issues such as peak broadening, poor resolution, and suspected carry-over, particularly in PUFA runs. Additional trials changing aspects of the buffer matrix, voltage, and separation time may improve this analysis and make it a viable option for fatty acid separation and quantification in seaweeds. Despite the challenges in the present study, the CE method has potential as an alternative to more traditional analysis techniques like GC-MS for fatty acid analysis. Future work should focus on method optimization specific to seaweed matrices, including sample clean-up steps and buffer refinement to optimize separation and reproducibility.

The presence of favourable n-6/n-3 ratios in giant kelp and sugar kelp suggests a potential application of these species as cattle feed additive for the aim of improving animal health and enhancing the nutritional quality of animal by-products. Prior research has shown that omega-3 enriched diets can increase the content of beneficial PUFAs in beef and milk which

is beneficial for consumers, improve the reproductive performance of the cattle, and reduce inflammation in the livestock (Daley et al., 2010; Fabjanowska et al., 2023). In addition, fatty acid supplementation has been linked to enteric methane reduction in cattle, with LC-PUFAs such as linolenic acid associated with reductions of up to 15 % (Patra, 2013; Grainger & Beauchemin, 2011).

Although the present study did not directly measure methane production and suppression, the identification of fatty acid-abundant seaweeds, particularly with high omega-3 content and low n-6/n-3 ratios, offers a promising starting point for integrating sea-based nutritional solutions in the agricultural system while using a local and abundant source. Further research linking these fatty acid profiles to rumen fermentation dynamics and methane suppression is warranted.

Limitations and Future Directions

This study provides a foundation for fatty acid analysis of seaweeds using a rapid and low-solvent method, but several limitations should be noted. Sample size was limited to three species, and fatty acid composition was not validated against traditional GC-MS methods. Additionally, *M. japonica* presented challenges in both detection and quantification, highlighting the need for improved extraction or concentration techniques for low-lipid species.

Future work should include:

- Expansion to additional seaweed species, particularly other brown seaweeds that are likely to be high in omega-3s;
- Cross-validation of ^{1}H NMR findings with GC-MS or LC-MS for improved accuracy;
- *In vivo* trials assessing animal health outcomes and methane emissions following the supplementation with seaweed-derived fatty acids.

CONCLUSION

These analyses demonstrated that low-solvent ^{1}H NMR is a viable method for rapidly detecting and estimating the omega-6 to omega-3 (n-6/n-3) ratios in seaweed samples, particularly in species with higher fatty acid content such as giant kelp and sugar kelp. Dehydration was identified as the preferred drying method, yielding clearer spectra and influencing n-6/n-3 fatty acid ratio quantification in giant kelp. While the pilot trials for the quantification of SFAs and PUFAs using CE methods proved inconclusive at this time, these initial findings highlight the potential of brown seaweeds to be functional feed additives. Their favourable fatty acid ratios, coupled with their abundance and importance as local marine biomass support further investigation into their role in improving cattle nutrition and reducing enteric methane emissions.

LITERATURE CITED

Bradbury, K. E., & Appleby, P. N. (2019). Dietary fats and cardiometabolic disease: Mechanisms and effects on risk factors and outcomes. *Nature Reviews Cardiology, 16*(4), 213–229. https://doi.org/10.1038/s41569-019-0206-1

Daley, C. A., Abbott, A., Doyle, P. S., Nader, G. A., & Larson, S. (2010). A review of fatty acid profiles and antioxidant content in grass-fed and grain-fed beef. *Nutrition Journal*, *9*(1), 10. https://doi.org/10.1186/1475-2891-9-10

Duarte, C. M., Wu, J., Xiao, X., Bruhn, A., & Krause-Jensen, D. (2017). Can seaweed farming play a role in climate change mitigation and adaptation? *Frontiers in Marine Science*, *4*. https://doi.org/10.3389/fmars.2017.00100

Fabjanowska, J., Kowalczuk-Vasilev, E., Klebaniuk, R., Milewski, S., & Gümüş, H. (2023). N-3 polyunsaturated fatty acids as a nutritional support of the reproductive and immune system of cattle—A review. *Animals*, *13*(22), 3589. https://doi.org/10.3390/ani13223589

Fernand, F., Israel, A., Skjermo, J., Wichard, T., Timmermans, K. R., & Golberg, A. (2017). Offshore macroalgae biomass for bioenergy production: Environmental aspects, technological achievements and challenges. *Renewable and Sustainable Energy Reviews*, *75*, 35–45. https://doi.org/10.1016/j.rser.2016.10.046

Grainger, C., & Beauchemin, K. A. (2011). Can enteric methane emissions from ruminants be lowered without lowering their production? *Animal Feed Science and Technology*, *166–167*, 308–320. https://doi.org/10.1016/j.anifeedsci.2011.04.021

Gunstone F.D. (2001). *Fatty acid and lipid chemistry*. Springer.

Gurr, M. I., Harwood, J. L., Frayn, K. N., Murphy, D. J., & Michell, R. H. (2016). *Lipid biochemistry* (6th ed.). Oxford, UK: Wiley-Blackwell.

Kapoor, B., Kapoor, D., Gautam, S., Singh, R., & Bhardwaj, S. (2021). Dietary polyunsaturated fatty acids (Pufas): Uses and potential health benefits. *Current Nutrition Reports*, *10*(3), 232–242. https://doi.org/10.1007/s13668-021-00363-3

Liu, Y., Shen, N., Xin, H., Yu, L., Xu, Q., & Cui, Y. (2023). Unsaturated fatty acids in natural edible resources, a systematic review of classification, resources, biosynthesis, biological activities and application. *Food Bioscience*, *53*, 102790. https://doi.org/10.1016/j.fbio.2023.102790

Lunn, J. (2007). Monounsaturates in the diet. *Nutrition Bulletin*, *32*(4), 378–391. https://doi.org/10.1111/j.1467-3010.2007.00669.x

Martínez-Pinilla, E., Oñatibia-Astibia, A., & Franco, R. (2016). The relevance of bioactive lipids in human nutrition and health. *Food & Function, 7*(12), 2432–2449. https://doi.org/10.1039/C6FO00367C

McDonald, P., Edwards, R. A., Greenhalgh, J. F. D., Morgan, C. A., Sinclair, L. A., & Wilkinson, R. G. (2011). *Animal nutrition* (7th ed.). Harlow, UK: Pearson Education.

National Research Council. (1989). *Diet and health: Implications for reducing chronic disease risk* (Executive Summary). Washington, DC: National Academies Press. https://doi.org/10.17226/1222

Nelson, D. L., & Cox, M. M. (2017). *Lehninger principles of biochemistry* (7th ed.). New York, NY: W. H. Freeman and Company

Patra, A. K. (2013). The effect of dietary fats on methane emissions, and its other effects on digestibility, rumen fermentation and lactation performance in cattle: A meta-analysis. *Livestock Science*, *155*(2–3), 244–254. https://doi.org/10.1016/j.livsci.2013.05.023

Prema, D., Jensen, J., Pilfold, J. L., Turner, T. D., Donkor, K. K., Cinel, B., & Church, J. S. (2016). Rapid determination of *n* -6 and *n* -3 fatty acid ratios in cereal grains and forages by^{1} H NMR spectroscopy. *Canadian Journal of Plant Science*, *96*(5), 730–733. https://doi.org/10.1139/cjps-2015-0302

Rocha, C. P., Pacheco, D., Cotas, J., Marques, J. C., Pereira, L., & Gonçalves, A. M. M. (2021). Seaweeds as valuable sources of essential fatty acids for human nutrition. *International Journal of Environmental Research and Public Health*, *18*(9), 4968. https://doi.org/10.3390/ijerph18094968

Sacks, F. M., Lichtenstein, A. H., Wu, J. H., Appel, L. J., Creager, M. A., Kris-Etherton, P. M., … Rudel, L. (2017). *Dietary fats and cardiovascular disease: A Presidential Advisory from the American Heart Association.* Circulation, 136(3), e1–e23. https://doi.org/10.1161/CIR.0000000000000510

Schmid, M., Guihéneuf, F., & Stengel, D. B. (2014). Fatty acid contents and profiles of 16 macroalgae collected from the Irish Coast at two seasons. *Journal of Applied Phycology*, *26*(1), 451–463. https://doi.org/10.1007/s10811-013-0132-2

Silva, A. M. S., Silva, C. M., Silva, T. A., & Silva, J. R. (2020). *Omega fatty acids and their role in human health: A comprehensive review*. *Journal of Lipid Research*, 61(8), 1023–1039.

Soliman, L. C., Donkor, K. K., Church, J. S., Cinel, B., Prema, D., & Dugan, M. E. R. (2013). Separation of dietary omega-3 and omega-6 fatty acids in food by capillary electrophoresis. *Journal of Separation Science*, *36*(20), 3440–3448. https://doi.org/10.1002/jssc.201300738

Types of fat. (2014, June 9). *The Nutrition Source*. https://nutritionsource.hsph.harvard.edu/what-should-you-eat/fats-and-cholesterol/types-of-fat/

CHAPTER 3: PHLOROTANNIN ANALYSIS

ABSTRACT

Phlorotannins are a type of polyphenol unique to brown seaweeds that have gained attention for their potential to mitigate enteric methane emissions and improve ruminant nutrition. This analysis aimed to identify and quantify phlorotannins in two locally abundant species in British Columbia, giant kelp (*Macrocystis pyrifera*) and sugar kelp (*Saccharina latissima*), using three analytical methods: ^{1}H nuclear magnetic resonance (NMR), Folin-Ciocalteu (F-C) assay, and capillary electrophoresis (CE). F-C assay was able to provide a general estimate of total polyphenolic content, with sugar kelp showing slightly higher mean concentrations than giant kelp, however; high variability (% RSD > 39 %) limited the methods reliability. Phloroglucinol aromatic H peaks were successfully identified using ^{1}H NMR near 8.4 ppm, however, quantification was impeded by small signals resulting from high insolubility in standard NMR solvents. CE trials showed consistent retention times for phloroglucinol standards but failed to yield linear calibration curves for quantification. These results highlight both the promise and current limitations of low-solvent analytical techniques for phlorotannin detection. Further refinement and standardization are necessary to enable accurate characterization of phlorotannins in brown seaweeds used for sustainable cattle feed.

INTRODUCTION

Phlorotannins are a type of bioactive polyphenol uniquely produced by brown macroalgae that are synthesized at the cellular level via the acetate-malonate (polyketide) pathway and assembled within the Golgi apparatus (De Bhowmick et al., 2023; Meng et al., 2021). Structurally, phlorotannins are oligomeric or polymeric derivatives of phloroglucinol (1,3, 5-trihydroxybenzene) polymerised monomer units connected by aryl (C-C), ether (C-O-C), or mixed bonds (Vissers et al., 2017). The type of bond between monomers determines the classification of the phlorotannin into one of four main classes: fuhalols and phlorethols (ether linkages), fucols (phenyl linkages), fucophlorethols (ether and phenyl), and eckols/carmalols (dibenzo-dioxin linkages) (Imbs et al., 2018).

Phlorotannins are biologically active, playing an important role throughout algal development and have various ecological functions such as structural reinforcement, antioxidant defense, and protection against microbial and/or herbivory colonization (Li et al., 2017; Meng et al., 2021). The content of phlorotannins within macroalgal tissue can fluctuate greatly depending on the species of seaweed, habitat, light availability, seasonal changes, salinity, and depth grown (De Bhowmick et al., 2023). Among seaweed varieties, brown seaweeds have the highest phlorotannin content with reported values ranging from 20 to 140 g/kg dry weight (Min et al., 2021). A depiction of common phlorotannin structures, including the monomer unit phloroglucinol, as well as classes phlorethols, fucols, and eckols, are shown in Figure 3.1 (Ford et al., 2019).

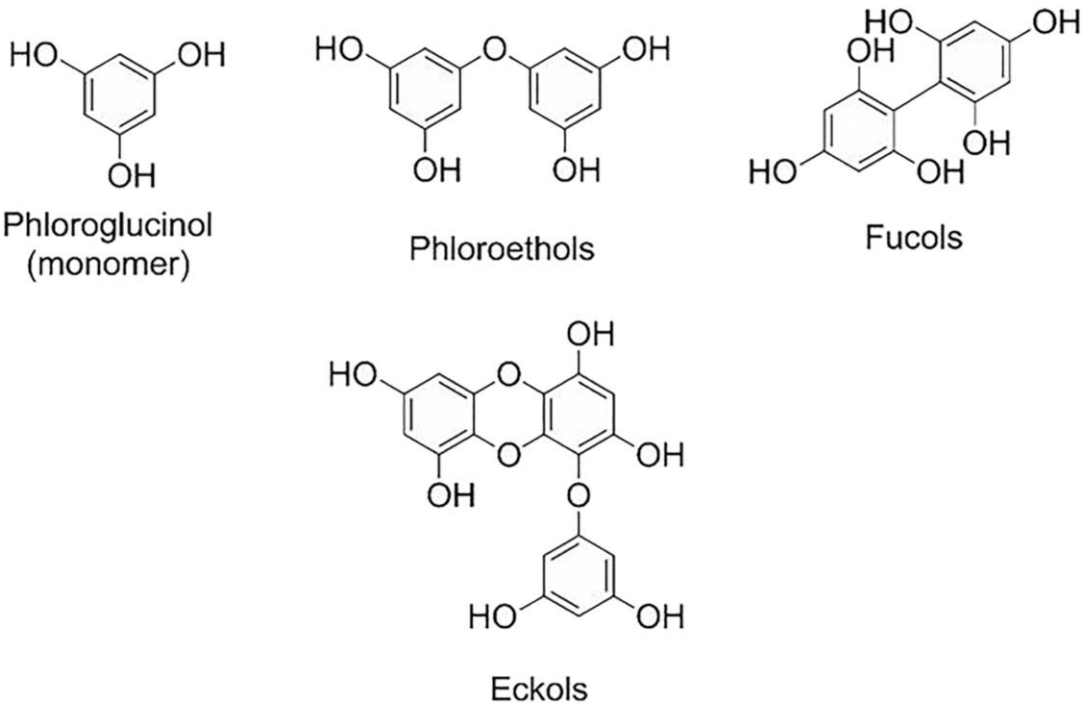


Figure 3.1 Examples of Common Phlorotannin Structures Including Phloroglucinol, Phloroetholds, Fucols, and Eckols (Ford et al., 2019).

Recently, phlorotannins have gained attention within the scientific community for their potential applications across food, pharmaceutical, environmental, and agricultural sectors. When applied to livestock feed, particularly in ruminants, phlorotannins have shown promise for their ability to improve protein utilization during the digestive process, as well as reduce enteric methane emissions (Abbott et al., 2023). Due to their abundance of hydroxyl groups, phenolic rings, and ether linkages, phlorotannins have a high capacity to bind to dietary proteins and in turn, protect the proteins from hydrolysis during enteric fermentation and promote post-ruminal absorption (Vissers et al., 2018). As a protective mechanism, the protein binding capacity of phlorotannins promotes nitrogen retention and modifies rumen fermentation patterns, contributing to reductions in methane production (Mueller-Harvy et al., 2019; Barry & Manley, 1984).

Vissers et al. (2018) demonstrated this effect *in vitro* where phlorotannin supplementation in cattle-feed at 40 g/kg led to a reduction in methane emissions from 24.5 mL/g to 15.2 mL/g of organic matter without compromising the production of total ruminal volatile fatty acids (VFAs). The findings from this study suggest that phlorotannins may alter hydrogen

utilization in the rumen or reduce the abundance of methanogenic archaea (Vissers et al., 2018). As brown seaweeds appear to typically produce negligible amounts of bromoform, a known methane-supressing compound, the possibility for phlorotannins to reduce methane production is becoming increasingly popular to investigate (Machmüller et al., 2022; Roque et al., 2021). Phlorotannin research is of particular interest and popularity in regions where bromoform-rich species are not predominant. Although further *in vivo* studies are needed, the potential of phlorotannins to facilitate the enhancement of ruminant nutrition and suppression of enteric methane supports their potential use as a sustainable feed additive.

Despite potential benefits, the extraction and quantification of phlorotannins from brown seaweeds are challenging due to their structural diversity, high molecular weight, and sensitivity to environmental conditions (Kumar et al., 2022). Phlorotannins can possess a variety of molecular weights from simple phloroglucinol units (126 Da) to large polymers (650 kDa), resulting in complex mixtures that can be difficult to separate and purify (Kumar et al., 2022). The absence of standardized extraction methodologies leads to significant variability in phlorotannin yield and is heavily dependent on factors including solvent type, temperature, drying method, and extraction duration (Meng et al., 2021; Ivane et al., 2025). These compounds are also prone to oxidation and degradation during processing, making analytical accuracy and consistency difficult (Ivane et al., 2025). Due to the complications associated with the extraction of phlorotannins, rapid and selective detection methods are increasingly sought.

Typical extraction techniques include complex solvent systems like solid-liquid extraction (SLE), with an additional purification step for the isolation of polyphenols separated from other components in the mixture (Pérez-Jiménez & Saura-Calixto, 2013). However, the development of rapid extraction techniques may be a useful tool as the utilization of phlorotannins becomes increasingly popular. To enable for a rapid and practical analysis, this study employed proton nuclear magnetic resonance (^{1}H NMR) to detect phloroglucinol (the monomer unit of phlorotannins) in two local brown seaweed species. Folin-Ciocalteu (F-C) assay was used to estimate the total polyphenol content of each species as a broad initial indicator of total polyphenols. Finally, capillary electrophoresis (CE) was also explored as a

complementary technique to assess the separation and potential quantification of phloroglucinol.

F-C assay is a colorimetric method that uses a mixture of tungsten and molybdate (F-C reagent) and involves the electron transfer from phenolic compounds to phosphomolybdic/phosphotungstic acid complexes (Li et al., 2017). The resulting blue complex that is formed can be quantified spectrophotometrically at 700 nm (Singleton et al., 1999). The reduction of the F-C reagent by polyphenols is depicted in Figure 3.2 (Ford et al., 2019). Although the method is nonspecific as it reacts with a variety of reducing compounds and often leads to an overestimation of polyphenolics, F-C assay is the current widely accepted method for evaluating total phenolic content in plant and algal materials (Rajauria et al., 2016; Ford et al., 2019). In this study, F-C assay served as a comparative baseline for the alternative analyses and provided a general understanding of polyphenolic abundance across species.

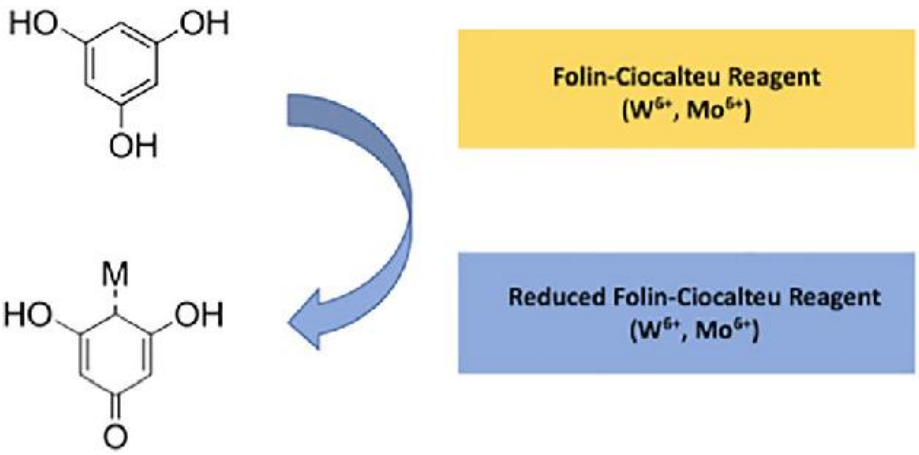


Figure 3.2 Reduction of F-C Reagent by Polyphenols (Ford et al., 2019)

The use of ^{1}H NMR is advantageous as it allows for the non-destructive and rapid analysis of complex organic materials, often without the involved solvent-intensive sample preparation common in other analytical techniques (Li et al., 2017). The ^{1}H nuclear spin response to electromagnetic radiation in a magnetic field in the NMR instrument can allow for the identification of chemical compounds or even the determination of chemical structure (Raja & Barron, 2016). As reported by Gager et al. (2020), the phenolic OH hydrogen peak of the

polyphenols typically appear between 5.5 - 6.5 ppm on a ^{1}H NMR spectrum (Figure 3.3). The location of polyphenol peaks will vary depending on what NMR solvent is used.

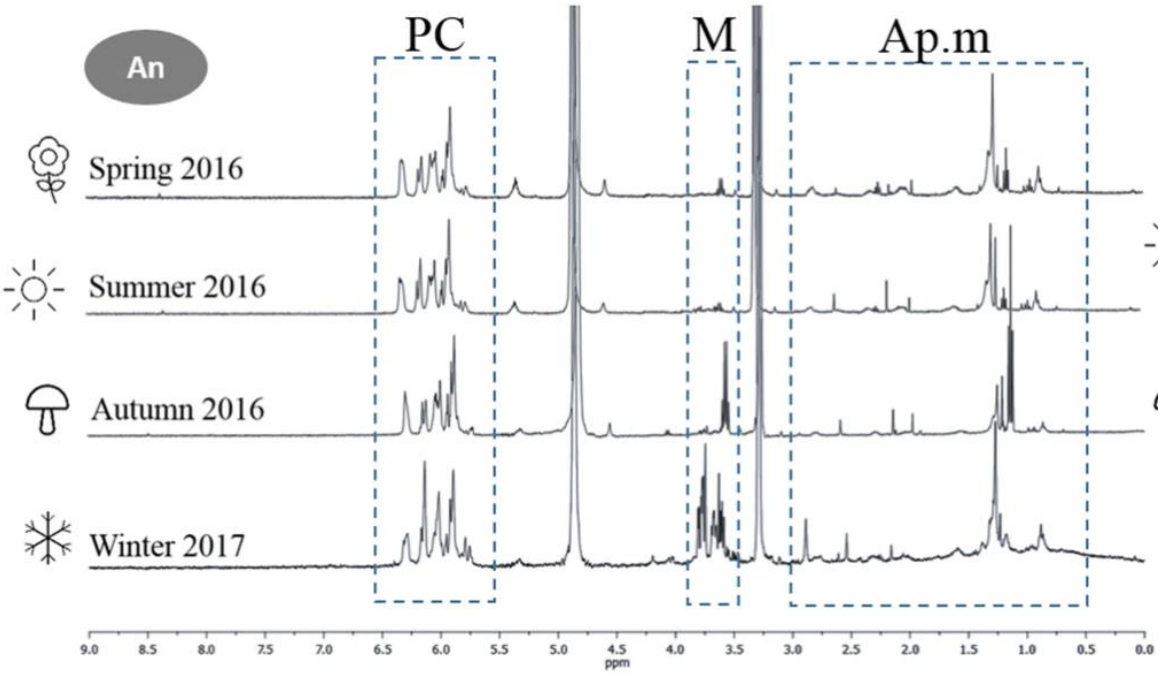


Figure 3.3 ^{1}H NMR Spectra of Brown Seaweed Species Ascophyllum nodosum (An) from the Ethyl Acetate Fraction (Gager et al., 2020)

The objective of this work is to evaluate the utilization of different analysis techniques for the detection of phlorotannins in two brown seaweed species, sugar kelp and giant kelp, with the aim of assessing their potential as sustainable cattle feed additives for enteric methane mitigation and nutritional enhancement. For these trials, a combination of analytical approaches were tested and optimized, including F-C assay for total polyphenol content estimation, ^{1}H NMR for rapid detection of phloroglucinol, and CE for separation and detection of phloroglucinol.[1] By comparing results of these techniques and the relative phlorotannin content in each species, this study aimed to contribute to the development of efficient low-solvent analytical methodologies for the extraction and characterization of phlorotannins from macroalgal biomass.

[1] For CE analysis, *Mazzaella japonica* was also evaluated as samples had been collected at this point in the analyses, however, the species was not analysed using other techniques due to general low phlorotannin content.

MATERIALS AND METHODS

Biological Materials Sampling

The study species for this analysis included the investigation of two brown seaweed species, Macrocystis *pyrifera* (giant kelp) and *Saccharina latissima* (sugar kelp). Fresh seaweed samples were collected by Cascadia Seaweed Inc. (Cascadia) from their farm site in Clayoquot Sound B.C. (approximate location shown in Figure 3.4). Harvesting took place at approximately 17:00 on June 5, 2024. According to Cascadia's documentation, live specimens were cut from the stalk with a knife, and visible biofouling was removed. The cleaned samples were wrapped in damp towels and transported in coolers containing seawater. They were shipped via air transport to Kamloops, B.C., and were received by TRU on June 7, 2024.

Figure 3.4 Clayoquot Sound B.C. Approximate Location of Sugar Kelp and Giant Kelp Harvest (image created via iMap BC).

Phlorotannin content in *Mazzaella japonica M. japonica* was investigated as part of the CE trials. *M. Japonica* was hand collected by TRU as storm-cast from the beaches of Deep Bay, Vancouver Island, B.C. on August 26th, 2024. The fresh red seaweed samples were packed in coolers with ice and transported to the TRU laboratory over the subsequent days.

Seaweed Sample Storage and Preparation

Upon arrival at the TRU laboratory, all macroalgal samples were sorted by species, rinsed with deionized water to remove impurities, and stored in Ziploc bags at -80 ºC until further processing. Prior to analysis, the samples were removed from the freezer for drying. Samples were dried at low temperatures, by a vacuum chamber or by dehydration using a Hamilton Beach household dehydrator set at 40 ºC, until dry. Oven drying was not used for sample preparation as phlorotannins are known to degrade at temperatures exceeding 50 °C, and it should be noted that initial decline of content can occur above 25 °C. Following drying, all samples were ground to a fine powder using an electric mill grinder. The resulting powders were stored in Ziploc bags, categorized by species and kept at room temperature until analysis. Dried samples were prepared in small ~100 g batches at a time to ensure freshness.

Chemical Supplies

Methanol (HPLC grade, ≥99.9%) and phloroglucinol (>99%) were used in ^{1}H NMR, CE, and F-C Assay protocols and were obtained from Millipore Sigma Canada Ltd. (Oakville, Ontario, Canada). High-purity water (18 MΩ·cm), used throughout sample preparation and analysis, was produced using a Milli-Q purification system (Millipore, Billerica, MA, USA). For ^{1}H NMR analysis, deuterium oxide (D_2O) and deuterated dimethyl sulfoxide (DMSO-d_6) used for phlorotannin extraction and spectral acquisition was sourced from Millipore Sigma Canada Ltd. Folin-Ciocalteu phenol reagent consisting of phosphotungstic acid and phosphomolybdic acid used for spectrophotometric analysis of phenolic compounds was procured from Millipore Sigma Canada Ltd. Other reagents used in CE, including buffer components acetonitrile, tetraborate, and dodecyl sulfate (SDS) were acquired from Millipore Sigma, and 0.1 M and 1.0 M sodium hydroxide (NaOH) conditioning solutions were prepared in volumetric flasks using 18 MΩ water.

Phloroglucinol Determination by ^{1}H NMR

^{1}H NMR Instrumentation

^{1}H NMR spectra were acquired using a Bruker Ultrashield 500 MHz spectrometer (Bruker BioSpin, Rheinstetten, Germany) equipped with a proton-optimized inverse detection (PULPROG: zg30) probe. The instrument was operated at 298 K and controlled the TopSpin software platform (version 3.5 pl 7, Bruker Biospin), which was also used for spectral processing and integration. IconNMR (component of Topspin) automation software was also utilized for automated acquisitions of sample sets. Each spectrum was collected using 16 scans with a sweep width of 15 ppm and relaxation delay (d_1) of 1 second.

Identification of Phloroglucinol by ^{1}H NMR

The sample preparation for the initial identification of phloroglucinol using ^{1}H NMR largely followed the procedure outlined by Jegou et al. (2015), with the intention of optimizing such methodology for the simplification of the analysis. Initially, the solubility of phlorotannins in seaweeds were trialled in various NMR solvents including acetone-d_6, acetonitrile-d_3, $CDCl_3$, DMSO-d_6, and D_2O. From this initial trial, DMSO-d_6 and D_2O were found the most promising for phlorotannin detection and as such, the ability for ^{1}H NMR instrumentation to detect phloroglucinol in these two NMR solvents was evaluated. For this, a few phloroglucinol crystals were added to glass vials and dissolved in approximately 1 mL of either of D_2O or DMSO-d_6. Once dissolved, the mixtures were filtered through fibreglass filter paper into NMR tubes. The samples were analysed using a Bruker Ultrashield 500 MHz ^{1}H NMR over 16 scans. The phloroglucinol peaks were identified at 5.87 ppm (phenolic OH) and 8.34 ppm (aromatic H) in D_2O and at 5.67 ppm (phenolic OH) and 8.96 ppm (aromatic H) in DMSO-d_6. Once the evidence of a phloroglucinol peak was determined in each NMR solvent, D_2O was chosen for further analysis based on its availability, cost and ease of use as an NMR solvent for phlorotannin analysis.

Dried samples of the two seaweed species, sugar kelp and giant kelp, were weighed to approximately 200 mg using an analytical balance (see **Appendix B.1¸Table B.1** for exact weights). Each sample was transferred to a glass vial (wrapped in aluminum foil to prevent photodegradation) and 4 mL of 50:50 v/v methanol/18 ΩM water was added. Then, the

samples were placed into a Bransonic Ultrasonic Cleaner (50/60 Hz) for two successive 2-hour extractions. The two extracts were pooled into aluminum-covered round bottom flasks, and the solvent was removed via rotary evaporation to isolate the crude product. After this initial extraction, different solvents were tested for their ability dissolve phlorotannins in the crude extract. First, 2 mL of D_2O was added to round bottom flasks and a pipette was used to siphon approximately 700 µL of the solution and filtered through Kimwipe directly into NMR tubes for analysis. After the remaining D_2O was evaporated from the round bottom flasks under vacuum, the residual crude polyphenol product was re-dissolved with DMSO-d_6 and filtered via pipette filtration. Samples were then analysed using ^{1}H NMR spectroscopy to analyse phloroglucinol peaks in the respective samples.

Identification of Phloroglucinol by ^{1}H NMR using an Acidified Extraction Solution

The two seaweed species were also subjected to an acidified extraction process to evaluate the effectiveness of different solid-liquid extraction techniques for phlorotannins. For this, 500 mg of finely ground seaweed sample was weighed and extracted successively with 15 mL of 1:1 v/v methanol: H_2O acidified to pH 2 with 2 N HCl, followed by 15 mL of 7:3 v/v acetone: H_2O (acidified with 2N HCl to pH 2) for two hours at a time in a Bransonic Ultrasonic Cleaner (50/60 Hz). The supernatants from each successive extraction were pooled together in a round bottom flask and alcohol/water components were removed by rotary evaporation. The crude product was then redissolved into D_2O and filtered through a Kimwipe directly into an NMR tube for analysis. Samples were then analysed using ^{1}H NMR spectroscopy to analyse phloroglucinol peaks, spectra from this extraction were compared to the original, and non-acidic extraction process.

Confirmation of Polyphenol Presence by F-C Assay

Spectrophotometer Instrumentation

The Thermo Scientific (a division of Thermo Fisher Scientific Inc.) GENESYS 20 visible spectrophotometer (Ottawa, Ontario, Canada) was used to generate calibration curves and quantify total polyphenolic content in the seaweed samples. The instrument utilizes a 4-step measurement process in which the wavelength selected for the analysis was 700 nm, the

mode used was Abs (absorbance), blanks were set to 0 Abs, and samples were inserted and absorbances were observed.

F-C Assay Methodology

Due to the trial basis of phloroglucinol identification using ^{1}H NMR in this study, the confirmation of polyphenol presence in the two brown macroalgae species was necessary. As such, the F-C assay method was employed to determine the total polyphenol content of the two seaweed species. The F-C reagent consists of phosphomolybdic and phosphotungstic acid that appear yellow in their oxidized state and form a blue complex when in the presence of polyphenols. The intensity of the blue is directly proportional to the polyphenolic content of the sample measured spectrophotometrically at 700 nm.

F-C Assay Analysis and Optimization

As the formation of the blue complex develops over time, the optimal development time prior to reading the absorbance spectrophotometrically was tested. For the F-C assay analyses, only dehydrated sugar kelp and giant kelp samples were used. Sample preparation began by weighing approximately 0.50 g of ground samples of each species (**Appendix B.2**, **Table B.2.1**) using an analytical balance and placing them in a 50 mL beaker with 40 mL of deionized H_2O for extraction. The solution was stirred and left to extract while the standards were prepared (approximately 1 hour). Once extraction was complete, the two sample solutions were filtered through 45 μm filter paper into beakers to separate the solid seaweed grounds from the extraction solution.

For standards preparation, a 500 ppm phloroglucinol stock solution was first prepared by dissolving ~ 0.050 g of phloroglucinol in 100 mL of deionized H_2O in a volumetric flask. Then, five standard phloroglucinol solutions of concentrations varying approximately 0.05 – 0.25 mg/mL were prepared, as well as sample solutions, by pipetting specific volumes of phloroglucinol stock solution, F-C reagent, and deionized water into volumetric flasks, as stated in **Appendix B.2**, **Table B.2.2**.

Once prepared, the five standard solutions and two samples were left to develop for 2, 4, and 6 hours, with absorbances recorded at each time interval. Full results of the time trials are

presented in **Appendix B.2, Table B.2.3.** Based on the time optimization results, it was determined that two hours of colour development yielded calibration curves with the highest R^2 value and therefore the most accurate quantification of polyphenolic quantity in seaweed samples and as such, the two-hour development experiment was performed two more times for an evaluation of the methods consistency and reliability. For the three two-hour development trials, total polyphenol content was calculated using the line equations from the resulting calibration curves and a dilution factor of 20 (10 mL/ 0.50 mL) for each species.

Capillary Electrophoresis for Phlorotannin Determination

In addition to ^{1}H-NMR spectroscopy and F-C Assay, the potential for capillary electrophoresis (CE) to quantify phloroglucinol in seaweed species was also investigated as the instrumentation uses minimal solvent quantities and can operate quite rapidly compared to more traditional analysis techniques. At the time of CE analysis, a red seaweed species, *M. japonica*, had been collected and added to the analysis.

CE Instrumentation

CE analysis was conducted utilising a SCIEX P/ACE MDQ Plus Capillary Electrophoresis system (Fullerton, California). Analytes were detected with an ultraviolet (UV) detector at 214 nm wavelength using direct absorbance, and data was acquired and processed using 32Karat software. For fatty acid separation, uncoated, fused-silica capillaries (Polymicro Technologies, Phoenix, AZ) of an inner diameter of 50 μm were used. The length of the capillary was 50.0 cm, with an effective length of 40.0 cm.

Sample Preparation/Buffer Preparation for CE

Prior to the attempt of phloroglucinol identification and quantification in the seaweed samples, a buffer trial was conducted with the aim of producing an optimal standard curve. For this, multiple buffers were prepared and ran with prepared standards and samples, and the resulting peaks were compared. Buffers prepared and tested included sodium tetraborate, sodium tetraborate with sodium dodecyl sulfate (SDS) for micellar electrokinetic chromatography (MEKC), 60:40 acetonitrile: 18 MΩ water with 6.5 mM NaOH, 70:30

acetonitrile: 18 MΩ water with 6.5 mM NaOH, and sodium tetraborate with acetonitrile at pH 11.

The sample preparation for CE followed the same process as that prepared for ^{1}H NMR analysis but now include *M. japonica*. Dehydrated samples of all three species were weighed to approximately 200 mg and extracted with 50:50 v/v methanol/18 MΩ water in a Bransonic Ultrasonic Cleaner (50/60 Hz) for two hours. The difference being that only one extraction (rather than two successive extractions) took place. A 500 ppm phloroglucinol stock solution was prepared by dissolving ~0.0125 g of phloroglucinol in 25 mL of deionized H_2O in a volumetric flask. Six concentrations of working phloroglucinol standards were prepared at 5, 10, 30, 30, 40, and 50 ppm from the 500-ppm stock solution using 50:50 v/v methanol/18 MΩ water. Specific volumes used to create each working standard are presented in **Appendix B.3**, **Table B.3.1**.

CE trials with each buffer were carried out under the same instrumental conditions. The capillary was initially rinsed for 4 min with 0.1 NaOH, 1 min with 18 MΩ water and 5 min with buffer solution all at 20 psi prior to analysis. Standards and samples were injected at 1.0 psi for 5.0 s and separated at 20.0 kV for 25.0 min each. For each buffer trial, a new set of standards and samples were prepared to avoid phlorotannin degradation over time.

Statistical Analysis

Due to the trial nature of ^{1}H NMR and CE analyses, no statistical analysis was applied to the results. Rather, these results serve as a framework for next steps and for the further development of the methods. However, as F-C assay analysis was conducted both on a development time trial basis and repeated as a complete methodology three times, statistical analyses were employed to assess the repeatability and accuracy of the method used.

As stated in **Section 2.3** of **Chapter 2**, Statistics Canada considers a relative standard deviation (% RSD) between 0% and 16.5% acceptable, between 16.6% and 33.3% marginal and greater than 33.3% unacceptable (Statistics Canada, 2025). These acceptable and unacceptable ranges were used when evaluating the validity of results obtained from the repeated 2-hour development F-C assay analyses. The % RSD for sugar kelp was determined to be 39.5% while giant kelp was 42.6%, both of which land in the unacceptable range

outlined by Statistics Canada. As such, the F-C assay method was not able to be validated, and the results presented should be interpreted with an account of method variability.

RESULTS

Identification of Phloroglucinol by ^{1}H NMR

The initial solubility trial in various NMR solvents determined that further extractions would proceed with D_2O and DMSO-d_6 based on an observed higher solubility and a better signal/noise ratio. Multiple spectra were attained from the phloroglucinol detection trials using ^{1}H NMR in various deuterated solvents using different extraction techniques. Prior to integration, all spectra were subjected to an automatic phase correction and referenced to residual protons in either in D_2O (4.79 ppm), or DMSO-d_6 (2.50 ppm). Aromatic H phloroglucinol peaks were evident at approximately 8.4 ppm in D_2O and 8.9 ppm in DMSO-d_6 for both sugar kelp and giant kelp samples, however, peaks closer to 6 ppm indicative of phenolic OH peaks were not found. An NMR spectrum of giant kelp in D_2O is shown in Figure 3.5 where the suspected phloroglucinol aromatic H peak at approximately 8.4 ppm is present, but not the phenolic OH peak.

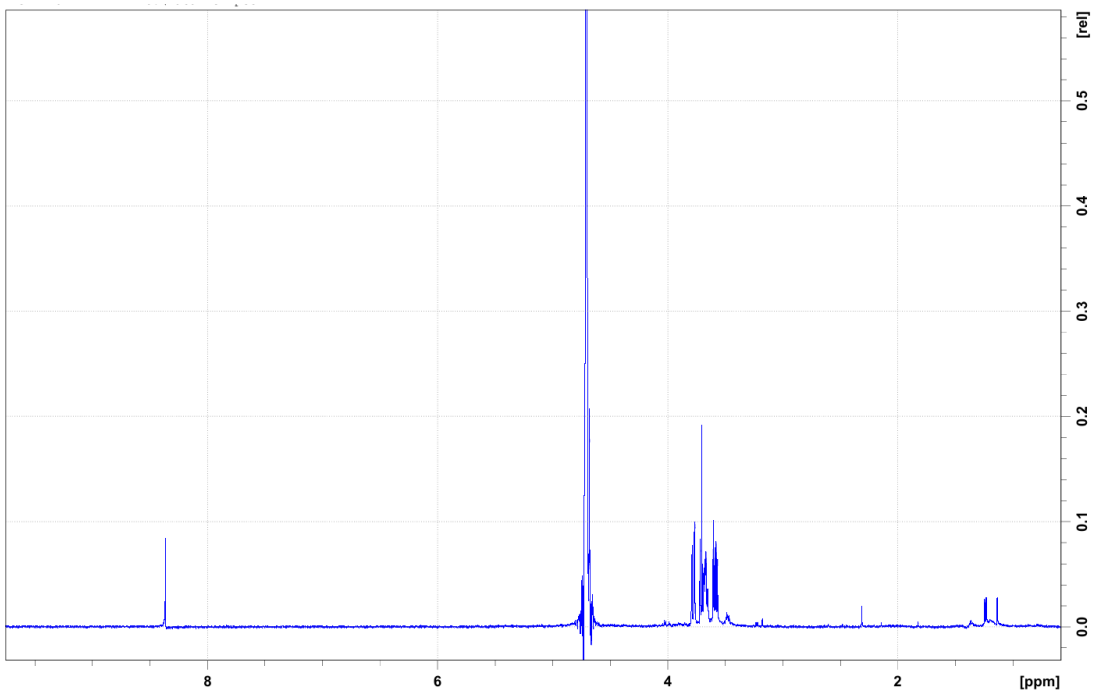


Figure 3.5 ^{1}H NMR Spectra of Giant Kelp in D_2O with Suspected Phloroglucinol Aromatic H Peak Evident at ~8.4 ppm

In contrast, spectra attained utilizing DMSO-d_6 did not show suspected phloroglucinol aromatic H peaks and overall did not indicate the presence of phloroglucinol. A ^{1}H NMR spectra showing this result is presented in **Appendix B.1**, **Figure B.1.1,** along with sugar kelp in D2O and Giant kelp in DMSO-d6 (**Figure B.1.2** and **B.1.3**, respectively). Spectra resulting from the acidified extraction process of seaweeds that were dissolved in D_2O for ^{1}H NMR analysis yielded similar findings compared to the non acidified extraction results. The spectra attained by the acidified extraction trial are appended in **Appendix B.1**, **Figure's B.1.4**, and **B.1.5**.

Determination of Total Polyphenolic Content Using F-C Assay

Initial development time trials revealed that the formation of a blue complex driven by F-C reagent was optimal around 2 h. This produced the best-suited calibration curve for polyphenol quantification in seaweed samples compared to 4 h and 6 h of development. A trend observed in longer development times was that the progression of colour formation in working standards persisted while colour progression in samples slowed after a certain amount of time. This resulted in sample absorbances falling within a more dilute range of the

calibration standards and therefore a less accurate quantification, compared to 2 h of development which led to sample absorbances falling mid-range within the calibration.

As such, the 2-h development time trial for F-C assay was repeated two more times for the evaluation of method validity, and to quantity total polyphenolic content in each species. The polyphenol content in each species was determined using the equation of the line produced by the corresponding calibration curves with the absorbance measured spectrophotometrically, and by applying a dilution factor of 20 (0.50 mL samples added to a 10 mL volumetric flask). The calibration curves for each 2-h F-C Assay trial can be found in Appendix B.2, Figure's B.2.1 – B.2.3, and the concentration of polyphenols (mg/mL) in each species, along with average concentrations are presented in Table 3.1, below (full results including % RSD results in **Appendix B.2, Table B.2.4**).

Table 3.1 *Total Polyphenolic Content in Sugar Kelp and Giant Kelp (mg/mL) as Determined by a 2-Hour Development F-C Assay*

Trial #	Total Polyphenolic Content in Sugar Kelp (mg/mL)	Total Polyphenolic Content in Giant Kelp (mg/mL)
1	2.47	2.79
2	1.30	1.13
3	3.07	1.92
Mean	2.28	1.95

Overall, the analysis showed that the average total polyphenolic content in sugar kelp was higher than giant kelp at 2.28 mg/mL and 1.95 mg/mL, respectively. However, it should be noted that % RSD calculations revealed the method used to obtain these measurements was not considered valid and therefore, the accuracy of these measurements may be uncertain. Additionally, throughout the initial time development trial, it was found that giant kelp consistently produced a higher absorbance – and therefore polyphenolic content – than sugar kelp. The discrepancy between results should be considered, however, total polyphenolic content was found to be rather similar in either species.

Phloroglucinol Determination by CE Buffer Trials

Pilot buffer trials for the determination of phloroglucinol by CE resulted in variable results for the seaweed samples. The buffer that produced the most stable and interpretable calibration electropherograms was 60:40 v/v acetonitrile: 18 MΩ water with 6 mM NaOH. However, although the phloroglucinol peak appeared at the same time in each standard and sample, around 8.6 min, the peak areas of the standards were not steadily increasing in size with concentration and therefore could not be used to quantity the samples accurately. Further optimization of the potential buffer needs to be performed to produce interpretable calibration curves with high R^2 values in order to accurately quantify phloroglucinol in seaweed samples.

DISCUSSION

Evaluation of ^{1}H NMR as a Method for Phlorotannin Detection

^{1}H NMR was explored as a rapid screening tool for phlorotannin detection as spectra can be acquired quickly and it requires minimal sample preparation. While the peaks evident near 8.4 ppm are indicative of phloroglucinol aromatic protons, the peaks were proportionally very small compared to the solvent peak making quantification difficult and inaccurate. For example, D_2O's water peak (~4.79 ppm) may mask nearby signals, and complex matrices like seaweed extract can cause overlapping peaks from other compounds such as mannitol and lipids (Gager et al., 2020; Imbs et al., 2018). Additionally, phenolic OH signals expected between 5.5-6.5 ppm were absent. This is likely due to using D_2O as the NMR solvent, which promotes hydrogen-deuterium exchange and can suppress acidic proton signals (Raja & Barron, 2016; Jegou et al., 2015).

The use of DMSO-d_6 showed no observable phlorotannin peaks, which likely reflects poor solubility. High molecular weight phlorotannins also suffer from peak broadening and reduced sensitivity in NMR (Kumar et al., 2022). The samples extracted using the acidified method did not show significantly different results, with phloroglucinol aromatic H peaks still evident at 8.4 ppm but no phenolic OH peak present in D_2O. As the aromatic H peak was still proportionally too small to quantify accurately, it was determined that further use of this method was unnecessary as it took substantially longer yet yielded similar results. Overall,

although ^{1}H NMR is promising for quick screening, it requires further optimization of solvents, extraction technique, and possibly further extract purification prior to use, particularly for quantification.

Evaluation of F-C Assay for Total Polyphenolic Content Estimation

The F-C assay successfully provided a general estimate of total polyphenol content and showed slightly higher average values in sugar kelp compared to giant kelp. However, high variability among replicate trials (with % RSDs exceeding 39%) indicated poor reproducibility, placing the method outside Statistics Canada's threshold for method validity (%RSD > 33.3%) (Statistics Canada, 2025).

This variability likely stems from the F-C assay's lack of specificity, as the reagent reacts with alternative reducing agents like certain sugars, and not solely with phenolic groups (Singleton et al., 1999; Rajauria et al., 2016). The assay is also sensitive to extraction conditions and reaction timing. While the 2-h development time produced more consistent standard curves than 4 or 6 h, variability in seaweed extract matrix composition may still have impacted the blue-complex formation and absorbance readings. Despite these limitations, the F-C assay served as a useful baseline indicator of polyphenol presence and supported findings from other methods. However, it should not be used alone for quantitative comparisons between species or samples without further method validation.

CE Trials for Phloroglucinol Separation and Quantification

CE was also explored as an alternative method for the detection of phloroglucinol in seaweed extracts. Multiple buffer systems were trialed, with the most stable separation occurring in 60:40 v/v acetonitrile:water with 6 mM NaOH. However, peak areas for the phloroglucinol standards did not scale proportionally with concentration, preventing reliable quantification. The variation may be attributed to matrix effects from the crude seaweed extracts, which can cause inconsistent migration or peak suppression due to variable conductivity and interactions with the capillary (Poole, 2003). Additionally, the structural diversity of phlorotannins and their high polarity can complicate separation in non-optimized systems (Kumar et al., 2022).

Although CE has potential for the separation of phlorotannins, successful application requires sample cleanup (e.g., alternative extraction techniques like solid-phase extraction), the inclusion of an internal standard, and refined buffer selection. At this stage, CE served as a qualitative tool with future potential for phlorotannin profiling but was not suitable for accurate quantification in its current state.

CONCLUSION

This study explored the detection and estimation of phlorotannins in two brown seaweed species, giant kelp and sugar kelp, using three analytical techniques: ^{1}H NMR, F-C assay, and CE. M. japonica was also briefly analysed in a trial basis using CE. While ^{1}H NMR successfully detected resonances consistent with phloroglucinol H protons, full confirmation was difficult due to the absence of characteristic aromatic OH peaks. The F-C assay indicated higher total phenolic content in sugar kelp compared to giant kelp, but the method's poor repeatability compromised result validity. CE demonstrated potential for separation; however, further buffer and calibration optimization is required to enable reliable quantification.

Collectively, these findings highlight both the potential and current limitations of low-solvent phlorotannin analysis methods. Sugar kelp and giant kelp remain promising sources of bioactive phlorotannins for methane mitigation and nutritional enhancement in livestock feed, particularly in regions like B.C. where bromoform-rich species are not readily available. Continued analytical development and *in vivo* studies will be critical to translate biochemical potential into practical and scalable feed applications.

LITERATURE CITED

Abbott, D. W., Aasen, I. M., Beauchemin, K. A., Grondahl, F., Gruninger, R. J., Hayes, M., ... & Huws, S. A. (2023). Seaweed and seaweed bioactives for mitigation of enteric methane: Challenges and opportunities. *Animal Feed Science and Technology, 301*, 115177. https://doi.org/10.1016/j.anifeedsci.2022.115177

Barry, T. N., & Manley, T. R. (1984). The role of condensed tannins in the nutritional value of Lotus pedunculatus for sheep: 4. Sites of carbohydrate and protein digestion as influenced by dietary reactive tannin concentration. *British Journal of Nutrition, 51*(3), 493–504. https://doi.org/10.1079/BJN19840054

De Bhowmick, G., Sahoo, D., & Sarkar, A. (2023). Current advances and emerging prospects in the extraction, characterization and biological activities of seaweed phlorotannins. *Marine Drugs, 21*(2), 115. https://doi.org/10.3390/md21020115

Ford, L., Lu, J. Y., & Zhou, K. (2019). Structural and functional properties of polyphenols in seaweed. In *Marine Nutraceuticals* (pp. 215–242). CRC Press.

Gager, L., Dufour, D., Gall, E. A., & Potin, P. (2020). Characterization of phenolic compounds in brown algae using ^{1}H NMR and UHPLC-ESI-MS. *Marine Drugs, 18*(12), 645. https://doi.org/10.3390/md18120645

Imbs, T. I., Skriptsova, A. V., & Zvyagintseva, T. N. (2018). Phlorotannins from brown algae: Structure, distribution, and biological activity. *Marine Drugs, 16*(7), 207. https://doi.org/10.3390/md16070207

Ivane, S. E., Chisale, M. R., Akhtar, N., & Chikowe, I. (2025). Seaweed-derived polyphenols: Challenges in extraction and analytical characterization. *Phycological Research, 73*(1), 12–27. https://doi.org/10.1111/pre.12467

Jegou, C., Bondon, A., Pichon, L., Le Lann, K., & Yvin, J. C. (2015). Investigation of seaweed phlorotannins by NMR spectroscopy. *Analytical and Bioanalytical Chemistry, 407*(1), 91–102. https://doi.org/10.1007/s00216-014-8257-3

Kumar, K. S., Ganesan, K., & Subba Rao, P. V. (2022). Challenges in extraction and quantification of phlorotannins from seaweeds. *Journal of Applied Phycology, 34*, 53–67. https://doi.org/10.1007/s10811-021-02541-y

Li, Y. X., Wijesekara, I., Li, Y., & Kim, S. K. (2017). Phlorotannins as bioactive agents from brown algae. *Process Biochemistry, 46*(12), 2219–2224. https://doi.org/10.1016/j.procbio.2011.09.015

Machmüller, A., Schären, M., & Huws, S. A. (2022). Impact of bromoform and other halogenated methane inhibitors on rumen fermentation and emissions: Implications for future strategies. *Frontiers in Animal Science, 3*, 881967. https://doi.org/10.3389/fanim.2022.881967

Meng, Y., Liu, Y., Geng, Q., Liu, H., Liu, T., & Wang, C. (2021). Seaweed-derived polyphenols: Recent advances in extraction, identification, and bioactivities. *Marine Drugs, 19*(8), 409. https://doi.org/10.3390/md19080409

Min, S. K., Kim, S. M., & Kang, K. (2021). Quantification of phlorotannins in brown seaweeds: Recent advances and challenges. *Food Chemistry, 342*, 128331. https://doi.org/10.1016/j.foodchem.2020.128331

Mueller-Harvey, I., Hart, K. J., Foan, L., Ascough, J., & Ramsay, A. (2019). Phlorotannins as novel rumen modifiers: Potential for enhancing protein utilization and methane mitigation. *Animal Feed Science and Technology, 251*, 14–25. https://doi.org/10.1016/j.anifeedsci.2019.02.004

Pérez-Jiménez, J., & Saura-Calixto, F. (2013). Solid-liquid extraction of polyphenols, with purification steps like liquid–liquid extraction and chromatographic techniques. *Molecules*, *18*(3), 2328–2379. https://doi.org/10.3390/molecules18022328

Poole, C. F. (2003). *The essence of chromatography*. Elsevier.

Raja, S., & Barron, L. D. (2016). Solvent effects in ^{1}H NMR spectroscopy: A practical guide. *Spectroscopy Europe, 28*(2), 10–13.

Rajauria, G., Jaiswal, A. K., Abu-Ghannam, N., & Gupta, S. (2016). Antimicrobial, antioxidant and free radical-scavenging capacity of brown seaweed Himanthalia elongata from western coast of Ireland. *Journal of Food Biochemistry, 37*(3), 322–335. https://doi.org/10.1111/jfbc.12042

Roque, B. M., Venegas, M., Kinley, R., de Nys, R., & Kebreab, E. (2021). Mitigating enteric methane emissions from cattle using a red seaweed. *Journal of Cleaner Production, 291*, 125987. https://doi.org/10.1016/j.jclepro.2021.125987

Singleton, V. L., Orthofer, R., & Lamuela-Raventós, R. M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods in Enzymology, 299*, 152–178. https://doi.org/10.1016/S0076-6879(99)99017-1

Statistics Canada. (2025). *Laboratory quality assurance and method validation guidelines*. Retrieved from [URL or access point if applicable]

Vissers, A. M., Hessle, A., & Olsson, J. A. (2018). Seaweed polyphenols and their potential for methane mitigation in ruminants – A review. *Journal of the Science of Food and Agriculture, 98*(14), 4965–4973. https://doi.org/10.1002/jsfa.9095

CHAPTER 4: TRACE ELEMENTS & HEAVY METALS ANALYSIS

ABSTRACT

This research investigates the presence and concentrations of trace elements including heavy metals in three seaweed species harvested in British Columbia (B.C.), *Macrocystis pyrifera* (giant kelp), *Saccharina latissima* (sugar kelp), and *Mazzaella japonica* (*M. japonica*), with an emphasis on implications for their use as cattle feed additives. Given the growing interest in seaweeds as sustainable methane-mitigating feed supplements, trace metal accumulation poses a potential toxicity risk to livestock. Using inductively coupled plasma-mass spectrometry (ICP-MS), concentrations of 17 trace elements were quantified and compared to regulatory Action Levels set by the Government of Canada. Five metals of toxicological significance, aluminum (Al), arsenic (As), cadmium (Cd), lead (Pb), and mercury (Hg), were statistically analysed for interspecies differences. All species had concentrations below regulated thresholds, supporting their potential use as feed additives. However, heavy metal content varied significantly across species, and site-specific conditions such as proximity to industrial activities, which emphasizes the need for careful sourcing and monitoring.

INTRODUCTION

“Heavy metals” is a term that is diversely defined but is generally used as a generic label for metals and metalloids that are correlated with environmental pollution and toxicity (Baker et al., 2012). Traditionally, heavy metals and metalloids are categorized by their high atomic weight, high density of > 5g/cm^3, and their toxicity to organisms even at low concentrations (Ali & Khan, 2018). Due to debate amongst the scientific community, and to encompass all elements of interest, heavy metals analyses are commonly referred to as an investigation of trace elements including heavy metals. Common trace elements including heavy metals include: arsenic (As), aluminum (Al), cadmium (Cd), chromium (Cr), lead (Pb), mercury (Hg), manganese (Mn), nickel (Ni), copper (Cu), and zinc (Zn). Some of these elements like Zn, Cu, and Mn are essential for biological survival at low concentrations but become toxic at higher concentrations (Fisher & Gupta, 2025). In contrast, heavy metals such as As, Cd, Pb, and Hg are toxic even at low concentrations and serve no biological function (Fisher & Gupta, 2025). These trace elements and heavy metals are of particular concern due to their persistence, bio-accumulative nature, and potential to cause detrimental human and ecological health effects even at relatively low concentrations.

Although trace elements including heavy metals contamination can be found in many areas surrounding industrial processes, a significant consideration is the presence of these elements in coastal environments due to the complex dynamics and ecological significance of coasts (El-Sharkawy et al., 2025). Trace elements including heavy metals can enter coastal and marine environments through natural processes such as weathering of rocks and volcanic activity, and through anthropogenic sources including industrial discharge, agricultural run-off, and wastewater from urban environments (Rajeshkumar et al., 2018). The coastal regions of British Columbia (B.C.) have experienced significant trace elements and heavy metals contamination through the industrialization of many coastal towns and ports. Notably, areas such as Haida Gwaii, Vancouver Island's coast, the Strait of Georgia, and B.C.'s north and central coasts have been identified as hotspots for metals and metalloids including Hg, Cd, As, Ni, Cu, and Pb (Comox Valley Record, 2023). Comprehensive studies assessing trace elements including heavy metals in sediment samples have been conducted across these

regions and continue to be conducted as industrial activities progress through time (Li et al., 2023).

The primary anthropogenic sources contributing to this contamination include historical and ongoing mining activities, industrial operations, and urban runoff. For example, the Britannia Mine which produced copper ore until 1974, was one of North America's largest metal pollution sources, leading to extensive heavy metal discharge into surrounding waterways (Zhou, 2023). Urban development and associated infrastructure have been linked to increased heavy metal levels in local water bodies, and industrial activities have been identified as contributors to Cr contamination in waters and sediments (Wu et al., 2025). Collectively, these anthropogenic sources contribute to a large amount of trace elements including heavy metals contamination along B.C.'s coasts which needs to be carefully considered prior to the usage of marine biological materials in livestock feed that may pose an affinity for these contaminants.

The pacific northwest is home to approximately 650 macroalgal species, of which about 530 can be found off the coast of B.C. (Gabrielson et al., 2000). All three major groups of seaweeds, green, red, and brown, can be found in B.C. with brown species dominating the biomass in the intertidal and upper subtidal zones (Bates, 2004). In marine ecosystems, seaweeds can sequester heavy metals through two mechanisms: biosorption and bioaccumulation, making them highly effective natural bio-remediators. Biosorption is the process in which metal ions bind to the functional groups present in the cell walls of the seaweeds that have a high affinity for metal cations such as alginate, fucoidan, and carrageenan (Gupta & Balomajumder, 2015) (Figure 4.1). Alternatively, bioaccumulation involves the active uptake and internal storage of heavy metals within cellular compartments of seaweed, which can be influenced by species-specific metabolic processes and surrounding environmental conditions (Bolan et al., 2021).

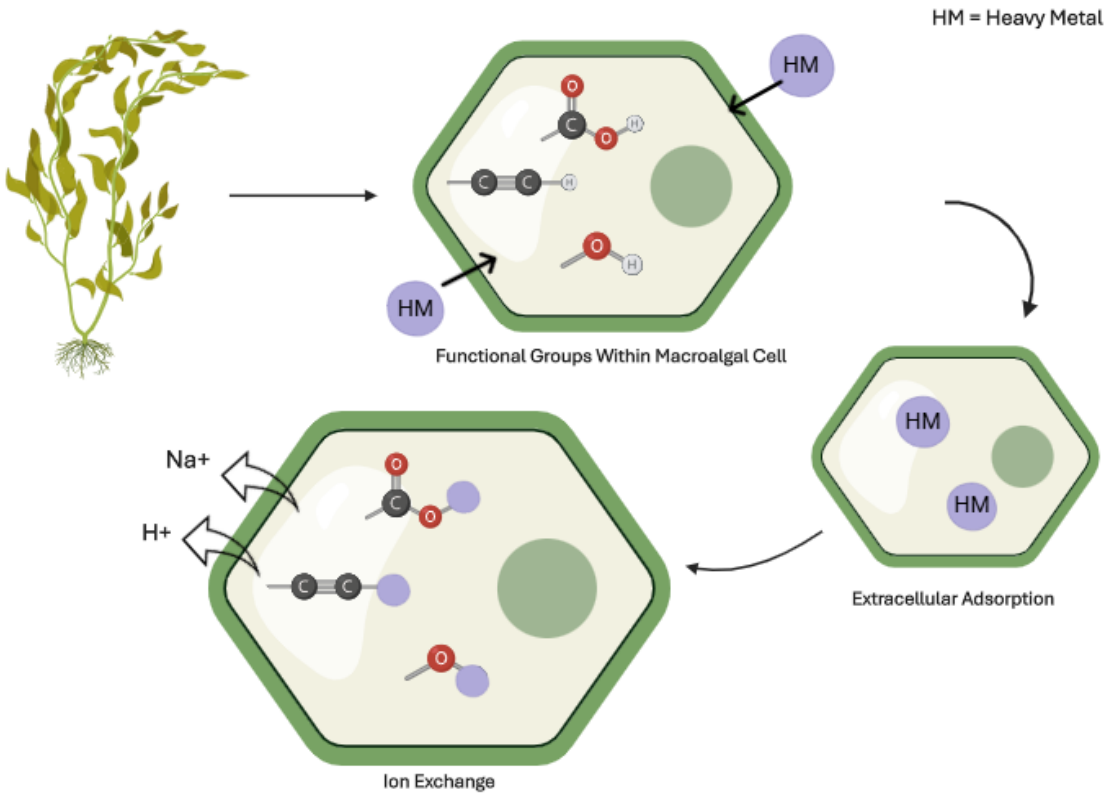


Figure 4.1 Example of Heavy Metal Biosorption Process in a Macroalgal Cell (created via Biorender)

The ability of seaweeds to absorb and retain heavy metals can be regarded as beneficial as it helps reduce trace elements and heavy metals concentrations in surrounding seawater and mitigates the impact of nearby polluting sources. As such, seaweeds are often employed as phytoremediators in attempt to clean coastal waters as they can efficiently capture metals without the use of energy-intensive remedial technologies (Torres et al., 2019). Additionally, the sequestration of trace elements including heavy metals by seaweeds reduces their bioavailability to marine organisms at higher trophic levels, thereby reducing risks of further bioaccumulation and biomagnification within the food chain (Baker et al., 2018). While this function is ecologically valuable, the potential transfer of heavy metals to organisms who ingest harvested seaweed necessitates careful monitoring and regulation of metals contamination in edible species.

The incorporation of seaweed in cattle-feed is gaining attention for its nutritional benefits and potential to reduce enteric methane emissions, however, the presence of sequestered trace elements including heavy metals in seaweeds raises concerns about potential toxic and metabolic effects on livestock (Maguire et al., 2023; Pierceg et al., 2020). When contaminated seaweeds are ingested by cattle, trace elements including heavy metals can be absorbed into the gastrointestinal tract and distributed to various organs and issues of the ruminant, particularly the liver and kidneys where detoxification attempts take place (Comerford et al., 2021). Some metals of concern, including Cd and Pb, have limited excretion pathways leading to bioaccumulation and the potential for nephrotoxicity, hepatotoxicity, and enzymatic function disruption over time (Ramos et al., 2016). Due to toxicity risks, if seaweeds are being processed for incorporation into cattle-feed, an analysis of trace elements including heavy metals within the seaweed tissue must be conducted prior to feed trials.

Due to this concern of contaminated livestock feed, the Government of Canada has established “Action Levels” for certain contaminants of interest. These are levels at which if exceeded, the contaminant may cause health risks due to metals toxicity for the animal itself, or unacceptable levels of contaminants found in animal by-products (C.F.I. Agency, 2015). Action Levels have been defined for Al, As, Cd, Pb, and Hg (only measured in fish by-products), and can be found in Table 4.1, below (C.F.I. Agency, 2015). Feed products are deemed acceptable if metals contamination is found below the determined Action Level, which applies to total livestock diet.

Table 4.1 *Action Levels for Metals Contaminants in Total Livestock Diets as Defined by the Government of Canada RG-8 Regulatory Guideline: Contaminants in Feed*

Metal Contaminant	Action Level	Reporting Limit/Limit of Quantification
Aluminum (Al)	Non ruminant: 200 ppm Ruminant: 1,000 ppm	3.2 ppm
Arsenic (As)	8 ppm	5.2 ppm
Cadmium (Cd)	Horses: 0.2 ppm Other Livestock: 0.4 ppm	0.05 ppm
Lead (Pb)	8 ppm	5 ppm

Heavy metals have been successfully identified and quantified by inductively coupled plasma mass spectrometry (ICP-MS) in a range of biological materials and as such, ICP-MS is widely recognized as the gold standard in heavy metals analysis (Beauchemin, 2018). ICP-MS has excellent sensitivity, accuracy, and multi-element detection capabilities (Beauchemin, 2018). ICP-MS operates by nebulizing the digested sample into an argon plasma where high temperatures (typically ranging from 6,000-10,000 K) ionize the metal species into their elemental forms (Ramos-Miras et al., 2020). The ions go through a series of electrostatic lenses and a quadrupole where they are separated based on their mass-to-charge ratio (m/z) and then quantified using a detector (Leopold et al., 2021).

Compared to alternative analysis techniques such as atomic absorption spectroscopy (AAS) or ICP optical emission spectrometry (OES), ICP-MS allows for the determination of lower detection limits down to parts per trillion (ppt) and can carry out rapid, simultaneous detection of multiple trace metals in a range of complex biological matrices (Laborda et al.,

2020). In seaweed analysis, ICP-MS enables precise quantification of toxic trace metals and heavy metals such as As, Cd, Hg, and Pb, supporting environmental monitoring and food safety assessments.

The objective of this work is to determine concentrations of trace elements including heavy metals in three seaweed species, sugar kelp, *M. japonica*, and giant kelp, comparing concentrations of key elements to RG-8 Regulatory Guidance: Contaminants in Feed, and to assess if the concentrations of trace elements including heavy metals were significantly different among the species. In addition, the methodology for analyzing trace elements including heavy metals in seaweed samples using ICP-MS will be optimized and validated through statistical analyses.

METHODS AND MATERIALS

Biological Materials and Sample Preparation

The study species for this analysis included two brown seaweed species, giant kelp and sugar kelp, and one red seaweed species*, M. japonica*. Fresh brown seaweed samples were collected by Cascadia Seaweed Inc. (Cascadia) from their farm site in Clayoquot sound, B.C. (approximate location shown in Figure 4.2). Harvesting took place at approximately 17:00 on June 5, 2024. According to Cascadia's documentation, live specimens were cut from the stalk with a knife, and visible biofouling was removed. The cleaned samples were wrapped in damp towels and transported in coolers containing seawater. They were shipped via air transport to Kamloops, B.C., and were received by TRU on June 7, 2024. The *M. Japonica* was hand collected by TRU as storm-cast from the beaches of Deep Bay, Vancouver Island, B.C. on August 26th, 2024 (Figure 4.2). The fresh red seaweed samples were packed in coolers with ice and transported to the TRU laboratory over the subsequent days.

Figure 4.2 Approximate Location of Sugar Kelp, Giant Kelp, and *M. japonica* in Clayoquot Sound, B.C and Deep Bay B.C (image created via iMap BC).

Upon arrival at the TRU laboratory, all macroalgal samples were sorted by species, rinsed with deionized water to remove impurities, and stored in Ziploc bags at -80 ºC until further processing. Prior to analysis, the samples were removed from the freezer and dried using low-temperature dehydration with a Hamilton Beach household dehydrator set at 40 ºC for approximately three hours (or until dry). Following drying, the samples were ground to a fine powder using an electric mill grinder, stored in Ziploc bags separated by species, and kept at room temperature until analysis.

Chemical Supplies

Trace metal grade nitric acid (HNO_3) (68-70% purity) and analytical reagent grade hydrochloric acid (HCl) (37% purity) were sourced from Millipore Sigma Canada Ltd. (Oakville, Ontario, Canada). Multi-elemental stock solutions used for ICP-MS specific functions including the environmental calibration standard, environmental calibration spike mix, and internal standard mix were purchased from Agilent Technologies Inc. (Mississauga,

ON, Canada). High purity water (18 MΩ cm^{-1}) used throughout preparation and analyses was generated using a Milli-Q purification system (Millipore, Billerica, MA, USA).

ICP-MS Instrumentation

The trace elements including heavy metals analysis was performed using an Agilent 7900 single quadrupole ICP-MS operated with the MassHunter software platform developed by Agilent Technologies Inc. The ICP-MS was operated in both He and H_2 modes and utilized the SPS 4 autosampler for sample uptake. Sample introduction included: sample uptake through a peristaltic pump, nebulization into a fine aerosol by a concentric Micro Mist nebulizer, removal of larger droplets through a Scott-type quartz double-pass spray chamber, exposure to a standard quartz ICP torch for atomization and ionization of the sample, a Ni-plated sampler and skimmer cones for extracting ions from the plasma, and a x-Lens ion lens for focusing the ions into a narrow beam. The start-up and operating parameters of the ICP-MS were optimized for maximised precision, accuracy and efficiency, and tuning solution (Agilent Technologies Inc.) consisting of 1 µL/mL of cerium (Ce), cobalt (Co), lithium (Li), magnesium (Mg), thallium (Tl), and yttrium (Y) in 2% HNO_3 was used for both hardware and auto tuning of the instrument.

Trace elements including heavy metals concentrations of interest were determined using external calibration for accurate quantification. An internal calibration standard (ISTD) (Agilent Technologies Inc.) consisting of 100 µg/mL of bismuth (Bi), germanium (Ge), indium (In), Li^6, lutetium (Lu), rhodium (Rh), scandium (Sc), and terbium (Tb) in 10% HNO_3 was employed to correct for instrumental drift. Calibration curves were generated using the Agilent MassHunter software and verified within +/- 10% error using a mid-range calibration standard (standard 3 at 10 µg/L) prepared from the 200-ppb intermediate stock solution. The mid range calibration standard was analysed every three samples to monitor for instrumental drift, and an instrument blank sample (2% HNO_3) was analysed at regular intervals to ensure no sample carry-over occurred.

Sample and Standards Preparation for ICP-MS

For sample preparation, ground samples of all three seaweed species, *M. japonica*, giant kelp, and sugar kelp, were each weighed to approximately 0.500 g using an analytical balance and placed into High Voltage Temperature (HVT) Teflon microwave digestion vessels (Anton Paar Inc., St-Laurent, Quebec, Canada) (actual seaweed masses reported in **Appendix D.1, Table D.1**). Then, approximately 5 mL of trace-metal grade HNO_3 was added to each tube prior to microwave digestion. The 35 min digestion process was performed in an Anton Paar Multiwave GO Microwave Digestion Platform. Once cooled, an additional 10 mL of 2% HNO_3 was added to each vessel and the mixture was subjected to gravimetric filtration through 0.45 µm Nylon filters into 50 mL Falcon tubes. The filtered sample solutions were then diluted to 50 mL with 2% HNO_3.

For the preparation of calibration standards,[2] a 200-ppb intermediate stock solution was first prepared by pipetting 1 mL of environmental calibration standard (Agilent Technologies Inc.) into a Falcon tube and diluting to 50 mL with 18-ΩM water. Subsequently, five trace elements including heavy metals standards with concentrations ranging between 0.1 and 1000 ppb were prepared using varying volumes of 200 ppb intermediate stock solution and 2% HNO_3. The prepared calibration standards were used to assess method linearity and produce reliable calibration curves for each trace metal and heavy metal (**Appendix C, Table C.1**).

Method Validation

To validate the ICP-MS trace elements including heavy metals methodology, samples were run in triplicate on three separate days to produce results for an inter- versus intra-day precision comparison. For each metal analysed on a specific day, the mean concentration, standard deviation, and percent relative standard deviation (% RSD) was determined using Microsoft Excel for each species. Due to the high sensitivity of the instrument and concentrations being reported in parts-per billion (ppb), a % RSD < 20% was considered acceptable. A % RSD for each species within the same day were compared as well as %

[2] Separate standards were prepared for trace elements and mercury analysis. The intermediate stock solution and standards were prepared using the same method for both sets of standard solutions.

RSD across the multi-day analysis. Based on the appointed acceptable % RSD, the mean concentration of beryllium (Be), selenium (Se), molybdenum (Md), and thallium (Tl) were found to be unacceptable as they produced % RSDs > 20% on one or more occasions. For this reason, Be, Se, Mo, and TI were excluded from further analyses as the means produced may not be precise. All other trace elements including heavy metals produced means with an acceptable % RSD across trials and therefore were considered precise and used in subsequent analyses. Full results from the % RSD analysis are provided in **Appendix C**, **Table C.2**.

Spike recovery studies were done in lieu of standard reference materials to assess the accuracy of the analysis, and as such, "spiked" and "non-spiked" samples were prepared and analysed. "Spiked" sample preparation was the same as the previously outlined sample preparation method, with the addition of 0.100 mL of Agilent Technologies Environmental Spike Mix containing 1000 µg/mL calcium (Ca), iron (Fe), potassium (K), magnesium (Mg), sodium (Na) and 100 µg/mL silver (Ag), Al, As, barium (Ba), Be, Cd, Co, Cr, Cu, Mn, Mo, Ni, Pb, Sb, Tl, uranium (U), vanadium (V), and Zn in 5% HNO_3 to each of the samples. The spiked samples were analysed against a "non-spiked" sample set and the difference in trace elements including heavy metals content was compared to the concentration in the Environmental Spike Mix (Agilent Technologies Inc.). Percent recovery was then calculated for each trace element and heavy metal analysed and compared to the accepted range of 80 – 120 % recovery. All percent recovery values were within the accepted range showing acceptable accuracy for the method used and the results obtained.

Statistical Analysis

Statistical analyses were conducted using R (version 4.4.3; R Core Team, 2024) within RStudio (version 2024.09.0+375) to assess the variability of significant trace elements including heavy metals content in each species and determine whether variances were significantly different. Five "key" elements including one essential trace element (Al) and four nonessential trace elements including heavy metals (As, Cd, Pb, and Hg) were appointed with reference to the Government of Canada metals contamination indices for livestock feed and were the focus of statistical analyses. These five elements are considered significant due to their relative toxicity to livestock and appointed threshold values of toxicity.

Prior to an analysis of variance (ANOVA), a Shapiro-Wilk test to assess the normality ($p > 0.05$) of each element of interest found in each species was conducted and the results are presented in **Appendix C**, **Table C.3**. The test revealed that all key elements in all species had a p value of > 0.05 indicating data normality, except for mercury in sugar kelp where $p = 0.01$, indicating possible abnormal distribution. After distribution normality was determined, a Z-score outlier detection test was applied to the data with a threshold of +/- 3 (**Appendix C**, **Table C.4**). Pb and As datasets both produced Z-scores above or below the appointed threshold and therefore outliers were detected. As both datasets showed normal distribution, both parametric (ANOVA) and non-parametric (Kruskal-Wallis) analyses were applied for comparative purposes. A non-parametric Kruskal-Wallis test followed by a pairwise Wilcoxon post-hoc test was applied to the mercury data, while a one-way ANOVA followed by a Tukey's Honestly Significant Difference (HSD) post hoc analysis was applied to the remaining key element data that showed normal distribution. These analyses of variance followed by a corresponding post hoc analysis are reported in **Appendix C**, **Table C.5** and **Table C.6,** respectively.

RESULTS

As determined by % RSD observations during method validation, mean values of Be, Se, Mo, and Tl were removed from subsequent analyses due to unacceptable % RSD's (>20%) on one or more occasions. As such, three sample species (one of each of sugar kelp, giant kelp, and *M. japonica*) were analysed in triplicate on three separate occasions for 17 trace elements including heavy metals. Full results tables with the mean concentrations of trace elements including heavy metals from each trial day are provided in **Appendix C, Table C.7**. Of the 17 elements analysed, five "key" trace elements including heavy metals (Al, As, Cd, Pb, and Hg) were appointed with reference to the Government of Canada metals contamination indices for livestock feed. Mean concentrations of key trace elements and heavy metals are presented in Table 4.2, and mean concentration ranges are illustrated by Figure 4.3**,** below.

Table 4.2 *Average Concentrations of Key Trace Elements and Heavy Metals in Three Seaweed Species*

Species	**Aluminum (ppb)**	**Arsenic (ppb)**	**Cadmium (ppb)**	**Lead (ppb)**	**Mercury (ppb)**
Sugar Kelp	466.60	1032.66	53.21	96.28	9.00×10^{-3}
Giant Kelp	526.21	539.78	13.55	50.85	4.00×10^{-3}
M. japonica	275.36	29.58	1.70	1.13	4.00×10^{-3}

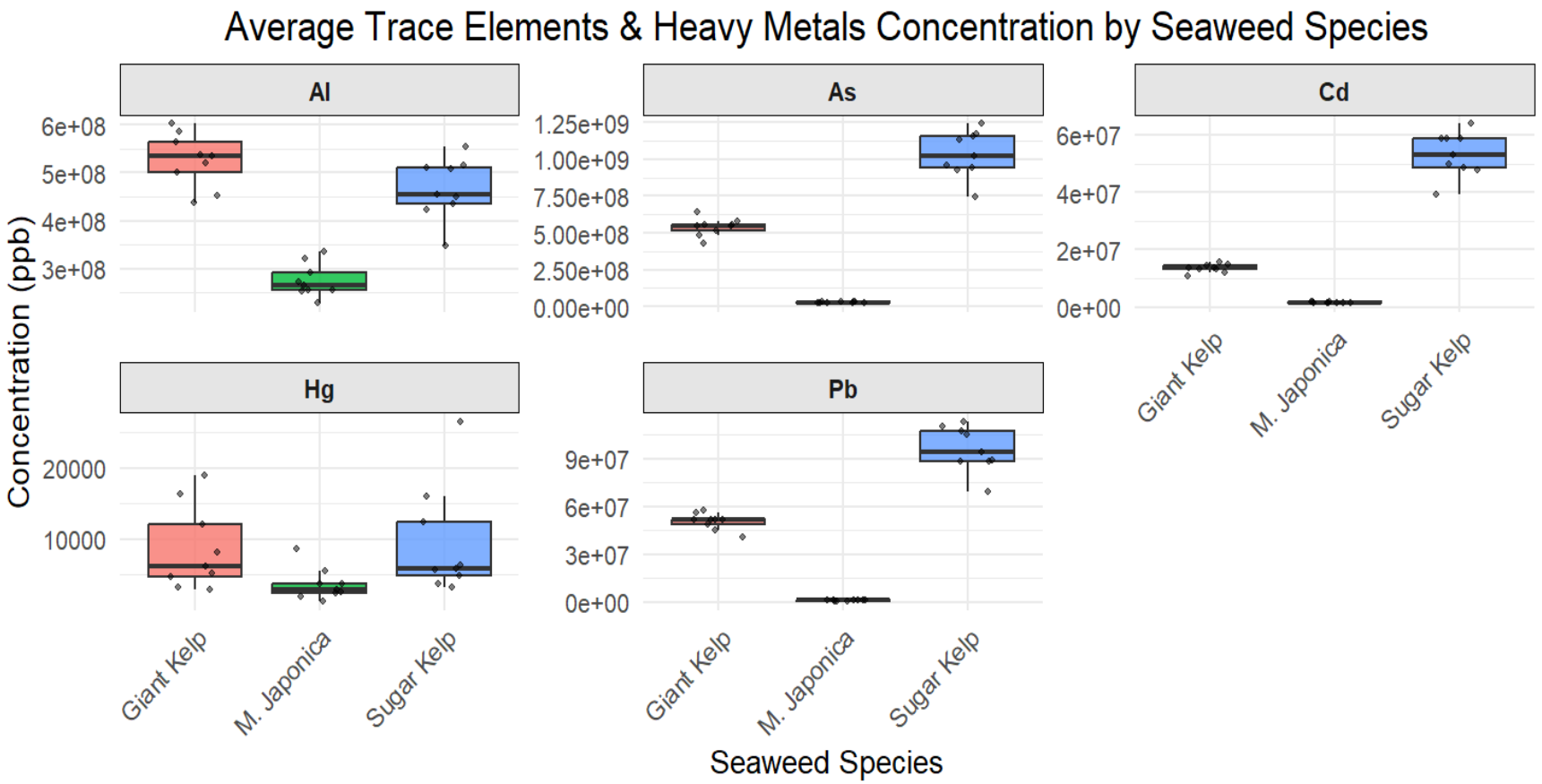


Figure 4.3 Average Concentration Ranges of Key Trace Elements and Heavy Metals in Three Seaweed Species

Although outliers were detected for Pb and As the above reported means are that of the entire dataset as the outliers detected were not the result of computational mistakes but more likely a result of cross-contamination or a natural extreme. In addition, the Pb and As datasets both showed normal distribution when Shapiro-Wilks analysis was applied and as such, all data was used in contribution to the means presented in the Table above. Overall, sugar kelp appeared to have the highest mean concentrations of key elements excluding Al which had a mean concentration of 466.60 ppb in sugar kelp and 526.21 ppb in giant kelp. Conversely, *M. japonica* consistently showed the lowest mean concentration of key elements, as well as generally lower concentrations of all trace elements including heavy metals analysed.

These key elements were subjected to one-way ANOVA's and post hoc analyses to determine if concentrations of key elements significantly differed among the three seaweed species. Shapiro-Wilks analyses identified normal distribution in all key elements except for Hg, and as such, Kruskal-Wallis test followed by a pairwise Wilcoxon post-hoc test was applied. Due to outliers detected in Pb and As datasets, Kruskal-Wallis analysis was also applied to these datasets for comparison against ANOVA but resulted in similar findings. The analyses of variance revealed that significant difference in concentrations of key elements were present between the three seaweed species. Results showing the p-values generated by the analyses of variance are presented in **Appendix D**, **Table D.4**.

To further assess which seaweed species specifically differed significantly from one to another, post hoc analyses (Tukey's HSD and Wilcoxon pairwise tests) were applied. The quantities of Pb, As, and Cd were found to be significantly different in all seaweed species. The quantities of Al in sugar kelp and giant kelp produced a $p = 0.059$, while sugar kelp, *M. japonica* and giant kelp, *M. japonica* returned values close to 0. Lastly, the pairwise Wilcoxon test applied to Hg data revealed non-significant differences in Hg concentration between sugar kelp and giant kelp. The results of the post-hoc analyses can be found in **Appendix D, Table D.5**.

DISCUSSION

The use of local seaweeds for methane suppression and improved animal nutrition is an attractive and plausible solution for combatting greenhouse gas (GHG) emissions associated with the cattle industry. Three local seaweed species, sugar kelp, giant kelp, and *M. japonica*, appear to be adequate candidates based on their abundance in B.C. However, seaweeds have an affinity for heavy metals and often sequester them through biosorption and bioaccumulation mechanisms (Gupta & Balomajumder, 2015; Bolan et al., 2021). As such, prior to use as livestock feed-additives, seaweeds must be analysed for trace elements including heavy metals to determine if concentrations surpass Action Levels outlined by the Government of Canada's RG-8 Regulatory Guidance: Contaminants in Feed (C.F.I. Agency, 2015), and to help assess which species may be most suitable for feed applications based on relative concentrations.

This analysis determined that compared to the RG-8 Regulatory Guidance, all three seaweed species analysed had concentrations of key heavy metals well below the determined Action Levels. For example, mean concentrations of Al were approximately 2000 times lower than the appointed Action Level in sugar kelp and giant kelp, and almost 4000 times lower in *M. japonica.* Cd, which has both the lowest Action Level (0.4 ppm) and limit of quantification (0.05 ppm), was present at a maximum mean of 0.053 ppm in sugar kelp. These values suggest low imminent toxicological risk and support the application of these species in cattle-feed systems. These findings are consistent with studies investigating similar brown seaweed species from less industrialized coastal zones, where Cd and Pb were also detected at relatively low levels (Torres et al., 2019).

Although the present study showed low toxicological risk, heavy metal content in seaweed is known to be directly proportional to the concentrations in the surrounding seawater (van Ginneken & de Vries, 2018), emphasizing the importance of site selection. The sugar kelp and giant kelp used in this study were harvested from Clayoquot Sound, B.C., currently a region with limited industrial presence, where concentrations of As, Cd, and Pb were found to be relatively low. If harvesting continues in this region under unchanged environmental conditions, it is likely that trace elements including heavy metals concentrations will remain within the acceptable thresholds. Conversely, M. *japonica* was collected as storm-cast from

the beaches of Deep Bay, B.C. Although this red species showed the lowest overall concentration of trace elements including heavy metals, it is difficult to trace the origin of samples. This is a potential issue for long term monitoring if this species, harvested using this method, is incorporated into commercial feed stocks.

Although concentrations measured in this study were below regulatory thresholds, chronic exposure to trace elements such as Cd and Pb can lead to bioaccumulation in cattle, particularly in hepatic and renal tissues (Ramos et al., 2016). These metals are poorly excreted, and sustained dietary intake even at low levels, and can contribute to toxicological effects such as nephrotoxicity, hepatotoxicity, and reproductive disruption (Comerford et al., 2021). Therefore, while the concentrations appear acceptable in isolated samples, cumulative effects over time must be considered before large-scale implementation in cattle-feed systems.

Additionally, while total arsenic (^{75}As) was quantified, the speciation of arsenic was not assessed. This is a critical limitation, as inorganic arsenic species (such as arsenite and arsenate) are considerably more toxic than organic species like arsenosugars, which dominate in most seaweeds (Torres et al., 2019; Laborda et al., 2020). Without speciation, it is difficult to determine the true toxicological relevance of total As values. Future analyses should employ HPLC-ICP-MS or similar techniques to differentiate between arsenic species in seaweed samples intended for animal consumption (Laborda et al., 2020).

To mitigate risks and implement seaweeds into feed-systems in the future, regular spatial monitoring of harvesting sites, inclusion of arsenic speciation in analysis protocols, and potential blending of species or site-sourced material are necessary strategies. These actions will help ensure seaweed feed additives remain within safe trace element including heavy metals contaminant levels and can be confidently introduced as methane-mitigation and nutritional supplementation strategies for cattle.

CONCLUSION

This work provided a foundational assessment of trace elements including heavy metals in three local species, and confirmed that concentrations of key elements, Al, As, Cd, Pb, and Hg, which fell below the Canadian regulatory Action Levels for livestock feed. Among the species analysed, M. *japonica* consistently demonstrated the lowest metal concentrations, suggesting it may be the most conservative choice in terms of feed safety. The results further show that sugar kelp and giant kelp, though higher in some metals, remain well within safe consumption thresholds and are still viable for feed applications if harvested from similar uncontaminated environments. These findings support the growing interest in seaweeds as a sustainable supplement in cattle feed and highlight the importance of spatial monitoring and regular contaminant analysis of biological materials used in feed additive. Moving forward, site-specific environmental assessments and species selection based on local contamination profiles will be critical for the safe and responsible use of seaweeds in livestock feed.

LITERATURE CITED

Ali, H., & Khan, E. (2018). What are heavy metals? Long-standing controversy over the scientific use of the term 'heavy metals' – proposal of a comprehensive definition. *Toxicological & Environmental Chemistry, 100*(1), 6-19. https://doi.org/10.1080/02772248.2017.1413652

Agency, C. F. I. (2015, April 9). *Rg-8 regulatory guidance:contaminants in feed(Formerly rg-1, chapter 7)* [Reference material]. http://inspection.canada.ca/en/animal-health/livestock-feeds/regulatory-guidance/rg-8

Baker, D., Karalliedde, L., Murray, V., Maynard, R., & Parkinson, N. H. (2012). *Essentials of toxicology for health protection: A handbook for field professionals*. Oxford University Press.

Baker, J. M., Handley, H. K., Christidis, G. E., & Gadd, G. M. (2018). Metal accumulation in seaweed: Implications for monitoring and bioremediation of polluted waters. *Environmental Pollution, 239*, 165-180. https://doi.org/10.1016/j.envpol.2018.04.039

Bates, Colin. 2004. An Introduction to the Algae of British Columbia. In: Klinkenberg, Brian.(Editor) 2004. E-Flora BC: Atlas of the Plants of British Columbia [www.eflora.bc.ca]. Lab for Advanced Spatial Analysis, Department of Geography, University of British Columbia, Vancouver.

Beauchemin, D. (2018). Inductively coupled plasma mass spectrometry (ICP-MS). In *Comprehensive Analytical Chemistry* (Vol. 81, pp. 503-534). Elsevier. https://doi.org/10.1016/B978-0-444-64026-0.00017-5

Bolan, N., Hoang, S. A., Beiyuan, J., Gupta, S., Hou, D., Karak, T., & Kumar, M. (2021). Contaminant removal from stormwater runoff using bioretention systems: A review. *Science of the Total Environment, 789*, 147725. https://doi.org/10.1016/j.scitotenv.2021.147725

Comerford, K. B., Miller, G. D., Kapsak, W. R., & Brown, K. A. (2021). The science of food and nutrition: Current advances and future directions. *Nutrition Reviews*, *79*(1), 6–22. https://doi.org/10.1093/nutrit/nuaa082

Comox Valley Record. (2023, March 15). Toxin pockets along B.C. coast raising red flags for salmon and orcas. *Comox Valley Record*. https://www.comoxvalleyrecord.com/news/toxin-pockets-along-bc-coast-raising-red-flags-for-salmon-and-orcas-7115744

El-Sharkawy, M., Alotaibi, M. O., Li, J., Du, D., & Mahmoud, E. (2025). Heavy metal pollution in coastal environments: Ecological implications and management strategies: a review. *Sustainability*, *17*(2), 701. https://doi.org/10.3390/su17020701

Fisher, R. M., & Gupta, V. (2025). Heavy metals. In *StatPearls*. StatPearls Publishing. http://www.ncbi.nlm.nih.gov/books/NBK557806/

Gabrielson PW, Widdowson TB, Lindstrom SC, Hawkes MW, and Scagel, RF (2000) Keys to the Benthic Marine Algae and Seagrasses of British Columbia, Southeast Alaska, Washington and Oregon. Phycological Contribution #5, University of British Columbia, Department of Botany. 189p.

Ginneken, V. van, & Vries, E. de. (2018). Seaweeds as biomonitoring system for heavy metal (Hm) accumulation and contamination of our oceans. *American Journal of Plant Sciences*, *9*(7), 1514–1530. https://doi.org/10.4236/ajps.2018.97111

Gupta, V. K., & Balomajumder, C. (2015). Biosorption technology for removal of heavy metals. In P. Singh & S. Kumar (Eds.), *Advances in Bioremediation and Phytoremediation* (pp. 89-118). Springer.

Laborda, F., Trujillo, C., Lobinski, R., & Monperrus, M. (2020). A tutorial on the validation of methods for quantification of metal species by HPLC/ICP-MS in biological samples. *Analytica Chimica Acta, 1104*, 1-16. https://doi.org/10.1016/j.aca.2020.01.045

Leopold, K., Foulkes, M., & Worsfold, P. (2021). Methods for the determination of heavy metals and metalloids in environmental samples. *Analytical Methods, 13*(5), 538-556. https://doi.org/10.1039/D0AY02034G

Li, Y., Ross, P. S., & Dangerfield, N. (2023). Sediment spatial distribution and quality assessment of metals in coastal British Columbia, Canada. *Environmental Science and Pollution Research, 30*(5), 12345-12360. https://doi.org/10.1007/s11356-022-22345-6

Maguire, J., Tiwari, B. K., & Garcia-Vaquero, M. (2023). *Anti-methanogenic potential of seaweeds and seaweed-derived compounds in ruminant feed: Current perspectives, risks and future prospects.* **Journal of Animal Science and Biotechnology, 14**, Article 145. https://doi.org/10.1186/s40104-023-00946-w

Pierceg, K., Slesak, J., & Ferrier, M. D. (2020). Seaweed as a sustainable animal feed: Opportunities and challenges. *Frontiers in Sustainable Food Systems, 4*, Article 128. https://doi.org/10.3389/fsufs.2020.00128

Ramos-Miras, J. J., Gil, C., Boluda, R., & Rodríguez-Martín, J. A. (2020). ICP-MS determination of heavy metals and rare earth elements in environmental samples: Challenges and considerations. *Microchemical Journal, 154*, 104610. https://doi.org/10.1016/j.microc.2020.104610

Ramos, S. J., Dinali, G. S., Oliveira, C., Martins, G. C., Moreira, C. G., & Guilherme, L. R. (2016). Heavy metal contamination in animal-based food products and its toxic effects in humans. *Environmental Science and Pollution Research, 23*(16), 16472-16487. https://doi.org/10.1007/s11356-016-6779-0

Rajeshkumar, S., Liu, Y., Zhang, X., Ravikumar, B., Bai, G., & Li, X. (2018). Studies on seasonal pollution of heavy metals in water, sediment, fish and oyster from the Yangtze River Estuary, China. *Marine Pollution Bulletin, 133*, 218-227. https://doi.org/10.1016/j.marpolbul.2018.05.028

Torres, F. M., Ulloa, P. A., Nejamkin, P., & Aedo, C. A. (2019). Accumulation and speciation of arsenic in brown algae from polluted sites in northern Chile. *Marine Pollution Bulletin, 138*, 429-437. https://doi.org/10.1016/j.marpolbul.2018.11.028

Wu, X., Yang, K., Lu, J., Li, B., Li, Y., Zhang, Y., & Ye, L. (2025). Contamination and ecological risk of heavy metals in sediments of urban rivers in a typical economic development zone, southern China. *Journal of Environmental Sciences*, *153*, 264–274. https://doi.org/10.1016/j.jes.2024.09.006

Zhou, T. (2023). Heavy metal water pollution in British Columbia, Canada. *Master of Land and Water Systems*. University of British Columbia. https://lfs-mlws-2020.sites.olt.ubc.ca/files/2023/11/Tianxuan-Zhou-MLWS-2023.pdf

CHAPTER 5: GENERAL DISCUSSION

Summary of Research Findings

This study investigated the chemical profiles of three locally abundant seaweeds, *Macrocystis pyrifera* (giant kelp), *Saccharina latissima* (sugar kelp), and *Mazzaella japonica* (*M. japonica*), for their potential use as cattle feed additives to reduce enteric methane emissions in British Columbia (B.C.). Through the analysis of omega-6 to omega-3 (n-6/n-3) fatty acid ratios, phlorotannin content, and heavy metals accumulation, this research provided a local assessment of seaweed suitability for methane mitigation and nutrition enhancement of animal derived products in the cattle industry using various analytical approaches.

Fatty acid analysis via ^{1}H NMR revealed that both giant kelp and sugar kelp possess favourable n-6/n-3 ratios, contributing to improved animal health and reduced inflammation, which may also reduce the amount of methane expelled from ruminants (Patra, 2013; Daley et al., 2010). Of the three species, giant kelp was found to contain the highest overall quantity of omega 6's and omega 3's, positioning this species as a promising fatty acid supplement and feed-additive. In contrast, *M. japonica* displayed low extractable lipid content using the method at hand and as such, fatty acid characterization by ^{1}H NMR was difficult and limited in scope.

Phlorotannin analysis highlighted the presence of polyphenols in both brown seaweed species. Phlorotannins have been shown to alter rumen enteric fermentation, supress methanogens, and improve nitrogen utilization in cattle, suggesting overall methane mitigation (Min et al., 2021; Abbot et al., 2023). Analysis varied by method and species, indicating the need for further optimization of extraction and detection protocols using ^{1}H NMR and CE instrumentation. F-C assay revealed the presence of total polyphenols in both species with higher concentrations shown in sugar kelp, however, variation between trials prevented method validation.

Trace elements including heavy metals analysis confirmed that concentrations in all three species were within acceptable and safe thresholds for livestock feed as defined by the Government of Canada's RG-8 Regulatory Guidance: Contaminants in Feed (CFIA, 2015). Species-specific accumulation differences were noted, specifically sugar kelp showing slightly higher levels of arsenic compared to giant kelp, both of which were cultivated and

harvested from the same location. This emphasizes the variability in trace elements including heavy metals uptake based on growing location, tissue type, and other environmental factors (Banach et al., 2020).

Collectively, these findings suggest that among these three species, giant kelp and sugar kelp may offer the most promise as a feed additive for further *in vitro* and *in vivo* analyses due to their favourable fatty acid content/profile, moderate phlorotannin content, and low trace elements including heavy metals accumulation. *M. japonica's* potential may be limited without further characterization and optimization of analysis techniques.

Contributions to Environmental Science

The integration of seaweed into cattle-feed systems aligns with global and national efforts to reduce agricultural greenhouse gas emissions (GHGs), and to promote the use of more sustainable and regenerative food systems. As agricultural practices account for about 10% of Canada's total GHG emissions, with enteric methane as a leading contributor (Government of Canada, 2023), innovative feed strategies are essential. Seaweeds abundant on the coast of B.C. offer a sustainable, non-competitive biomass that does not require arable land or freshwater resources, positioning them as viable supplements to pre-existing feed and forage systems (Duarte et al. 2017; Buschmann et al., 2017).

The increased cultivation of seaweeds, if done in a sustainable and regenerative manner, can promote nutrient absorption (nitrogen and phosphorus; typically introduced to marine environments through industrial and agricultural practices) that prevents eutrophication (Zheng et al., 2019). Kelp biomass cultivation supports many marine ecosystem services such as the enhancement of biodiversity, CO_2 sequestration, shore erosion protection from storm systems, and nutrient cycling (Chung et al., 2017). In B.C., kelp aquaculture is gaining interest as a blue economy solution, and this research supports its potential integration into regional agricultural systems from a sustainability perspective.

However, as interest in seaweed-based solutions becomes more popular, it is crucial that cultivation and harvesting remain ecologically sustainable. Overharvesting can negatively impact marine ecosystems by altering species composition, damaging habitats, and reducing the reproductive capabilities of naturally occurring seaweed populations (Bermejo et al., 2022). For example, the unregulated harvesting of wild kelps in Chile has led to biodiversity

decline and has disrupted the habitats of key species, including shellfish, a very commercially important species (Vasquez et al., 2012). Additionally, dense monoculture farming can facilitate disease outbreaks and reduce ecosystem resilience (Park et al., 2018). These issues emphasize the importance of ecosystem-based management the potential integration of multi-trophic aquaculture systems to promote environmental balance. Ensuring seaweed farming contributes positively to local ecosystems is essential for it to be a viable long-term solution in livestock feed and other sea-based additives/applications.

Strengths and Limitations

A key strength of this research is the focus on regionally abundant species, contributing to the limited literature on North Pacific seaweeds in the context of application to ruminant feed. Additionally, the use of non-destructive analytical techniques like ^{1}H NMR, which require very little sample preparation, reagents and solvent, offers a sustainable and rapid method for chemical detection, and reliable data for n-6/n-3 ratio determinations in seaweeds. The research is limited by the inability to test *in vivo* emissions, which is required to confirm the efficacy of these seaweeds in ruminant diets and is the appropriate next step to progress this research. Seasonal variations, sample size, and aquaculture collection methods were not controlled, and only preliminary CE data for fatty acids were obtained, limiting direct comparison across analytical methods. Additionally, specific quantification of phlorotannins in each species remains to be evaluated.

Applications and Significance

Findings from this study may guide the future development of seaweed-supplemented feed for western Canadian cattle operations. Giant kelp, as well as sugar kelp, show potential as sustainable and regionally available feed additives to both enhance ruminant health and meat/dairy products and potentially supress methane emissions through both nutritional and biochemical mechanisms. Their implementation into feed systems may contribute to Canada's 2030 Emissions Reduction Plan while creating opportunities for local seaweed aquaculture expansion, as well as the formation of new partnerships between B.C. aquaculture and agriculture (Government of Canada, 2025).

Another important contribution this research makes is to the development of analytical methods. The successful use of ^{1}H NMR as a rapid and efficient tool for quantifying n-6/n-3

ratios provides a viable screening approach for future similar feedstock assessments and applications. While pilot trials with CE presented challenges, they provide valuable insights into the limitations of current methods and may inform future optimization efforts. These analytical trials offer preliminary data as stepping stones toward more efficient evaluation techniques for seaweed-based extracts.

Beyond direct scientific contributions, this research has practical relevance for the advancement of integrated agriculture-aquaculture systems in B.C. As public and governmental support grows for green agri-tech solutions, the incorporation of locally farmed seaweed into cattle feed could be facilitated through collaborative programs involving aquaculture operations and livestock producers. Small-scale implementation could commence with mixed-ration trials on coastally located farms through the utilization of surplus kelp biomass. Through the development of this supply chain over time, connections between seaweed harvesters and feed manufacturers could foster a new circular bioeconomy within the province, maximizing the value of marine resources without displacing existing feed industries (Kim et al., 2017; Purcell-Meyenrink et al., 2021). Governmental support, particularly through programs under the Sustainable Canadian Agricultural Partnership or Fisheries and Oceans Canada's Blue Economy Strategy, could further support this initiative (DFO, 2022).

Seaweed-fed cattle may also present new market opportunities to beef and dairy producers. As consumers are generally interested in low emission and sustainable raised animal products, producers who incorporate seaweed into cattle feed may be able to access eco-labels or climate-friendly certifications that add value to their products (Vijn et al., 2020; Abbott et al., 2023). Additionally, as described in Chapter 2: Fatty Acids, the fatty acid profile of feed consumed alters the fatty acid profile of resulting meat and dairy products. As such, feeding omega-3 and omega-6 rich seaweeds to cattle will likely influence omega-3 and omega-6 content in animal products, creating a "healthier" product for enhanced marketability. These market opportunities could be of particular benefit for B.C. producers who operate in competitive export markets. As seaweed applications in ruminant feed research expands, this research offers a regional foundation for future studies.

Future Research Directions

To advance the integration of local seaweeds in livestock feed systems, certain research priorities are essential. Giant kelp and sugar kelp should be assessed in both *in vitro* and *in vivo* methane suppression trials to validate their potential for anti-methanogenic mechanisms. Phlorotannin extraction and detection methods should be optimized for consistent and reliable results. Future research may also want to include an analysis of seasonal and spatial variability of bioactive compounds in seaweeds, the economic feasibility of seaweed inclusion in local agricultural structures and conduct a life cycle assessment of seaweed-supplemented feed products to analyse their net environmental impacts.

Concluding Remarks

This body of work provides an initial assessment of the chemical composition of giant kelp, sugar kelp, and *M. japonica,* with a focus on their suitability as livestock feed additives to combat enteric methane emissions. The analysis of fatty acid profiles, phlorotannin presence, and trace elements including heavy metals concentrations also offers a foundation for evaluating the potential nutritional and functional benefits of these species through a western Canadian agricultural lens.

Although the scope of this research was limited to laboratory-based chemical characterization, the findings contribute to a rapidly expanding body of literature supporting the exploration and potential inclusion of seaweeds into sustainable livestock feeding frameworks. The application of low-solvent and rapid analytical techniques such as ^{1}H NMR highlights the potential for more efficient and environmentally conscious approaches to detection and quantification of chemical compounds of interest.

Continued investigation, particularly through *in vitro* and *in vivo* studies, will be crucial to validating the impacts of these species on enteric methane emissions and animal health. Nonetheless, the present study provides a useful starting and reference point for future interdisciplinary work; and highlights the importance of localized, sustainable, and scalable solutions when addressing climate and sustainability challenges within the agricultural sector.

LITERATURE CITED

Abbott, J., Min, B. R., Renaud, D. L., & McAllister, T. A. (2023). Anti-methanogenic potential of seaweeds and seaweed-derived compounds in ruminant feed: Current perspectives, risks and future prospects. *Journal of Animal Science and Biotechnology*, 14(1), 47. https://doi.org/10.1186/s40104-023-00946-w

Banach, J. L., Hoek-van den Hil, E. F., & van der Fels-Klerx, H. J. (2020). Food safety hazards in the European seaweed chain. *Comprehensive Reviews in Food Science and Food Safety*, 19(2), 332–364. https://doi.org/10.1111/1541-4337.12524

Bermejo, R., Radulovich, R., & Buschmann, A. H. (2022). Sustainability of wild seaweed harvesting for food and industrial applications. *Reviews in Fisheries Science & Aquaculture*, 30(3), 379–397. https://doi.org/10.1080/23308249.2021.1917062

Buschmann, A. H., et al. (2017). Seaweed production: Overview of the global state of exploitation, farming and emerging research activity. *European Journal of Phycology*, 52(4), 391–406.

Chung, I. K., et al. (2017). Installing kelp forests/seaweed beds for mitigation and adaptation against global warming: Korean project overview. *ICRI Proceedings*, 22–28.

Daley, C. A., et al. (2010). A review of fatty acid profiles and antioxidant content in grass-fed and grain-fed beef. *Nutrition Journal*, 9(10), 1–12. https://doi.org/10.1186/1475-2891-9-10

DFO (Fisheries and Oceans Canada). (2022). *Canada's Blue Economy Strategy: What We Heard Report*. Government of Canada. https://www.dfo-mpo.gc.ca/campaign-campagne/bes-seb/index-eng.html

Duarte, C. M., et al. (2017). Can seaweed farming play a role in climate change mitigation and adaptation? *Frontiers in Marine Science*, 4, 100. https://doi.org/10.3389/fmars.2017.00100

Government of Canada. (2023). *National greenhouse gas inventory: 1990–2021*. Environment and Climate Change Canada.

Government of Canada. (2025). *2030 Emissions Reduction Plan*. https://www.canada.ca/en/services/environment/weather/climatechange/climate-plan.html

Kim, J. K., Kraemer, G. P., & Yarish, C. (2017). Development of a simple seedstring for improving the efficiency of sugar kelp (*Saccharina latissima*) aquaculture. *Journal of Applied Phycology, 29*(1), 411–420. https://doi.org/10.1007/s10811-016-0959-z

Min, B., Solaiman, S., & Gurung, N. (2021). Seaweed supplementation for methane mitigation in ruminants: A review. *Agronomy*, 11(3), 494. https://doi.org/10.3390/agronomy11030494

Patra, A. K. (2013). The effect of dietary fats on methane emissions from ruminants: A meta-analysis. *Animal Feed Science and Technology*, 178(2–4), 161–175. https://doi.org/10.1016/j.anifeedsci.2012.10.006

Purcell-Meyenrink, M., Walker, T. R., & Scheinberg, J. (2021). Seaweed aquaculture in British Columbia: A case study for sustainable coastal development. *Marine Policy, 134*, 104792. https://doi.org/10.1016/j.marpol.2021.104792

Vásquez, J. A., Piaget, N., & Vega, J. M. A. (2012). The lessonia nigrescens fishery in northern Chile: Management and sustainability of the resource. *Journal of Applied Phycology*, 24(3), 395–399. https://doi.org/10.1007/s10811-011-9721-4

Vijn, S., Compart, D., Dutta, N., Foukis, A., Hess, M., Hristov, A. N., ... & Tricarico, J. (2020). Key considerations for the use of seaweed to reduce enteric methane emissions from cattle. *Frontiers in Veterinary Science, 7*, 597430. https://doi.org/10.3389/fvets.2020.597430

Zheng, Y., Jin, R., Zhang, X., Wang, Q., & Wu, J. (2019). The considerable environmental benefits of seaweed aquaculture in China. *Stochastic Environmental Research and Risk Assessment*, *33*(4), 1203–1221. https://doi.org/10.1007/s00477-019-01685-z

APPENDIX A: SUPPLEMENTARY INFORMATION FOR CHAPTER 2 – FATTY ACID ANALYSIS

A.1. ^{1}H NMR Fatty Acid Analysis

Table B.1.1 *Seaweed Sample Weights for Initial ^{1}H NMR Analysis, Comparison of Oven-Drying (OV) and Dehydration Drying (DH)*

Drying Method	Trial #	Sugar Kelp (g)	Giant Kelp (g)	*M. japonica* (g)
DH	1	0.2051	0.2040	0.2043
	2	0.2045	0.2059	0.2052
	3	0.2043	0.2010	0.2024
OV	1	0.2005	0.2028	0.2034
	2	0.2042	0.2035	0.2019
	3	0.2046	0.2044	0.2016

Table A.1.2 *Seaweed Sample Weights for Secondary ^{1}H NMR Analysis, Dehydrated Samples Only*

Trial #	Sugar Kelp (g)	Giant Kelp (g)	*M. japonica* (g)
1	0.2011	0.2024	0.2040
2	0.2003	0.2044	0.2042
3	0.2019	0.2016	0.2081

Table A.1.3 *^{1}H NMR Chemical Shifts for Fatty Acids of Note Adapted from Siudem et al. (2022)*

Number	Compounds	Chemical Structure	δ (ppm)
1.	All fatty acids except linolenic acid	$-CH_2-CH_3$	0.89
2.	Linolenic acid	-CH=CH- CH_2-CH_3	0.97
3.	Acyl chains	-(CH)n-	1.31
4.	Acyl chains	$-CH_2-CH_2-COOH$	1.61
5.	Mono- and polyunsaturated fatty acids	$-CH_2-CH=CH-$	2.04
6.	Acyl chains in unsaturated fatty acids	$-CH_2-COOH$	2.31
7.	**Linoleic and linolenic acid**	**$-CH=CH-CH_2-CH=CH-$**	**2.77**
8.	Triacylglycerols	$-CH_2-OCO-$	4.27
9.	Triacylglycerols	-CH-OCO-	5.28
10.	Mono- and polyunsaturated fatty acids	-CH=CH-	5.35

Table A.1.4 *Standard Deviation and Coefficient of Variation of n-6/n-3 Ratios for Oven-Dried and Dehydrated Seaweed Sampling using ^{1}H NMR*

Drying Technique	*M.japonica*	Sugar Kelp	Giant Kelp
	Standard Deviation		
DH	0.068643402	0.041836226	0.029114547
OV	0.085683215	0.113580175	0.016127975
	Coefficient of Variation (%)		
DH	194.4538867	24.95312906	9.51575802
OV	138.406826	85.51910929	4.541608078

Table A.1.5 *Intra-day Precision (Repeatability) Analysis of Dehydrated Sugar Kelp and Giant Kelp*

Day	Sugar Kelp	Giant Kelp
	Standard Deviation	
1	0.051925363	0.040892909
2	0.037058332	0.036248034
3	0.022062638	0.019087518
	% RSD or Coefficient of Variation	
1	21.00823861	12.13439434
2	16.35407431	10.43409166
3	7.993709457	5.84908613

Table A.1.6 *Inter-day Precision Analysis across all Dehydrated Sugar Kelp and Giant Kelp Samples*

	Sugar Kelp	**Giant kelp**
Standard Deviation	0.0400	0.0303
CV or % RSD	16.0095	9.0069

Table A.1.7 *Full 1H NMR n-6/n-3 Fatty Acid Ratio Results for Initial Trial – Oven Dried and Dehydrated Seaweed Samples*

256 Scans			
Trial #	***M.japonica***	**Sugar Kelp**	**Giant Kelp**
1	-0.027	0.213	0.284
2	0.083	0.160	0.295
3	0.098	0.130	0.339
DH mean	0.051	0.168	0.306
1	0.035	0.054	0.348
2	0.157	0.078	0.344
3	-0.007	0.262	0.374
OV mean	0.062	0.131	0.355

Table A.1.8 *Full ^{1}H NMR n-6/n-3 Fatty Acid Ratio Results for Secondary 3-Day Trial – Dehydrated Seaweed Samples*

256 Scans- Phase Adjusted			
Day of Trial	Trial #	**Sugar Kelp**	**Giant Kelp**
Day 1	1	0.24	0.32
	2	0.30	0.31
	3	0.20	0.38
	Mean	0.25	0.34
Day 2	1	0.18	0.32
	2	0.25	0.33
	3	0.24	0.39
	Mean	0.23	0.35
Day 3	1	0.30	0.31
	2	0.27	0.32
	3	0.26	0.35
	Mean	0.28	0.33

Table A.1.9 *^{1}H NMR Fatty Acid Composition of Seaweeds Results from Initial Trial*

Drying Method	Trial #	I(2)/ (I(2) + I(1)	1/2((I(7)-4(ALA))	(I(9)-4LA-6ALA)/2	Oleic+LA+ALA
		ALA	LA	Oleic	Total Unsaturated
			M.japonica		
oven-dried	NA-1-40-1	0.1809	-0.3603	0.1792	-0.0002
	NA-1-40-2	0.1125	0.3600	-0.7559	-0.2834
	NA-1-40-3	0.1281	0.2754	-0.2341	0.1694
dehydrated	NA-1-40-4	0.2244	-0.2864	0.0252	-0.0368
	NA-1-40-5	0.2202	-0.2795	-0.0150	-0.0743
	NA-1-40-6	0.2507	-0.3339	-0.0119	-0.0951
			Sugar Kelp		
oven-dried	NA-1-40-7	0.4069	-0.2389	-0.5299	-0.3620
	NA-1-40-8	0.5234	-0.8558	0.1759	-0.1566
	NA-1-40-9	0.5312	-0.6199	-0.2420	-0.3308
dehydrated	NA-1-40-10	0.4295	-0.2522	-0.6129	-0.4356
	NA-1-40-11	0.3844	-0.2387	-0.5248	-0.3792
	NA-1-40-12	0.5035	-0.4931	-0.3805	-0.3701
			Giant Kelp		
oven-dried	NA-1-40-13	0.4009	-0.1686	-0.7608	-0.5284
	NA-1-40-14	0.4050	-0.5677	-0.0266	-0.1893
	NA-1-40-15	0.2691	0.1079	-0.9056	-0.5286
dehydrated	NA-1-40-16	0.3795	-0.1877	-0.6487	-0.4569
	NA-1-40-17	0.4054	-0.2005	-0.6897	-0.4848

	NA-1-40-18	0.4193	-0.5814	-0.0423	-0.2045

A2. CE Fatty Acid Analysis

Table A.2.1 *FA Stock Solution Concentrations (ppm)*

Fatty Acid	**Mass (g) or volume (µL) of FA**	**Total Stock Volume (L)**	**FA Stock Concentration (mg/L or ppm)**
Lauric acid	0.0039 g	0.020	195
Myristic acid	0.0031 g	0.020	155
Palmitic acid	0.0032 g	0.020	160
Stearic acid	0.0035 g	0.020	175
Linoleic acid	20.8 µL	0.075	249.6
α-Linolenic acid	6.8 µL	0.025	248.6
ɣ-Linolenic acid	6.8 µL	0.025	251.3
Arachidonic acid	12.2 µL	0.075	149.97
Stearidonic acid	4.02 µL	0.025	156.8
Eicosapentaenoic acid (EPA)	3.98 µL	0.025	150.2
Docosahexaenoic acid (DHA)	0.0025 g	0.016667	149.99

Table A.2.2 *Lauric Acid Standards – Concentration and Peak Area Results*

Concentration (ppm)	FA Peak Area/ IS Peak Area
0	0
6.53	0.1620
19.5	0.1543
39	0.1474
65.3	0.2593
97.5	0.6005
129.7	2.1915

Graph A.2.1 *Lauric Acid Calibration Curve via CE*

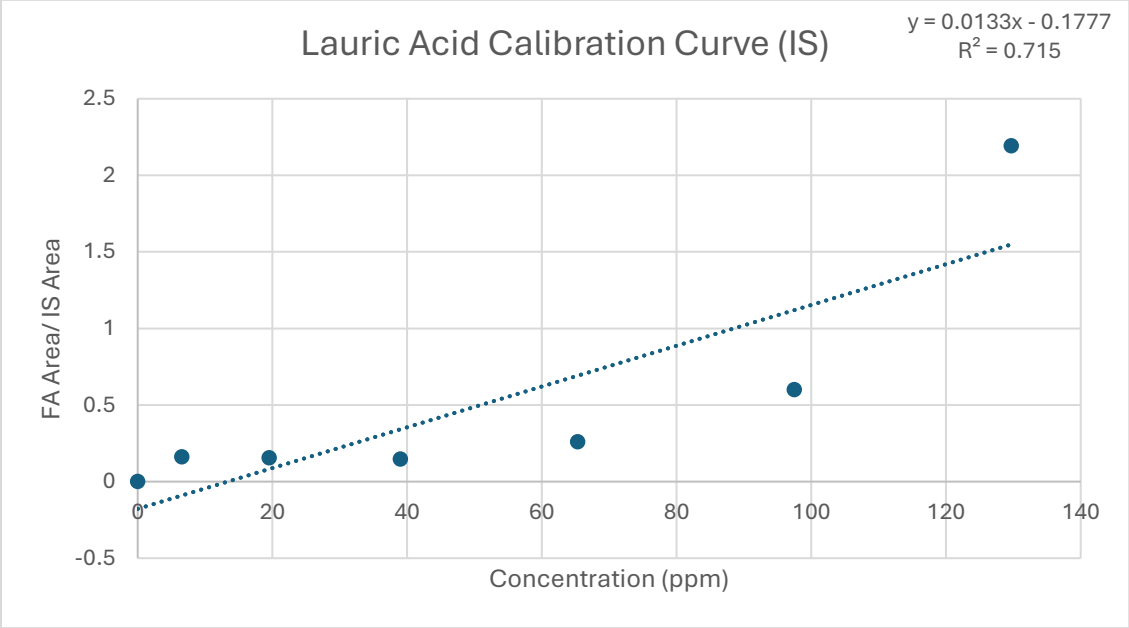
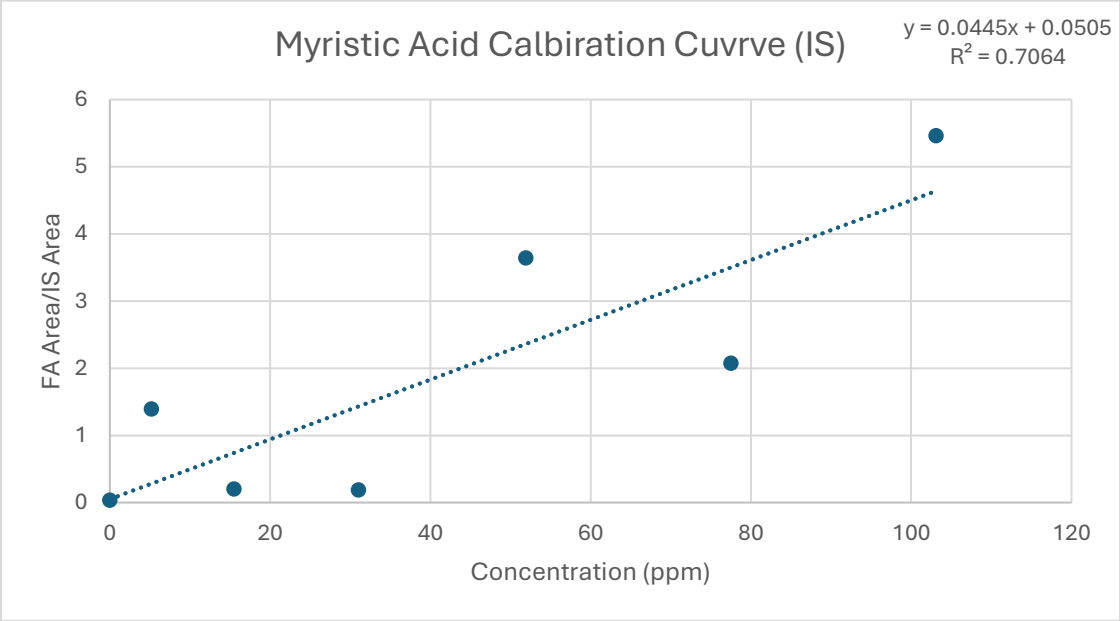


Table A.2.3 *Lauric Acid Standards – Concentration and Peak Area Results*

Concentration (ppm)	FA Peak Area/ IS Peak Area
0	0.0345
5.19	1.3954
15.5	0.2037
31	0.1879
51.9	3.6415
77.5	2.0739

103.1	5.4627

Graph A.2.2 *Myristic Acid Calibration Curve via CE*



APPENDIX B: SUPPLEMENTARY INFORMATION FOR CHAPTER 3 – PHLOROTANNIN ANALYSIS

B.1 Phloroglucinol Detection by ^{1}H NMR

Table B.1 *Dehydrated Seaweed Sample Weights for Phloroglucinol Determination via ^{1}H NMR*

Species	Sample Weight (g)
Sugar Kelp	0.1988
Giant Kelp	0.2047

Figure B.1.1 *^{1}H NMR Spectrum of Sugar Kelp in DMSO-d_6 Highlighting the Absence of Phloroglucinol Peaks*

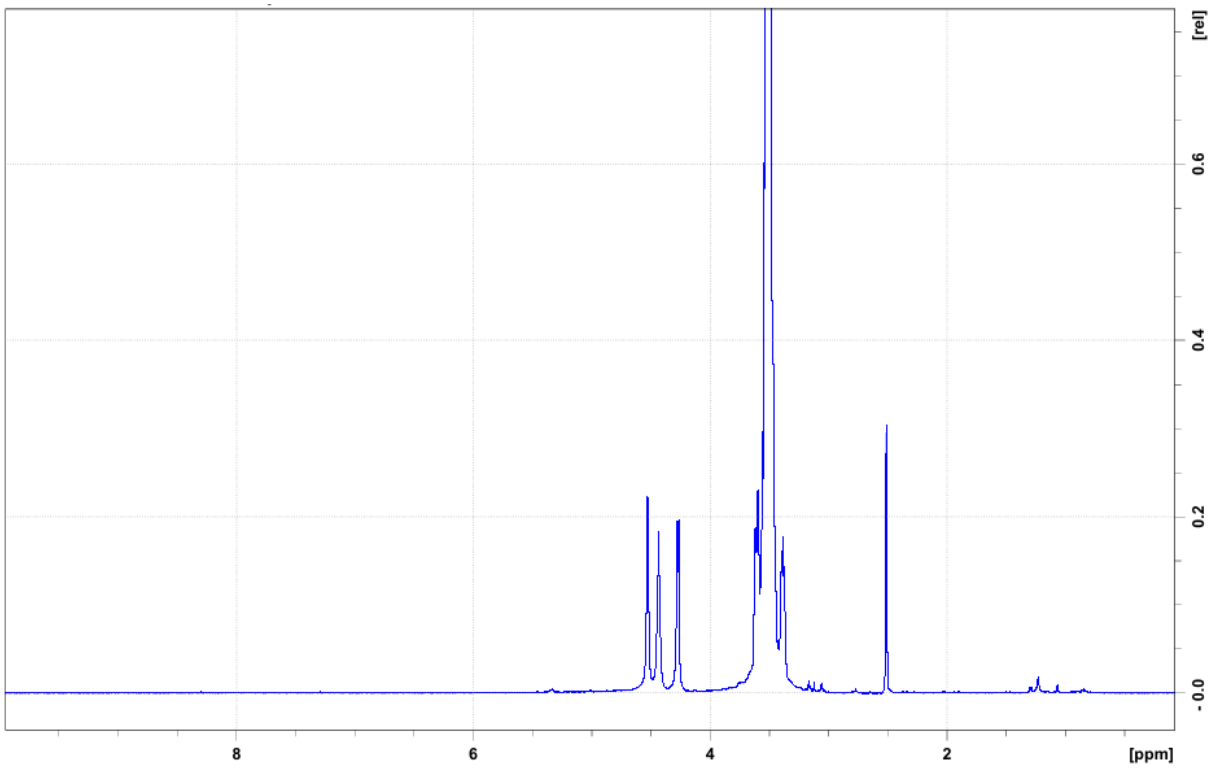


Figure B.1.2 *^{1}H NMR Spectrum of Sugar Kelp in D2O*

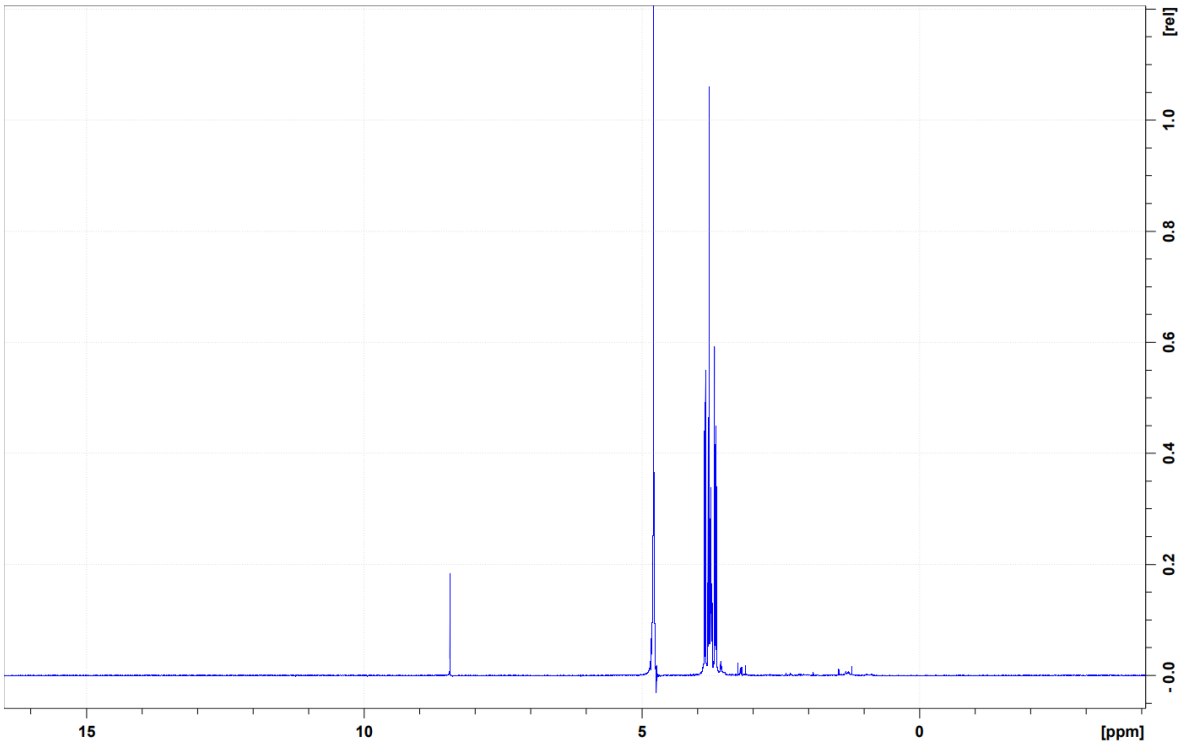


Figure B.1.3 *^{1}H NMR Spectrum of Giant Kelp in DMSO-d_6*

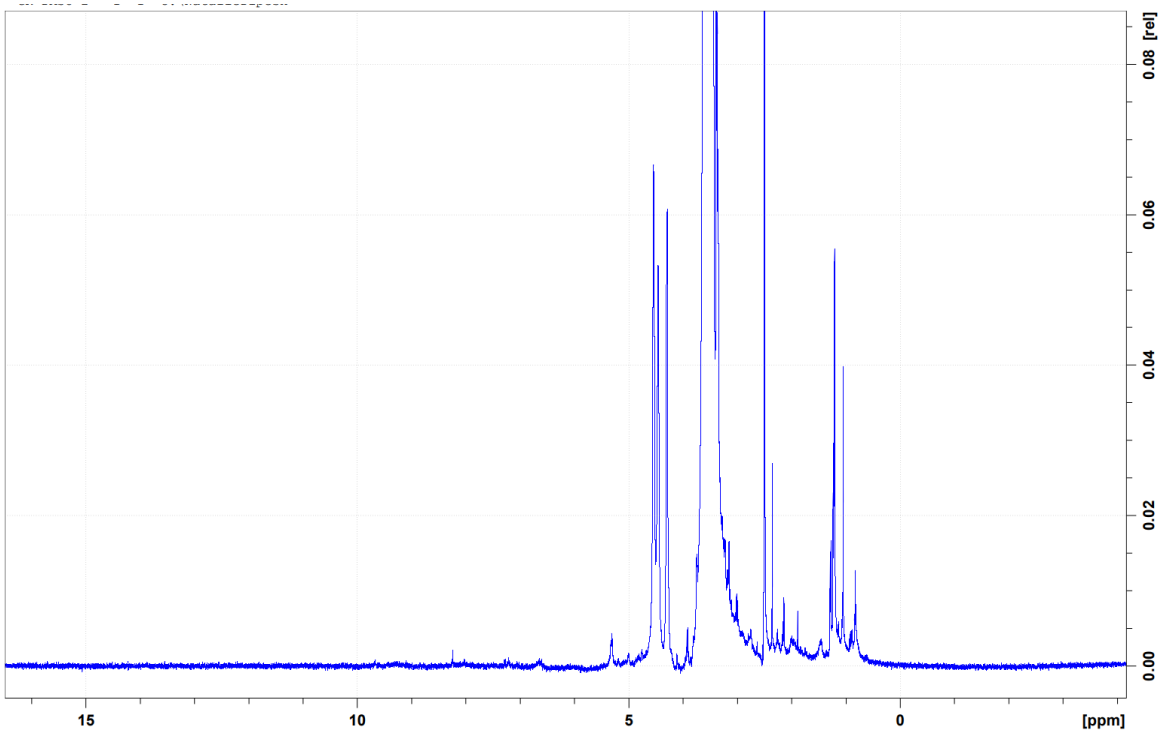


Figure B.1.4 *^1H NMR Spectrum of Sugar Kelp in D_2O after an Acidified Extraction*

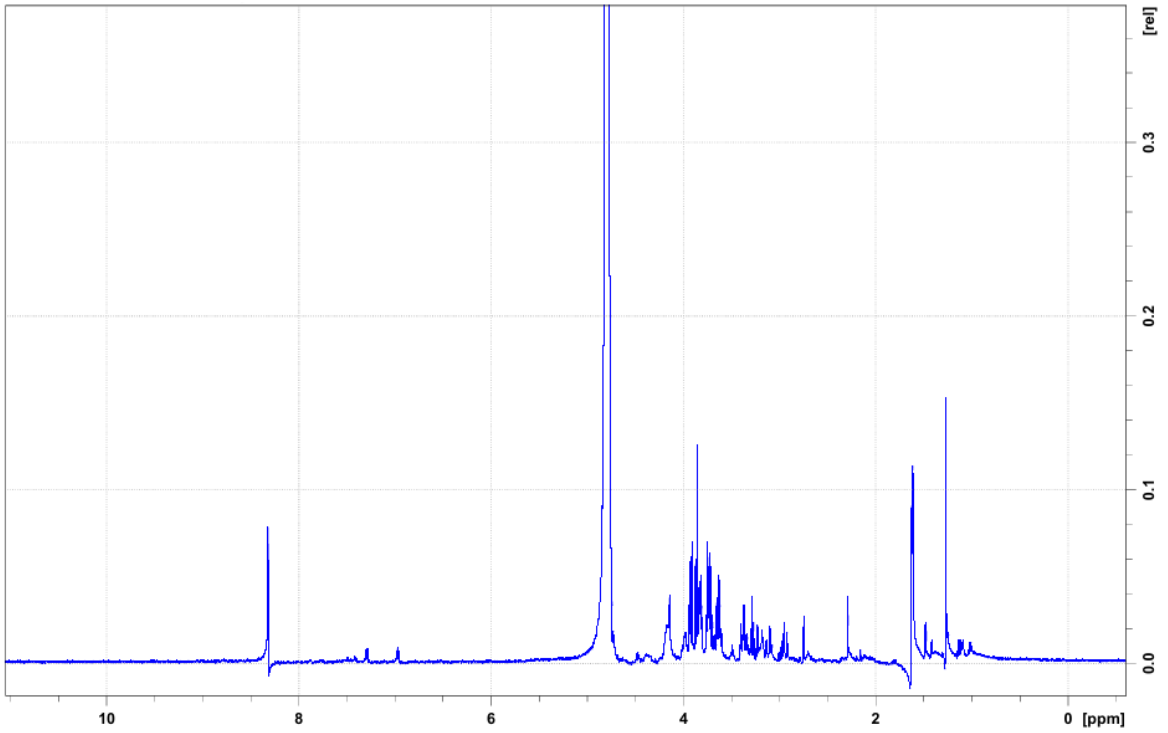
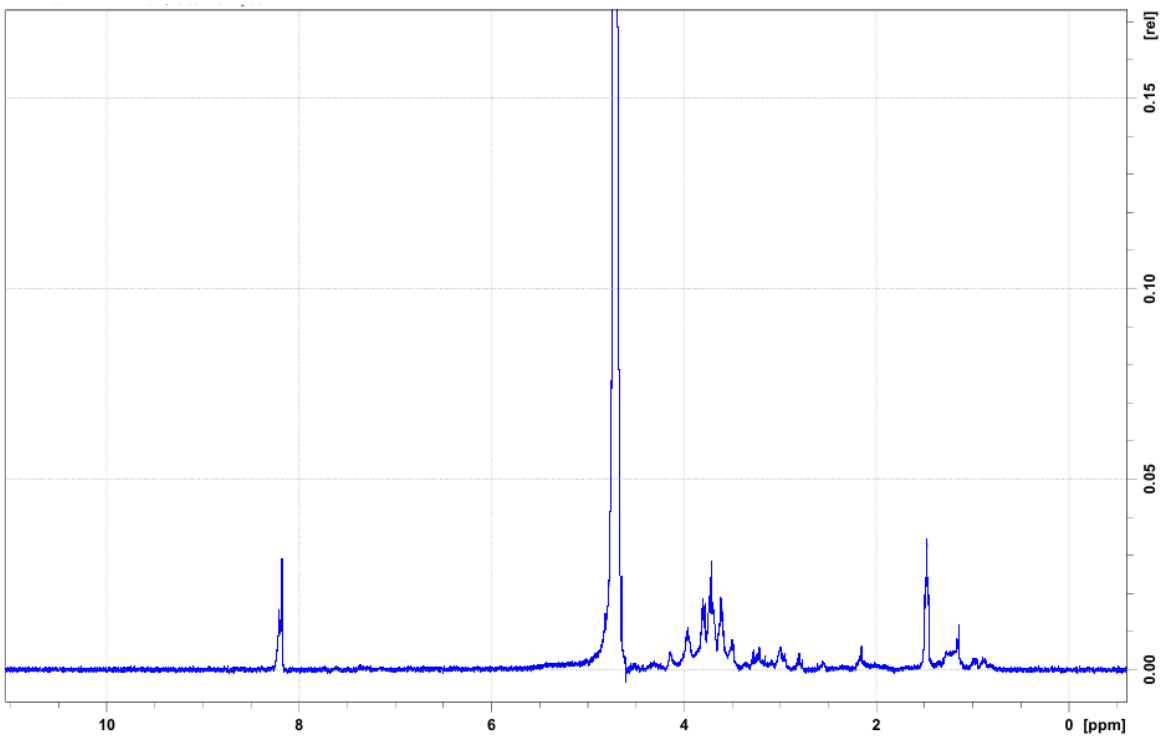


Figure B.1.5 *^1H NMR Spectrum of Giant Kelp in D_2O after an Acidified Extraction*



B.2 Total Polyphenol Content Determination by F-C Assay

Table B.2.1 *Dehydrated Seaweed Sample Weights for Total Polyphenol Content Determination using F-C Assay*

Trial	Sugar Kelp	Giant Kelp
Development time trial	0.5019g	0.5008g
2 hours – 2nd trial	0.5015g	0.5025g
2 hours – 3rd trial	0.5013g	0.5003g

Table B.2.2 *F-C Assay Phloroglucinol Standards and Samples Composition*

Standard #/ Samples	Volume of Phloroglucinol Standard (mL)	Volume of F-C Reagent (mL)	Volume of DH_2O (mL)	Volume of Sample Extract (mL)
S0	0	2	8	-
S1	1	2	7	-
S2	2	2	6	-
S3	3	2	5	-
S4	4	2	4	-

S5	5	2	3	-
SK	-	2	7	1
GK	-	2	7	1

Table B.2.3 *F-C Assay Development Time Trial Absorbances and Concentrations*

Time (hours)	**Blank**	**S1**	**S2**	**S3**	**S4**	**S5**	**SK**	**GK**
Absorbance								
2	0.000	0.042	0.066	0.086	0.103	0.117	0.067	0.074
4	0.000	0.073	0.111	0.141	0.167	0.185	0.081	0.102
6	0.000	0.089	0.133	0.169	0.199	0.221	0.089	0.114
Concentration (mg/mL)								
2	0	0.0513	0.1026	0.1539	0.2052	0.2565	2.4740*	2.7940*
4	0	0.0513	0.1026	0.1539	0.2052	0.2565	1.8616*	2.4712*
6	0	0.0513	0.1026	0.1539	0.2052	0.2565	1.5706*	2.1809*

Note: *Concentration of sugar kelp and giant kelp presented have been multiplied by a dilution factor of 20 (10 mL/0.50 mL)

Table B.2.4 *Mean, Standard Deviation, and % RSD of Phloroglucinol (mg/mL) in Sugar Kelp and Giant Kelp Determined by F-C Assay over a 2-Hour Time Development*

Trial #	Phloroglucinol in **Sugar Kelp** (mg/mL)	Phloroglucinol in **Giant Kelp** (mg/mL)
1	2.47	2.79
2	1.30	1.13
3	3.07	1.92
Mean	2.28	1.95
SD	0.9005	0.8313
% RSD	39.4765	42.6326

Figure B.2.1 *Concentration of Phloroglucinol (mg/mL) Standards after Two Hours of Development using F-C Assay – Trial 1*

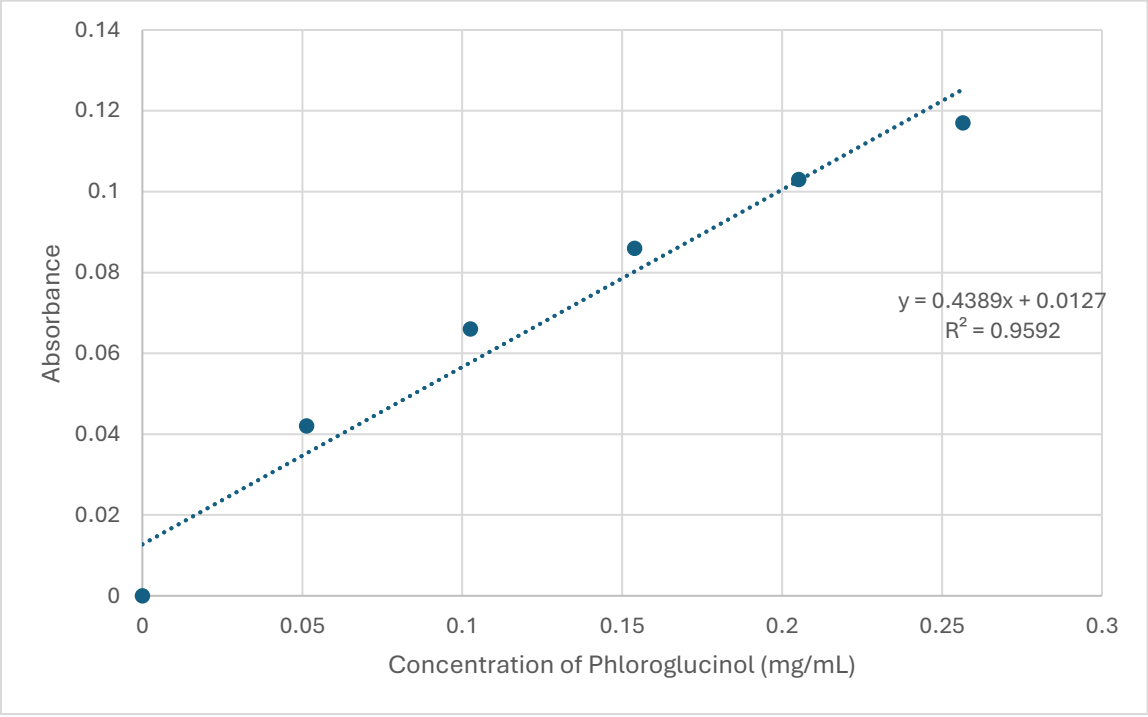


Figure B.2.2 *Concentration of Phloroglucinol (mg/mL) Standards after Two Hours of Development using F-C Assay – Trial 2*

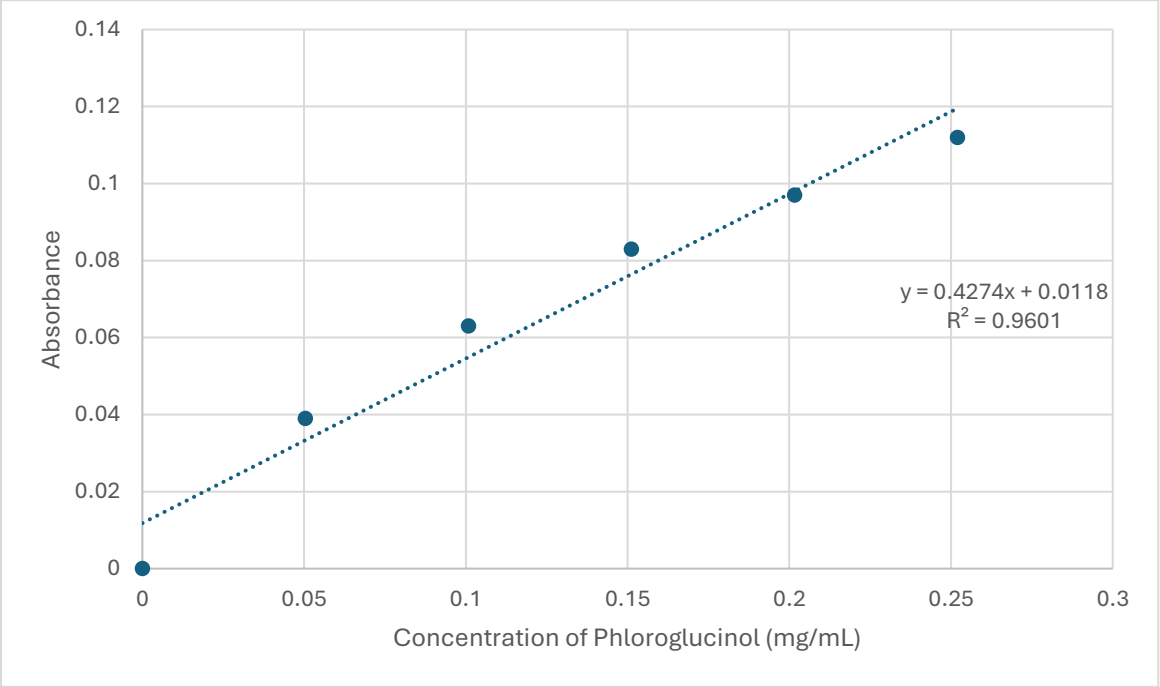
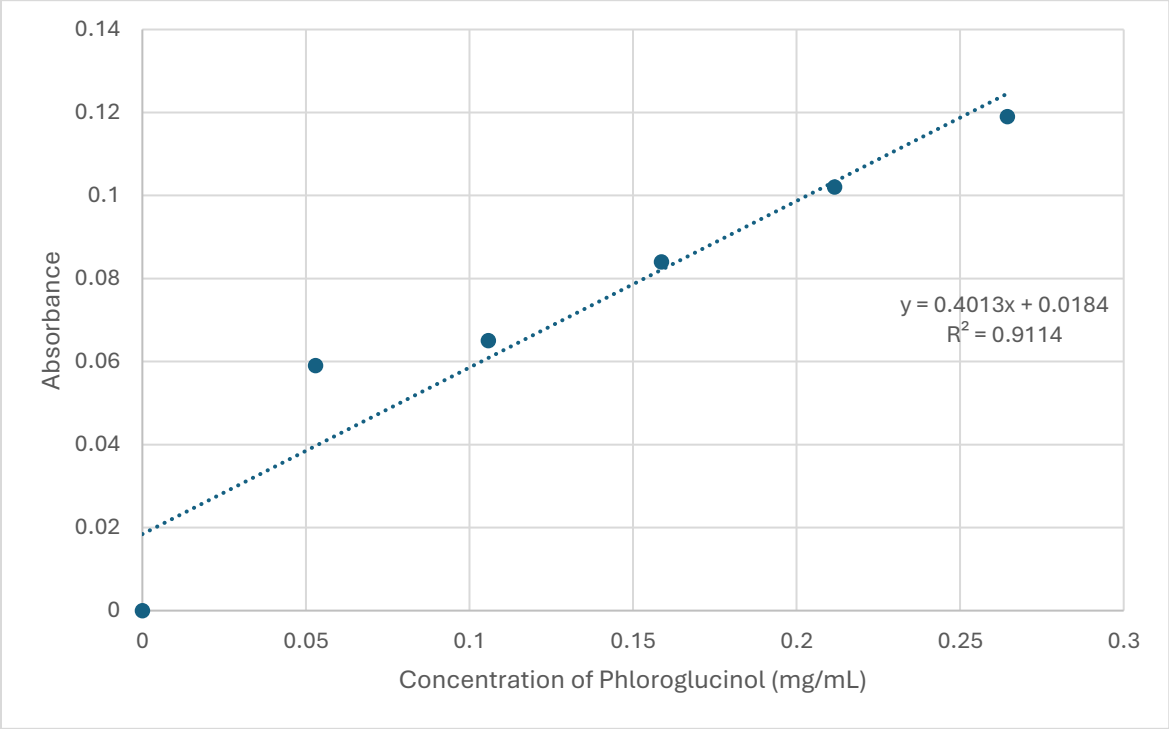


Figure B.2.3 *Concentration of Phloroglucinol (mg/mL) Standards after Two Hours of Development using F-C Assay – Trial 3*



APPENDIX C: SUPPLEMENTARY INFORMATION FOR CHAPTER 4 – TRACE ELEMENTS INCLUDING HEAVY METALS ANALYSIS

Table C.1 *Exact concentrations of trace metals including heavy metals standards, mercury standards, and sample species sugar kelp (SK), giant kelp (GK), M. japonica (MJ).*

Standard Name	**Volume of 200-ppb stock solution**	**Standard Concentration (ppb)**
S0	-	-
S1	25 µL	0.1
S2	250 µL	1
S3	2.5 mL	10
S4	12.5 mL	100
S5	25 mL	1000
Sample Species	Mass of Seaweed Sample (g)	Sample concentration (g/mL)
SK	0.5062	0.010
GK	0.5641	0.011
MJ	0.5261	0.011

Table C.2 % RSD Results for Inter- and Intra-Day Comparison Analysis – Method Validation

Element Analysed	**Trial Day 1 (% RSD)**			**Trial Day 2 (% RSD)**			**Trial Day 3 (% RSD)**		
	SK	GK	MJ	SK	GK	MJ	SK	GK	MJ
Beryllium	18.44	11.44	**35.99**	8.46	**39.35**	23.44	17.19	**37.18**	10.46
Magnesium	10.62	13.63	7.10	9.19	3.87	8.02	13.16	14.56	13.57
Aluminum	10.40	14.20	6.93	9.83	3.87	7.51	13.46	15.10	13.91
Vanadium	11.78	13.19	5.04	9.01	3.53	7.823	12.52	15.34	14.76
Chromium	11.93	12.87	5.74	9.34	3.33	8.47	12.16	15.43	15.05
Manganese	12.40	13.54	4.26	9.24	4.48	9.03	12.22	15.77	14.54
Iron	12.51	13.33	4.98	9.44	3.73	8.64	12.73	14.80	14.16
Cobalt	12.06	11.98	5.04	9.18	2.68	8.71	12.87	14.96	13.94

Nickel	11.50	12.44	5.08	8.61	3.51	8.82	13.97	15.30	13.55
Copper	12.60	15.52	6.27	8.96	4.42	11.90	5.82	6.90	5.60
Zinc	11.20	12.52	5.27	8.96	3.07	8.70	12.95	14.57	13.67
Arsenic	11.89	13.83	6.00	9.59	3.18	8.44	13.31	15.50	12.98
Selenium	**47.88**	7.14	50.30	9.79	**74.77**	**273.39**	10.62	5.74	**32.95**
Molybdenum	**23.60**	12.40	1.74	20.48	16.95	4.19	5.02	**23.67**	**21.13**
Silver	11.60	14.42	4.06	5.65	6.81	6.51	7.02	7.62	5.48
Cadmium	11.76	13.49	4.97	9.22	2.89	8.35	12.49	15.84	13.18
Strontium	15.11	13.20	5.82	12.25	3.55	2.86	13.23	14.68	13.25
Barium	11.06	13.90	5.21	9.29	3.58	9.00	12.45	15.03	13.55
Thallium	17.56	10.67	4.94	15.01	16.04	9.97	5.78	**26.55**	**20.98**
Lead	11.28	11.99	28.93	9.16	2.88	3.28	13.43	15.69	18.19
Uranium	11.63	12.82	6.29	10.39	4.02	8.46	13.30	16.00	12.91
***Mercury**									

Table C.3 *Shapiro Wilk Assessment of Normality Results for Key Elements of Interest*

Key Element	SK	GK	MJ
Al	0.6932	0.7602	0.4351
As	0.6377	0.7573	0.6062
Cd	0.6424	0.8335	0.4769
Hg	**0.01432**	0.1256	0.09299
Pb	0.3655	0.541	0.1786

Table C.4 *Z-Score Outlier Detection Results for Key Elements of Interest*

	Sugar Kelp	Giant Kelp	*M. japonica*
		As	
Min	**-436.3966**	**-164.6304**	**-8.8487**
Max	**59.3044**	**44.6923**	1.4091
		Al	
Min	-2.0173	-1.6902	-1.4443
Max	1.4911	1.4486	1.8278
		Cd	
Min	-1.9303	-1.8980	-1.5910
Max	1.4839	1.4852	1.5976
		Pb	
Min	**-40.1467**	**-14.4998**	-1.5750
Max	1.2767	2.4106	2.3440
		Hg	
Min	-0.8533	-1.0349	-1.1249
Max	2.3611	1.8743	2.3593

Table C.5 *ANOVA and Kruskal-Wallis Test Results for Key Elements of Interest*

Key Element	ANOVA/ Pr (>F)	Kruskal-Wallis (p-value)
Al	8.35×10^{-10}	-
As	$<2.00 \times 10^{-16}$	9.43×10^{-6}
Cd	$<2.00 \times 10^{-16}$	-
Pb	$<2.00 \times 10^{-16}$	9.43×10^{-6}
Hg	0.0889	0.02098

Table C.6 *Post-Hoc Analysis Results for Key Elements of Interest – Wilcoxon Pairwise analysis applied to Hg and Tukey HSD applied to all other elements of interest*

Key Element	*M. japonica* – Giant Kelp	Sugar Kelp – Giant Kelp	Sugar kelp – *M. japonica*
Al (Tukey HSD)	0.000	0.059	2.00×10^{-7}
As (Tukey HSD)	0.000	0.000	0.000
Cd (Tukey HSD)	2.52×10^{-5}	0.000	0.000
Pb (Tukey HSD)	0.000	0.000	0.000
Hg (Wilcoxon Pairwise)	0.056	1.000	0.043

Table C.7 *Average ICP-MS Trace Elements Including Heavy Metals Concentrations (ppb) for each species run in triplicate on three separate trial days*

Species	Trial Day	24 Mg	27 Al	51 V	52 Cr	55 Mn	56 Fe	59 Co	60 Ni
		Conc. [ppb]	Conc. [ppb]	Conc. [ppb]	Conc. [ppb]	Conc. [ppb]	Conc. [ppb]	Conc. [ppb]	Conc. [ppb]
Sugar Kelp	1	80177.1980	480.4593	10.0406	4.0074	72.1279	10.3038	1.3910	9.0742
	2	82104.5161	508.6881	10.3269	4.1070	75.5201	10.5214	1.4298	9.4036
	3	68189.6780	410.6482	7.9697	3.1415	57.1251	8.2459	1.1224	7.3832
Giant Kelp	1	85527.9760	525.1401	9.3167	3.1691	55.5526	12.1111	0.7446	4.7920
	2	82478.1265	524.5223	8.9276	2.9939	54.3535	11.5745	0.7147	4.5494
	3	84629.5634	528.9785	8.5418	2.8288	51.5844	11.1885	0.6923	4.3880
M. japonica	1	70863.5668	273.7226	9.8968	4.3054	104.7345	21.4901	5.3679	73.1443
	2	61447.9730	248.7117	8.6205	3.7139	93.4091	18.6074	4.6629	63.4299
	3	80275.3845	303.6525	10.4599	4.4837	112.1864	22.7848	5.6108	77.2567

Table C.7 *Average ICP-MS Trace Elements Including Heavy Metals Concentrations (ppb) for each species run in triplicate on three separate trial days*

Species	Trial Day	65 Cu	66 Zn	75 As	107 Ag	114 Cd	123 Sb	135 Ba	208 Pb	238 U
		Conc. [ppb]	Conc. [ppb]	Conc. [ppb]	Conc. [ppb]	Conc. [ppb]	Conc. [ppb]	Conc. [ppb]	Conc. [ppb]	Conc. [ppb]
Sugar Kelp	1	133.445	221.363	1089.392	0.342	55.101	1.147	211.512	102.319	1.658
	2	135.433	225.597	1131.253	0.411	58.578	1.226	225.067	104.336	1.733
	3	151.738	178.089	877.327	0.299	45.955	0.976	180.780	82.177	1.430
Giant Kelp	1	30.187	189.666	557.347	0.1603	13.602	0.827	131.720	51.833	1.621
	2	27.492	180.360	539.185	0.236	13.601	0.834	129.611	50.839	1.575
	3	46.655	176.811	522.858	0.175	13.444	0.858	129.440	49.875	1.654
M. japonica	1	25.873	129.539	30.385	0.218	1.702	0.109	8.040	1.334	2.096
	2	20.296	111.906	26.562	0.310	1.541	0.111	6.949	1.184	1.827
	3	36.709	135.744	31.795	0.229	1.853	0.134	8.604	0.863	2.299