Variation in chemical components of aquacultured *Ulva* (Chlorophyta) in response to environmental variables

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Plagiarism declaration

I know the meaning of plagiarism and declare that all of the work in the dissertation, save for that which is properly acknowledged, is my own. Aside from guidance from my supervisors, I have received no assistance, except as acknowledged.

Signed by candidate

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DATE: 19th September 2014

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Abstract

A detailed literature review of relevant *Ulva* biology and chemical composition is included. Marine algae are known to produce a wide range of volatile organic compounds that are primarily used in chemical communications. These compounds are released in seawater and act as either pheromones or allelochemicals. Aldehydes have been reported to be the main group of volatile compounds in green algae *Ulva*. Cultivation of *Ulva* as feed on abalone farms in South Africa has been a success but there has been little research on the chemistry of South African *Ulva*. This study aims to investigate the potential effects of environmental variables and grazing on the chemical profile, and specifically on the aldehyde-type natural products produced (δ_H 9.00 – 10.50) by laboratory cultured *Ulva* using ^{1}H NMR spectroscopy and multivariate statistical analysis. *Ulva armoricana* was cultured at different salinities: 5, 10, 20, 25 and 35 ‰ (all ± 0.1 ‰) and nutrient treatments: 100 % Provasoli ES medium (high nutrient supply) and 0 % Provasoli ES medium (low nutrient supply) at 10 °C and 15 °C for 6 days under constant light (39.2 ± 0.43 µmol photons m^{-2} s^{-1}) on a 16:8 hours light:dark photoperiod. Natural grazing (using *Tricolia capensis* Dunker) and artificial grazing (stimulated by scissors) was performed to determine their effects on the chemical composition of *Ulva armoricana*. Results obtained show that grazing and nutrient experiments mostly affected the aromatic, hydroxylic and carbonyl compounds regions, while salinity change mostly affected the alcohol, ester and phenolic regions. The aldehyde profiles included a prominent peak at δ_H 9.76 in almost all treatments that was provisionally identified as hexanal. *Ulva armoricana* grown at 10 °C under low nutrient condition and in a medium with salinity at 25 ‰ was found to be the ideal condition to produce a higher intensity of the main aldehyde.

Keywords: Aldehydes. Grazing. Multivariate Analysis. NMR. Nutrient. Salinity. *Ulva armoricana*

Chapter 1

Chapter 1: Literature Review

1.1 The genus *Ulva*

The genus *Ulva* (Phylum: Chlorophyta, Class: Ulvophyceae, Order: Ulvales, Family: Ulvaceae) was first described by Linnaeus in 1753 (Kong *et al.,* 2011). Predominantly marine, the Ulvales are a group of multi-cellular macroalgae combining features of terrestrial plants with those of unicellular microscopic organisms such as *Chlamydomonas* (Hori *et al.,* 1985; Lewis and McCourt, 2004). They have a very simple morphology and, in general, the thalli in *Ulva* species can be either foliose (blade-like) and distromatic, or tubular and monostromatic (Hayden *et al.,* 2003; Wolf et *al.,* 2012). The form of foliose species can be either erratically lobed, linear, roughly expanded, cuneate, lanceolate, oblanceolate or entirely divided into linear laciniae. Some species such as *Ulva reticulata* demonstrate regular perforations, while other species such as *Ulva rigida* and *Ulva taeniata* can show serrated edges (tooth-like; Wolf *et al.,* 2012).

Since the Linnaean period, numerous algal taxonomists have been engaged in the identification of *Ulva* species, as they are extremely difficult to classify based on morphological characters, but also due to the lack of relevant information distinguishing taxa (Heesch *et al.,* 2009; Kraft *et al.,* 2010; Wolf *et al.,* 2012). *Ulva* species show morphological variations with salinity, age of their thallus, season or whether they are attached or free-floating (Malta *et al.,* 1999; Loughnane *et al.,* 2008). *Ulva californica*, *Ulva angusta* and *Ulva scagelii* showed ecological, morphological and cytological similarities but varied in term of size, habit and distributions that could have been controlled by wave exposure, temperature, season and latitude (Tanner, 1986). The thalli of *Ulva fenestrata* Postels and Ruprecht showed significant variation in size, shape and thickness that may have been

controlled by vertical position and wave exposure, while *Ulva taeniata* (Setchell) Setchell and N.L. Gardner showed the thickening of the blade as well as decreased number of marginal teeth with increase in water temperature (Tanner 1979). This plasticity makes it difficult to identify these organisms based on morphological and cytological criteria (Blomster *et al*., 1998, 2000; Coat *et al*., 1998).

For the last two decades, there has been an increased use of molecular systematic techniques to identify and classify members of the *Ulva* genus (e.g. Blomster *et al.*, 1998; Tan *et al.*, 1999; Malta *et al.*, 1999; Hayden and Waaland 2002; Shimada *et al.*, 2003; Heesch *et al.*, 2009; Duan *et al.*, 2012). Recently, molecular studies have impelled the union of species with tubular forms ("*Enteromorpha*") and species with bladelike forms ("*Ulva*") to the genus *Ulva*, as it was demonstrated that the two genera were molecularly polyphyletic (Hayden *et al.*, 2003). At present, there are 568 *Ulva* species names in the Algaebase database of which 192 species are flagged as currently accepted taxonomically (Guiry and Guiry, 2013). The taxonomy of *Ulva* remains in a considerable state of flux. The name *Ulva lactuca* is widely used in the literature, particularly the aquaculture literature, without proper identification of the species involved (JJ Bolton, *pers. comm.*).

1.2 Distribution

The genus *Ulva* is cosmopolitan in its distribution, and species within this group are known to occur in oceans and estuaries along all coastlines around the world (Guiry and Guiry, 2013). These seaweeds are often conspicuous and dominant members of the intertidal and subtidal zones, and many *Ulva* species have the ability to tolerate a broad range of conditions (Heesch *et al.*, 2009; Kirkendale *et al.*, 2013). Although this

genus primarily occurs in saline waters, several species may proliferate in freshwater environments (van den Hoek *et al.,* 1995; Shimada *et al.,* 2008; Ichihara *et al*., 2009). For example, *Ulva flexuosa* was reported to be the main constituent of a bloom that occurred in Lake Michigan, USA in 2003 (Lougheed and Stevenson 2004). Several members of the Ulvales are famous as the most commonly transported and introduced species, since they are well-known biofoulers of ships' hulls and ballast water (Flagella *et al.,* 2007). Species of *Ulva* have often been introduced to new habitats, either accidentally or deliberately, through aquaculture activities, such as *Ulva pertusa* in the Venice lagoon (Sfriso and Curiel, 2007; Hewitt *et al.,* 2007; Manghisi *et al.*, 2011).

1.3 Life history of *Ulva*

Ulva species are often the first macroalgae to colonize open substrata, and their cosmopolitan presence is mainly attributed to their tolerance to a wide range of environmental conditions, but also to their high reproduction capacities (Littler and Littler, 1980; Beach *et al.,* 1995; Callow *et al*., 1997). This genus has either a haplontic life cycle or an isomorphic diplohaplontic life cycle (van den Hoek *et al.,* 1995).

In *Ulva*, haploid biflagellate gametes are produced by the male and female haploids that fuse to produce zygotes, which then develop to form diploid sporophytes (Figure 1; Alström-Rapaport *et al.,* 2010). During the formation of haploid quadriflagellate spores, meiosis occurs in the sporophyte that then grow to male and female haploid thalli (Alström-Rapaport *et al.,* 2010). *Ulva* can adopt another form of reproduction known as parthenogenesis, where unfertilized gametes may parthenogenetically

develop into gametophytes, or through the process of diploidization into parthenosporophytes (Hoxmark and Nordby, 1974). Additionally, these parthenosporophytes produce zoospores that then develop into gametophytes (Hiraoka and Yoshida, 2010).

Environmental factors such as light, temperature and salinity are considered to have the most effects on spore release (Azanza and Aliaza, 1999; Clifton and Clifton, 1999). During the reproduction phase in *Ulva*, the shedding of the motile cells was accompanied by a colour change of the thallus from yellow-green (wholly vegetative state) to dark olive and to white, indicating the release of reproductive cells (Han *et al.*, 2008a). The release of reproductive spores may be periodic, and driven by tidal/lunar cycles (Smith, 1947), but may also depend upon the season (Alström-Rapaport *et al.*, 2010).

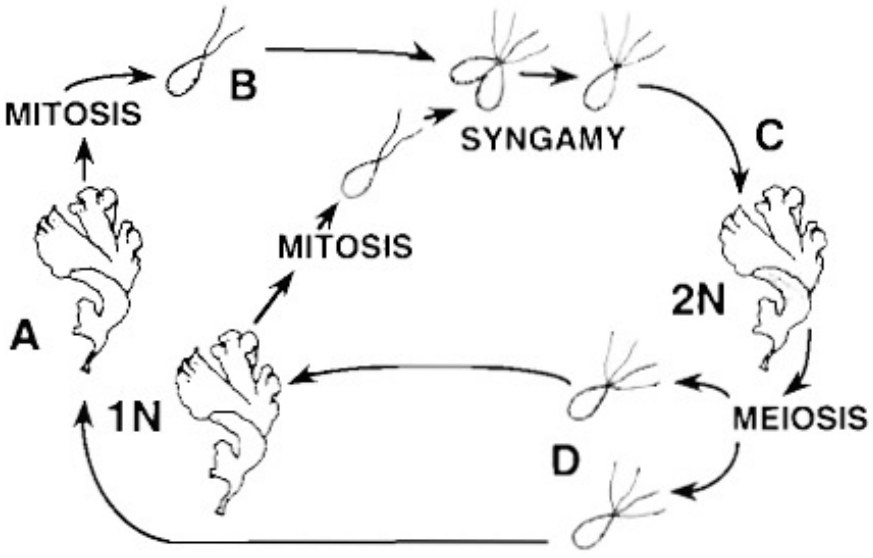

Figure 1. Typical sporic meiosis life history of *Ulva* (A) Male and female gametophyte; (B) Biflagellate gamete; (C) Sporophyte; (D) Quadriflagellate zoospore; 1N- haploid and 2N- diploid. (Source: Beach *et al.*, 1995).

1.4 Environmental factors affecting *Ulva*

Seaweeds are considered vital components of shallow marine ecosystems as primary producers, competitors and ecosystem engineers, but they are also commercially important in many regions in the world (Harley *et al.,* 2012; Govindasamy *et al.,* 2012). In nature, several environmental variables such as temperature (*e.g.* Raikar *et al.,* 2001), salinity (*e.g.* Steen, 2004), desiccation (*e.g.* Chu *et al.,* 2012), nutrients (*e.g.* Cronin and Hay, 1996), wave heights (*e.g.* Graham *et al.,* 1997), carbon dioxide concentration (*e.g.* Kroeker *et al.,* 2010) and pH (*e.g.* Martin and Gattuso, 2009), could affect the growth, survival and reproduction of seaweeds. Various parameters including salinity, irradiance, nutrient and temperature has been documented to controlled the growth of cultures seaweeds (*e.g.* Taylor *et al.,* 2001).

1.4.1 Light

Light is an essential component for photosynthetic organisms to assimilate carbon during the photosynthesis process (Zhang *et al.,* 2012). In the marine environment, seaweeds' vertical and horizontal distribution are dependent on their tolerance to high light stress (Hsu and Lee, 2012), since most of these organisms are sessile and are not capable of vertical motion to adapt themselves to changing irradiation conditions (Häder and Figueroa, 1997; Häder *et al.,* 1998). The light spectral composition and irradiance can vary in this environment and depends on: (1) the depth of the water column; (2) the absorption and scattering properties of the water (optical properties); (3) the angular distribution of the solar radiation; and (4) the presence of other components such as plankton, dissolved organic matter, particulate matter and yellow substances (Kirk, 1994; Krause-Jensen and Sand-Jensen, 1998).

Light quality may also have an impact on growth, pigmentation and photosynthetic and carbon mechanism of seaweeds (Lüning and Dring, 1985; Figueroa *et al.*, 1995a,b; Aguilera *et al.,* 2000). Although light represents the primary source of energy for photosynthesis, prolonged exposure of seaweeds to high irradiances could provoke damage to the photosynthetic system but also may contribute to the reduction of both the quantum efficiency and maximum rates of photosynthesis (Sampath-Wiley *et al.,* 2008; Dong *et al.,* 2012). Marine macroalgae are vulnerable to high solar irradiance, including short wavelength UV-B (280 to 315 nm; Gómez *et al.,* 2004; Han and Han, 2005). It could cause damage to the DNA (Pakker *et al.,* 2000), decrease of growth (Aguilera *et al.,* 1999), cause inhibition of the photosynthesis (Bischof *et al.,* 1998) and disturb enzymatic activities but could also modulate changes in carbon and nitrogen assimilation (Bischof *et al.,* 2000; Figueroa and Viñegla, 2001). Similar to higher plants, macroalgae use the mechanism of photoinhibition to protect themselves during excessive periods of irradiation (Franklin and Forster, 1997). For further protection, they make use of UV-absorbing compounds such as coumarins (*e.g.* Pérez-Rodríguez *et al.,* 2001), phenolics (*e.g.* Schoenwaeler *et al.,* 2003) and mycosporine-like amino acids (MAAs; *e.g.* Karsten *et al.,* 1998), that may act like a sunscreen during high radiation (Han and Han, 2005). Han and Han (2005) reported that *Ulva pertusa* Kjellman produced compounds that absorbed UV-B, and the maximum absorption was recorded at 294 nm with respect to the UV-B dosages. Furthermore, Dong *et al.,* (2012) reported that one LhcSR gene contributed to the photo-protection process in *Ulva linza* under high light and low temperature.

1.4.2 Temperature

Temperature represents a critical ecological factor influencing the growth and morphology, geographical distribution and seasonal growth pattern of seaweeds (Garbary, 1979; van den Hoek, 1984; Pakker *et al.,* 1994; Lee *et al.,* 1999). Temperature could also affect the rates of productivity in seaweeds since a rise in temperature would normally result in increase in metabolic rate (Rinde and Sjøtun, 2005). Most seaweed are able to grow and reproduce over a broad range of temperatures which is achieved by changing their metabolic rate (*e.g.* Rinde and Sjøtun, 2005), and the majority of them have geographic distributions that extend over a wide range of temperatures (Bolton and Lüning 1982; Schils and Wilson 2006). Staehr and Wernberg (2009) reported that *Ecklonia radiata,* occurring at lower (warmer) latitudes, has a 50% lower photosynthetic rates and a 90% lower respiration rates at their prime temperature than the same species occurring at higher (cooler) latitudes.

Seaweeds have developed biochemical and physiological adaptations, such as modification of the properties of their cell membranes, enzymatic adjustments and concentration of their proteins, which allow them to maximize their performance with reference to the temperatures they experience (Eggert, 2012). Vayda and Yuan (1994) reported that heat shock proteins (HSP70) and the ubiquitin polyprotein (UBI) were produced by *Plocamium cartilagineum* (Linnaeus) P.S. Dixon when incubated at 5 °C that repairs or removes damaged proteins.

On the other hand, higher temperature may cause algal death but may also include the denaturation of proteins and damage to heat-labile membranes or enzyme while lower temperature, lipids and proteins of cellular membranes may be destroyed due to the

formation of intracellular ice crystals (Lüning, 1990). Temperature may also control the uptake of nutrients through Q_{10} effects on algal metabolism (Raven and Geider, 1988), and several studies have shown the influence of temperature on the uptake of different nutrients (*e.g.* Gerard, 1997; Ozaki *et al.,* 2001). Fan *et al.,* (2014) showed that *Ulva prolifera,* collected from the coast of Qingdao, Shandong Province of China, had a higher N uptake rate at 20 °C while the lowest rates were recorded at 5 °C. In the same experiment, P uptake rates were found to correlate with increasing temperature. In a study on the influence of temperature on the infradian rhythm of growth of *Ulva lactuca*, Kalita and Titlyanov (2013) reported that the growth of the organism at 5 or 10 °C increased the prevalence of 3-day cycles and kept the seaweed in a vegetative growth stage, while growth at 15 or 20 °C triggered a predominance of the 2-day cycle and prompted reproduction.

1.4.3 Salinity

Next to light and temperature, salinity is regarded as one of the main abiotic factors affecting the growth and distribution of algae in numerous habitats (Kirst, 1996) Hyper/hypo-saline conditions occurring in intertidal zones and estuaries which are mainly associated to river run-off, evaporation and rainfall, could affect the osmotic regulation in seaweeds (Eggert *et al.,* 2007; Beauchamp, 2012). During hypersaline condition seaweeds have the tendency to accumulate compounds that participate in osmoregulation, in contrast to hyposaline condition (Hayashi *et al.,* 2011). Organic *β*-dimethylsulfoniopropionate (DMSP) contributes in the osmotic adjustment process in *Ulva (*as *Enteromorpha) intestinalis,* but also to be the main low molecular weight osmolyte present (Edwards *et al.,* 1987). However, Van Alstyne *et al.,* (2003)

suggested that osmotic acclimation was not the primary function of DMSP produced by *Ulva fenestrata*, but that it rather has other functions such as an antioxidant and herbivore deterrent.

Fluctuating salinities in the intertidal zone causes osmotic stress that may apply substantial oxidative stress on seaweeds, but also the production of reactive oxygen species (ROS; Ledford and Niyogi, 2005; Kumar *et al.*, 2010). Moreover, hypo/hyper osmolarities can disturb the external water potential and turgor pressure but also disturb distribution of ions and organic solutes inside the cell (Kumar *et al.*, 2010). Under these types of stress, seaweeds could reduce growth and may increase antioxidant enzymes and pigment concentrations (*e.g*. Kumar, *et al.*, 2010).

Imchen (2012) reported that recruitment (germination and growth) of zoospores from *Ulva flexuosa* Wulfen was dependent on light and salinity. On the other hand, lowered salinity may negatively affect the growth and nutrient uptake of some *Ulva* species (*e.g.* Fong *et al.*, 1996, Martins *et al.*, 2001). In a study on the responses of *Ulva lactuca* to salinity fluctuations, the changes in internal solute concentrations showed to reduce the changes in the turgor pressure following salinity fluctuations in the seaweed (Dickson *et al.*, 1982).

1.4.4 Nutrients

Nutrients are one of the most important factors regulating the growth, reproduction, development, morphology, productivity, biochemical components and distribution of seaweeds (DeBoer, 1981). Nitrogen and phosphorus are two of the most common macronutrients affecting macroalgae (DeBoer, 1981; Lapointe, 1987; Valiela *et al.*,

1997). Nitrogen is associated to be the main limiting nutrient for growth (*e.g.* Larned, 1998; Valiela *et al.,* 1997), while in some regions, macroalgal production may be controlled by phosphorus (*e.g.* de Casabianca *et al.,* 2002; Villares and Carballeir, 2004). Generally, seaweeds are able to utilise both inorganic forms of nitrogen (N) such as nitrate (NO_3), ammonium (NH_4), and phosphorus (P) such as phosphate (PO_4) (*e.g.* Hurd and Dring, 1991; Phillips and Hurd, 2003), but also organic nitrogen compounds such as urea and amino acids (*e.g.* Tyler *et al.,* 2005).

Seaweed genera such as *Ulva* have been described as opportunistic mainly due to the morphology of their thallus (thin and undifferentiated thalli), exhibiting rapid uptake of inorganic nutrients and rapid growth rates (Littler and Littler, 1980). *Ulva* could also take up nutrients efficiently at low substrate concentrations (Pedersen and Borum, 1997; Naldi and Viaroli, 2002). When compared to other seaweeds, *Ulva* species showed to possess a low capacity to store nutrients (Pedersen and Borum, 1997). These seaweeds have a high surface-area to volume ratio (SA:V ratio) which require more nutrients, and become more abundant at higher nutrient levels in the environment (Wallentinus, 1984; Karez *et al.,* 2004). Lilliesköld Sjöö and Mörk (2009) suggested that the low storage capacity could make *Ulva* inappropriate to be used as an indicator for historical nutrient loading at a specific site, since attributes for indicators depend on the nutrient uptake and storage of the species, as response time to variations in nutrients rely on the metabolic properties of the organisms.

Excessive development of human activities has increase nutrient inputs in coastal marine ecosystems, thus affecting the water quality and leading to eutrophication (Xin *et al.,* 2010). Aquatic ecosystems have encountered large scale nutrient input that has resulted in dissolved oxygen depletion, loss of submerged aquatic vegetation and fish

habitat, and increased occurrence and duration of toxic microalgal blooms (Bricker *et al.,* 2007; Howarth *et al.,* 2011). Special types of harmful algal blooms known as 'green tides', primarily caused by *Ulva* species, have been increasing in magnitude and geographic range due to elevated nutrient contents in seawater (Buapet *et al.,* 2008; Ye *et al.,* 2011). Species such as *Ulva linza, Ulva procera* and *Ulva prolifera* are reported to form 'green tides' in China (Leliaert *et al.,* 2009; Kim *et al.,* 2011). Species of *Ulva* can also be used to improve the water quality in integrated culture with abalone on aquafarms (Bolton, 2006; Robertson-Andersson *et al.,* 2008; Bolton *et al.,* 2009). *Ulva* species could be useful for bioremediation, to remove nutrients in shrimp cultures (Copertino *et al.,* 2009; Sánchez *et al.,* 2012) and could help to prevent the outbreaks of disease in these systems (Selvin *et al.,* 2011).

1.4.5 Effects of heavy metals

Metals such as iron, copper, cadmium, lead and mercury associated with anthropogenic activities like sewage, use of fertilizers, metal product manufacturing, petroleum refining and leather tanning, are considered to be the principal cause of heavy metal enrichment of the marine environment (Haritonidis and Malea, 1995; Lee and Wang, 2001; Kamala-Kannan *et al.,* 2008). Macroalgae such as *Ulva* have often been considered as valuable indicators of heavy metal pollution in coastal areas, mainly due to their thin sheet-like thalli but also due to their high accumulation capacity for metals (Haritonidis and Malea, 1999; Lee and Wang, 2001). Previously, *Ulva lactuca* and *Ulva rigida* were used as indicators for copper, manganese, iron, lead, and cadmium in coastal environments (Malea and Haritonidis, 2000; Gaudry *et al.,* 2007). *Ulva* species are normally considered to be resistant to a broad range of

environmental stressors, including metals (Correa *et al.,* 1996; Han *et al.,* 2008b). However, a study on *Ulva pertusa* indicated that metals such as copper, cadmium, zinc and lead could have adverse effects on the sporulation of the alga (Han and Choi, 2005).

Copper, which is a very important element for eukaryotic organisms, has been reported to induce phytotoxic symptoms in algae at a certain level, and these effects are mainly on algal growth, photosynthesis and fertility, interruption of development, and damage to membranes (Brown and Newman, 2003; Nielsen *et al.,* 2003). Another study concluded that copper affects pigmentation and chlorophyll fluorescence in algae (Cid *et al.,* 1995). Han *et al.,* (2008b) showed that *Ulva armoricana*, which is an introduced species on the east coasts of Korea, is more tolerant to copper exposure than the native species *Ulva pertusa* in terms of growth, pigmentation, photosynthesis, antioxidant and nitrate reductase capacity. Findings from this experiment also concluded that at a higher concentration of copper, uncoupling growth from photosynthetic activity provided sufficient energy to *Ulva armoricana* to induce protective measures in order to resist copper toxicity.

1.5 Volatile organic compounds

Marine organisms, including algae, are known to produce a wide array of volatile organic compounds from various biosynthetic origins (Ferraces-Casais *et al.*, 2013). These secondary metabolites consist mostly of volatile organic compounds with low molecular weight, low to moderate hydrophilicity and high vapour pressure. Molecules such as hydrocarbons, alcohols, esters, halogen or sulphur containing compounds, phenols, fatty acids, aldehydes, ketones and terpenes, have been recorded

and studied in macroalgae (Kladi *et al.,* 2004; Gressler *et al.,* 2009, 2011; Ferraces-Casais *et al.,* 2013).

In the marine ecosystem, these volatile compounds have fundamental roles in chemical communication and several of them have other biological functions (Fink, 2007; Gressler *et al.,* 2012). They can act either as pheromones (Pohnert and Boland 2002) or allelochemicals such as herbivore deterrents (*e.g.* Hay *et al.,* 1998; Pelletreau and Muller-Parker, 2002), possess anti-fouling activities (*e.g.* Da Gama *et al.,* 2002), and suppress or kill growth of neighboring competitors (*e.g.* Suzuki *et al.,* 1998). However, the levels of secondary metabolites in seaweeds can vary according to several environmental factors such as salinity (*e.g.* Pedersen 1984), light (*e.g*. Pavia *et al.,* 1997) and desiccation (*e.g.* Renaud *et al.,* 1990).

Aldehydes consist of a major group of volatile compounds in green algae such as *Ulva* (*e.g.* Fujimura *et al.,* 1990; Kajiwara *et al.,* 1992). In *Ulva pertusa*, long-chain aldehydes (C-15 and C-17) were formed from α-oxygenation of long-chain saturated fatty acids (C-14 and C-16) though the formation of 2-hydroperoxy acids (Akakabe *et al.,* 1999, 2000, 2001). Long chain aldehydes such as pentadecanal, (Z)-8-heptadecenal, (Z,Z,Z)-8,11,14-heptadecatrienal and (Z,Z)-8,11-heptadecadienal were isolated from *Ulva pertusa*, and these aldehydes consisted of the most important flavor components among a selection of volatile compound found in this alga (Fujimura *et al.,* 1990; Akakabe *et al.,* 2003). Short-chain aldehydes (C-6 and C-9) are said to have a fresh green and cucumber-like aroma, and may be produced from C-18 and C-20 fatty acids in seaweeds (Boonprab *et al.,* 2003a,b). Volatile aldehydes such as (2E,4Z)-2,4-decadienal and (2E,4E)-2,4-decadienal are reported in *Ulva conglobata* (Akakabe *et al.,* 2003). Diatom-derived 2,4-decadienal may act as a

potential chemotherapeutic agent against shell-infesting polychaetes that affect abalone *Haliotis midae* from South Africa (Simon *et al.,* 2010).

Dimethyl sulfoniopropionate, commonly known as DMSP, occurs in high concentrations mainly as an osmoregulatory solute in several green macroalgae and more particularly in *Ulva* (Edwards *et al.,* 1987; Van Alstyne *et al.,* 2001). DMSP could also improve the feeding efficiency and feed intake in shrimps (Cruz-Suárez *et al.,* 2009). Dimethylsulfide (DMS), which is a sub-product of DMSP degradation, may give negative flavours and tastes to several seafood products (Figure 2; Brooke *et al.,* 1968; Levasseur *et al.,* 1994; Smit *et al.,* 2010). DMSP can also affect the sensory quality (taste) of cultivated abalone in South Africa (Smit *et al.,* 2007). Seaweed-based abalone feed contain large amount of DMSP that is incorporated in the animals' tissue, and the degree of accumulation may depend on its concentration in the feed itself (Smit *et al.,* 2007). Abalones that were fed on an *Ulva* species diet had an "off taste" and "petroleum" or "seaweed- or kelp-like" smell during canning which is caused by DMS (Troell *et al.,* 2006; Smit *et al.,* 2007).

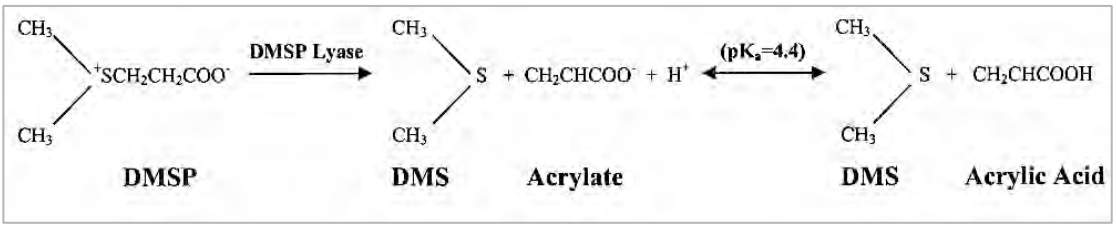

Figure 2. The cleavage pathway producing DMS and acrylic acid from DMSP by DMSP lyase in seaweed (Source: Van Alstyne *et al.,* 2001).

1.6 Nutritional contents

Globally, at least 145 out of the 221 macroalgal species that have been commercially exploited, have been used for human food (Zemke-White and Ohno, 1999). In Asian countries such as Japan and China, seaweeds are an important part of the staple diet and have been used for a long time compared to Europe and North America, where only a few species have been used (Chapman and Chapman, 1980). Seaweed usage in Western countries are mostly as a source of phycocolloids (agars, carrageeenans and alginates), thickening and gelling agents which can contribute up to 40% of the dry weight of some red and brown algae (Abbott, 1996). Over the past few decades, there has been a growing interest for the utilisation of seaweeds as food in Europe, but there are no European Union specific regulations established regarding their usage for human consumption (Rupérez, 2002; Dawczynski *et al.*, 2007). Since the 1990s, 13 species of seaweeds have been regulated in France as food products, including species of *Ulva* (Marfaing and Lerat, 2007).

For several years, there has been a growing consumer interest concerning seaweed products that support or even encourage health (Taboada *et al.*, 2010), and seaweeds have gained popularity and have been increasingly viewed as prospective sources of bioactive compounds with considerable pharmaceutical and biomedical potential (Smit, 2004; Veena *et al.*, 2006). In general, seaweeds have been recognized as being a valuable food source for humans and animals since they are low in fat and are rich in protein, vitamins, minerals and polysaccharides (Hong *et al.*, 2007; Taboada *et al.*, 2010). Additionally, they are known contain several bioactive substances that have proven to promote health (Burtin, 2003; Bocanegra *et al.*, 2009; Teas *et al.*, 2009). *Ulva* species, which are used in the preparation of “aonori” in Japan, are said to have antioxidant properties and contain high amounts of protein and amino acids, vitamins

and mineral such as calcium and magnesium (Nisizawa *et al.*, 1987; Fleurence, 1999; Qi *et al.,* 2005; MacArtain *et al.,* 2007; García-Casal *et al.,* 2009). Nutrient contents, especially protein content may vary according to species and season (Fleurence, 1999; MacArtain *et al.,* 2007, Shuuluka *et al.,* 2013).

Generally, the crude protein content of *Ulva* species may vary between 10 to 26 % of its dry weight (DW; Fleurence, 1999). The average crude protein obtained from South African *Ulva* species (*Ulva capensis, Ulva rigida* and '*Ulva lactuca*') ranged from 17.6 to 20.1 % DW (Shuuluka *et al.,* 2013). The '*Ulva lactuca*' studied by Shuuluka *et al.,* (2013) is the same South African material studied in the current study, and has been more recently referred to *Ulva armoricana*. *Ulva armoricana* has a protein content of 18 to 24 % DW (Fleurence *et al.,* 1999), and up to 27.2 % in *Ulva lactuca* (Ortiz *et al.,* 2006). Aspartic and glutamic acids constitute a major part of the amino acid fraction of seaweeds (Fleurence, 1999). Essential amino acids of *Ulva* proteins ranged from 30.8 to 33.6 % of the total amino acid content of *Ulva capensis, Ulva rigida* and '*Ulva lactuca*', and they are comparable to that of eggs and soybean amino acid contents (Shuuluka *et al.,* 2013). *Ulva* species also constitute a source of a complex sulfated polysaccharide known as ulvan, which gained interest as a potential source of functional biopolymer (Lahaye and Robic, 2007). Ulvan contents in *Ulva* species can vary between 8 to 29 % DW (Robic *et al.,* 2009). Ulvan extracted from *Ulva pertusa* may have an important role as a free radical scavenger *in vitro* and showed to possess antioxidant and antihyperlipidemic activities (Qi *et al.,* 2005, 2012).

Unlike proteins, the lipid contents of edible seaweeds are generally low and account for between 1 to 5 % of dry weight (Burtin, 2003). *Ulva lactuca* may also have lower

lipid content of 0.3g/100g DW (Ortiz *et al.*, 2006). In addition, some seaweed species are a rich source of minerals such as calcium and magnesium, which are necessary for human nutrition (Fleurence *et al.*, 2012). For example, *Ulva rigida* may have a calcium fraction of 5.245 g/kg DW, magnesium fraction of 20.941 g/kg DW and sodium fraction of 15.950 g/kg DW (Taboada *et al.*, 2010).

1.7 *Ulva* in aquaculture

In the aquaculture industry, aquafeeds rely strongly on fishmeal and fish oil to meet protein and lipid requirements of cultured animals, especially species which are carnivorous (Soler-Vila *et al.*, 2009). Fishmeal, represents the main ingredient and principal protein source in aquafeeds, and the increasing cost and insufficient supplies worldwide have created a need for alternative sources to ensure sustainability of the industry (Soler-Vila *et al.*, 2009). Recently, much effort has been dedicated to assessing an array of ingredients that can be either used as protein sources or feed additives in aquafeeds, and several plant protein resources have been identified that could be potentially used (Wassef *et al.*, 2013).

Algae (macro- and microalgae) have emerged as interesting alternative protein sources for fish feeds mainly due to their protein level and high rate of production (Güroy *et al.*, 2007). Seaweeds in general have been recognized as a good source protein, vitamins and minerals (*e.g.* Norziah and Ching, 2000; Wong and Cheung, 2000; Sánchez-Machado *et al.*, 2002), amino acids and fatty acids (*e.g.* Wahbeh 1997), coloring agents (*e.g.* Soler-Vila *et al.*, 2009, Cyrus *et al.*, 2014), and as a good source of biologically active compounds (*e.g.* Bansemir *et al.*, 2006). Previous studies showed that the inclusion of small amounts of algae meal to fish feed could resulted

in substantial effects on growth rate (*e.g.* Hashim and Saat, 1992, Cyrus *et al.,* 2014), lipid metabolism (*e.g.* Nakagawa, 1997), physiological condition and body composition (*e.g.* Nakagawa *et al.,*1997) and protein digestibility (*e.g.* Nandeesha *et al.,* 1998). Furthermore, algae in aquafeed could also act as a feed attractant (e.g. Silva-Neto *et al.,* 2012).

Ulva species have been frequently used in feed (*e.g.* Carefoot, 1980; Nakagawa *et al.,* 1987; Hashim and Saat, 1992), since they represent a good source of vitamins and minerals, and more importantly they are rich in ascorbic acid (Ortiz *et al.,* 2006; García-Casal *et al.,* 2007). Ascorbic acid is known to promote fish vitality through lipolysis, which may change the body composition and reduces the level of tissue lipid (Miyasaki *et al.,* 1995; Ji *et al.,* 2003). Addition of low-levels of *Ulva* meal could also promote growth performance, feed efficiency, nutrient utilization and body composition such as muscle quality and firmness (*e.g.* Wassef *et al.,* 2001; Ergün *et al.,* 2009). Fish species such as rainbow trout (*Oncorhynchus mykiss*; Yildirim *et al.,* 2009), Nile tilapia, (*Orechromis niloticus*; Ergün *et al.,* 2009) and common carp (*Cyprinus carpio*; Diler *et al.,* 2007) showed positive cultivation qualities when fed with an *Ulva* supplemented diet. Feeding trials using fish feed with *Ulva lactuca* on grey mullet (*Mugil cephalus*) fingerlings and on European seabass (*Dicentrarchus labrax* L.) fry with *Ulva lactuca* and red seaweed *Pterocladiella capillacea*, could be beneficial (Wassef *et al.,* 2001, 2013). In an experiment for the comparison of various seaweed-based diets and formulated feed on the growth rate of abalone in South Africa, abalone grew well on a diet of fresh seaweed combinations (*Gracilaria gracilis*, *Ulva lactuca*, and kelp; Naidoo *et al.,* 2006). The likely reason for that observation was that the green and red seaweeds in the fresh seaweed combinations were farm-grown, thus containing increased protein content (Naidoo *et al.,* 2006).

Furthermore, lutein which occurs as the principle carotenoid in *Ulva clathrata*, could be a good pigmentation precursor in white shrimp (*Litopenaeus vannamei*; Cruz-Suárez *et al.,* 2009). The inclusion of varying amounts of *Ulva* in protein-rich diets may be beneficial in term of weight, gonad size and colour in sea urchin (*Tripneustes gratilla*), and that the supplementation of the diet with 20 % *Ulva* weight/weight (w/w) could produce commercially acceptable size and colouration of gonads (Cyrus *et al.,* 2014). The culture of white-legged shrimp (*Litopenaeus vannamei*) with *Ulva clathrata* could be advantageous compared to shrimp monoculture since it allows *L. vannamei* to feed freely on *Ulva clathrata* thus reducing the need for commercial feed, but also it increases the market value of the final products in term of size, fatty acid profile and pigmentation (Cruz-Suárez *et al.,* 2010).

The co-cultivation of organisms at different trophic levels in aquaculture (IMTA: integrated multi-trophic aquaculture) may be beneficial in terms of production and sustainability (Naylor *et al.,* 2000; Lüning and Pang, 2003; Troell *et al.,* 2003). The wastes of one organism could be used by another organism, and in this way decrease the reliance on external ecosystems for food and energy, and reduce accumulation of wastes and negative impacts on the environment related to aquaculture (Troell *et al.,* 2006). The integration of seaweeds to marine animal aquaculture could also diversify and increase the income of aquaculture farms by reducing nutrient levels and provide significant return in term of biomass that could be used to feed the aquaculture species (Neori *et al.,* 1996, 1998; Troell *et al.,* 2003, 2006; Valente et *al.,* 2006; Neori, 2008). Some species of the *Ulva* genus have the ability of removing large amount of dissolved nitrogen (up to 90 %) from aquaculture discharge waters (Neori *et al.,* 1996, Robertson-Andersson *et al.,* 2008), while increasing their protein content (Shpigel *et al.,* 1999).

1.8 South African abalone industry

The South African cultivation of local abalone species, *Haliotis midae* L., has rapidly grown from its beginning in the 1990s to the largest producer outside Asia (Troell *et al.,* 2006; Robertson-Andersson *et al.,* 2008). The industry relies heavily on harvested kelp (mostly *Ecklonia maxima*) as feed (Troell *et al.,* 2006). Practically, all the South African abalone farms (ca. 20) are situated on the west of Cape Agulhas, very close to kelp beds while two farms situated much further to the east (Troell *et al.,* 2006; Bolton *et al.,* 2009). With the wild stock of kelp reaching its limit, the use of kelp as abalone feed has drastically decreased from 5,800 tonnes in 2005 to 3,800 tonnes in 2006, with farms now utilizing more formulated compound feeds and some farmed cultivated seaweed such as *Ulva* species (Troell *et al.,* 2006; Robertson-Andersson *et al.,* 2008).

Ulva cultivation has been increasing and it represents the most cultivated product by weight in South African aquaculture, with two abalone farms depending almost exclusively on farm-grown seaweed to meet their feed requirements (Bolton, 2006; Troell *et al.,* 2006; Robertson-Andersson *et al.,* 2008). The main species grown is *Ulva lactuca,* a name which has been widely used for aquacultured *Ulva* worldwide. Most aquacultured *Ulva* is not in fact true *Ulva lactuca*, and the South African material is genetically identical with *Ulva armoricana* (L. Kandjengo and JJ Bolton, *pers. comm.*). South Africa is one of the biggest world producers of *Ulva* in aquaculture (ca. 2,015 mt per annum; Amosu *et al.,* 2013).

The co-culture of abalone and seaweeds may have several advantages, such as reducing the expense of water pumping if the seaweed units are gravity-fed from the abalone tanks, reduction of nutrients through biofiltration by the seaweeds and

reduction of infestations of *Ulva* by epiphytic alga (Troell *et al.,* 2006; Nobre *et al.,* 2010). Protein-enriched *Ulva* grown on aquaculture farms has also showed to promote the growth several abalone species such as *Haliotis midae* (Naidoo *et al.,* 2006), *Haliotis discus hannai* (Shpigel *et al.,* 1999), *Haliotis tuberculata* (Neori *et al.,* 1998) and *Haliotis roei* (Boarder and Shpigel, 2001). The use of *Ulva* ponds in a re-circulating system could be a successful tool since they help in the partial-recirculation of the water, reduce pumping costs and especially increase the temperature level in abalone culture tanks from the improved light-absorbing properties of the seaweed (Bolton *et al.,* 2009), and could also have significant economic benefits both by increasing farm profits and also to the public with regard to carbon credits (Nobre *et al.,* 2010).

Chapter 2

Chapter 2

2.1 Introduction

The green algal genus *Ulva* Linnaeus (Phylum: Chlorophyta; Class: Ulvophyceae; Order: Ulvales), which includes species formerly placed in the genus *Enteromorpha* Link (Hayden *et al*., 2003), are ubiquitous rocky shore and estuarine macroalgae with more than 100 species taxonomically accepted (Guiry and Guiry, 2013). This group is well-known for its cosmopolitan presence in all oceans and estuarine ecosystems in the world (Guiry and Guiry, 2013). Compared to other macroalgae, *Ulva* species possess features that make them competitive for quick and successful colonization in eutrophic coastal waters, mainly due to their morphology (high surface area:volume ratio), high reproduction capacity, ability to rapidly take up inorganic nutrients, and rapid growth, but also due to their ability to tolerate a wide range of environmental conditions (Littler and Littler, 1980; Beach *et al.*, 1995; Callow *et al*., 1997).

Ulva species, also known as "sea lettuce", represent an important food source in many Southeast Asian countries (*e.g.* as 'aonori' in Japan; Nisizawa *et al.*, 1987) and are used as nutritional supplements in countries such as China, USA, Japan, France and Chile (Peña-Rodríguez *et al.*, 2011). They consist of an excellent source of proteins, essential amino acids, vitamins and minerals, and may possess multiple health benefits (Fleurence, 1999; Qi *et al.*, 2005; MacArtain *et al.*, 2007; Sathivel *et al.*, 2008; García-Casal *et al.*, 2009). These macroalgae have been easily cultured (*e.g.* De Busk *et al.*, 1986; Israel *et al.*, 1995; Neori *et al.*, 1991), including in integrated multi-trophic aquaculture systems (IMTAs) where they have been grown with other organisms (*e.g.* Jimenez del Rio *et al.*, 1994; Neori *et al.*, 2000; Robertson-Andersson *et al.*, 2008; Cruz-Suárez *et al.*, 2010). In South Africa, one of the biggest world

producers of *Ulva* in aquaculture (ca. 2,015 mt per annum; Amosu *et al.,* 2013), *Ulva* is mainly used as feed for abalone, *Haliotis midae* (Bolton *et al.,* 2009). Furthermore, the integration of the aquaculture of this seaweed with abalone, *Haliotis midae* could be a promising approach to remove dissolved nutrients level from effluent, reduce infestation of epiphytic alga and reduce cost of water pumping (Troell *et al.,* 2006; Bolton *et al.,* 2009, Nobre *et al.,* 2010). Additionally, the use of *Ulva* as feed or feed supplement may also to promote growth performance, feed efficiency, nutrient utilization, coloration and body composition such as muscle quality and firmness in some species (*e.g.* Wassef *et al.,* 2001; Ergün *et al.,* 2009; Cruz-Suárez *et al.,* 2009; Cyrus *et al.,* 2014).

Marine macroalgae produce a wide range of secondary metabolites, of which many have demonstrated a variety of bioactivities (*e.g.* Harder *et al.,* 2004; Yuan and Walsh, 2006; Engel *et al.,* 2006; Ferraces-Casais *et al.,* 2013). These secondary metabolites, which are mostly volatile organic compounds of low molecular weight, low to moderate hydrophilicity and high vapour pressure, are released by these algae into the seawater, and have vital roles in the chemical communication of the organisms (Kladi *et al.,* 2004; Fink, 2007; Gressler *et al.,* 2009, 2011, 2012; Ferraces-Casais *et al.,* 2013). In green algae such as *Ulva*, aldehydes consist the main group of volatile compounds (Fujimura *et al.,* 1990; Kajiwara *et al.,* 1992). Recently, several aldehydes such as pentanal, hexanal, (E)-2-octenal, (E)-2-nonenal, (Z,E)-2,6-nonadienal, (E,E)-2,4-decadienal, (Z,Z)-8,11-heptadecadienal, (Z,Z,Z)-8,11,14-heptadecatrienal and (Z)-8-heptadecenal, have been isolated from *Ulva*, and they have been confirmed to be important for the sensory characteristic aroma of this genus (Sugisawa *et al.,* 1990; Fujimura *et al.,* 1990; Akakabe *et al.,* 2003, Horincar *et al.,* 2014). Feeding tests and feeding preference studies using red, green and brown algae,

have concluded that *Ulva* was preferentially consumed compare to the other algae species, and the authors also suggested the presence of feed attractants in the essential oil of *Ulva* (Akakabe and Kajiwara, 2008). For years, *Ulva* utilisation as feed on South African abalone farms has shown to be very successful (Bolton *et al.*, 2009), and it would be of much interest to look for compounds that might have attractant- or stimulant-like properties in this seaweed which is widely used as feed. The present study was aimed to investigate the potential effects of environmental variables and grazing on the chemistry, and more specifically on the aldehyde profile of aquacultured *Ulva armoricana* using Nuclear Magnetic Resonance (NMR) techniques and a quantitative metabolomic approach using multivariate statistics.

The research questions were:

- Do different temperatures, salinities and nutrient levels affect the chemical components of aquacultured *Ulva armoricana*?
- Does natural and artificial grazing affect the chemical components of aquacultured *Ulva armoricana*?
- Do any of these environmental treatments have specific effects on the aldehyde profile of aquacultured *Ulva armoricana*?

2.2 Materials and Methods

2.2.1 Plant material

The *Ulva* species used in the present study is what in previous South African literature is known as *Ulva lactuca*, but it is identical in marker-gene sequences to that of the holotype of *Ulva armoricana* P.Dion, B.de Reviers and G.Coat, 1998, a representative of the *Ulva rigida* complex (L Kandjengo and JJ Bolton unpublished). The seaweed was initially obtained as material grown in an aquaculture system at Irvine and Johnson (I & J) Cape Abalone aquafarm in Gansbaai, South Africa (Robertson-Andersson *et al.*, 2008; Bolton *et al.*, 2009; Shuuluka *et al.*, 2013).

Fresh mature thalli of *Ulva armoricana* were obtained from the Department of Agriculture, Forestry and Fisheries Marine Research Aquarium, Cape Town, South Africa on the 8th November 2013. To prepare the seaweed for the different experimental treatments, it was first thoroughly washed in seawater and cleaned to remove any surface contaminants and epiphytes. The seaweed was equally divided by weight into two separate containers, and each was then acclimatised in walk-in controlled temperature rooms, one at 10 °C and the other at 15 °C (both ± 0.1 °C) under constant light (39.2 ± 0.43 µmol photons m^{-2} s^{-1}) provided by cool-white fluorescent tubes on a 16:8 hours light:dark photoperiod in containers with circulating seawater for 3 days. This was done to prepare the algae prior to the experiment. Irradiances were measured using a Skye Quantum Sensor.

2.2.2 Culture experiment

In order to determine the effects of environmental changes on the chemical composition, the seaweed was cultured at different temperatures, salinities and nutrient concentrations. All experiments were carried out in walk-in controlled temperature rooms, set at either 10 °C or 15 °C (both ± 0.1 °C) under constant light (39.2 ± 0.43 µmol photons m^{-2} s^{-1}) on a 16:8 hours light:dark photoperiod for 6 days.

In total, five different salinity experiments: 5, 10, 20, 25 and 35 ‰ (all ± 0.1 ‰) and two different nutrient concentration experiments: high nutrient supply (100 %) and low nutrient supply (0 %) Provasoli ES medium (Provasoli, 1968) were carried out in triplicate (n=3) at 10 °C and 15 °C. The salinity culture media were prepared by mixing natural seawater (salinity – 35 ‰) with deionised freshwater for treatments with salinity less than 35 ‰, while full seawater was used for the salinity: 35 ‰ treatment (note: no additional nutrient was added to the salinity culture media). The salinity for culture media of the salinity experiment was verified using a refractometer. For the nutrient culture experiment, full Provasoli ES medium was used for the high nutrient supply treatment (100%) and natural seawater was used for the low nutrient supply (0%). Prior to the culture experiments, all the culture media were prepared and stored at 10 °C and 15 °C.

Fresh *algae* was blot-dried prior to weighing, and for each treatment 15 g (± 0.1 g) fresh weight of the seaweed was cultured in conical flasks containing 500 ml (± 1 ml) of the culture medium. The conical flasks were placed on flask shakers (Stuart Scientific Co. Ltd., UK) at 80 rpm. Culture media were changed on the third day of the experiment, and on the sixth day all the plant material was removed, washed with deionised freshwater and stored at -80 °C prior to analysis.

2.2.3 Grazing experiment

2.2.3.1 Natural grazing

The herbivorous gastropod *Tricolia capensis* Dunker was used for the natural grazing experiment. The snails were collected in the intertidal zone in Simon's Town, Cape Town, South Africa during low tide on the 21th November 2013 and a confirmed identification was given by Prof. C. L. Griffiths of the Biological Sciences Department, University of Cape Town. After collection, the organisms were counted and placed in a 1000 ml conical flask with aerated seawater in a controlled room set at 15 °C under constant light (39.2 ± 0.43 µmol photons m^{-2} s^{-1}) and starved for 24 hours prior to the start of the experiment. The experiment was carried out in triplicate (n=3) using conical flasks with each containing 15 g (± 0.1g) of fresh algae, 500 ml (± 1 ml) of natural seawater and sea snails, *Tricolia capensis* Dunker (n=120). The flasks were placed in a temperature controlled room set at 15°C with constant light (39.2 ± 0.43 µmol photons m^{-2} s^{-1}) supply for 2 days. Aerators were used to ensure continuous water movement inside the flasks. At the end of the experiment, the snails were removed, and the plant materials were washed with deionised freshwater and stored at -80 °C until analysis.

2.2.3.2 Artificial grazing

A pair of scissors was used to simulate grazing for the artificial grazing experiment. For the experiment, about 15 g (± 0.1g) of fresh *Ulva* was placed in conical flasks (n=3) containing about 500 ml (± 1 ml) of natural seawater. The conical flasks were placed in a temperature controlled room set at 15 °C with constant light (39.2 ± 0.43 µmol photons m^{-2} s^{-1}) supply for 2 days. The algae were lightly trimmed, i.e. numerous incisions (≈ 20 – 25) of 3 – 5 mm in length, were made three times per day

to stimulate the grazing effects. Aerators were used to provide continuous water movement inside the flasks and at the end of the experiment the algae were removed, washed with deionised freshwater and stored at −80 °C until further use.

2.2.4 Sample preparation and NMR analysis

This was performed at the Faculty of Pharmacy, Rhodes University, Grahamstown, South Africa. In all, sixteen sets of samples in triplicate (n=3) from five different salinity treatments, two nutrient treatments and two grazing experiments were analysed. All the plant materials were washed with distilled water and blot-dried prior to the extraction process. About 2 g (± 0.1g) of each sample was placed into test tubes containing 3 ml methanol (CH_3OH). The test tubes were sonicated for 5 minutes using a bench top sonicator (Cole-Parmer ultrasonic bath, Chicago, Illinois, USA). Then, 6 ml dichloromethane (CH_2Cl_2) was added to the test tubes, and they were further sonicated for 5 minutes. The resultant organic phase of each sample was separated into pre-weighed vials, dried using a rotary evaporator (BÜCHI Rotavapor® R-215) at 40°C and stored for NMR analyses.

The NMR experiments were performed with a Bruker Avance 600 MHz spectrometer. The dried algal extracts were dissolved in 1 ml deuterated chloroform ($CDCl_3$) and analysed for ^1H NMR spectroscopy using dimethylformamide (DMF, C_3H_7NO) as an internal reference for 32 scans per spectrum. Bruker's TopSpin™ 2.1 software was used for the acquisition and elaboration of the NMR spectra.

2.2.5 Data Analysis

NMR spectra were further processed using MestReNova 9.0.1 software. After the manual phase and baseline corrections, the spectral region δ_H -0.20 – 12.20 was divided into bins with each integral having an equal width of 0.04 ppm. The regions δ_H 1.47 – 1.63, δ_H 2.87 – 2.95, δ_H 3.35 – 3.51, δ_H 5.27 – 5.55, δ_H 7.23 – 7.35 and δ_H 7.71 – 8.07 were discarded to remove residual water, dimethylformamide, dichloromethane, methanol and chloroform signals, respectively. In order to compensate for the intersample differences, each region was normalized to the sum of total spectral area. Normalized NMR data were exported to and rearranged in Microsoft® Excel® 2011 and then saved as ".csv" (comma delimited) files. The integration function was used to obtain the absolute intensity of the aldehyde peak at δ_H 9.76 and then expressed as the average ± standard deviation of the three replicates (Figure 3).

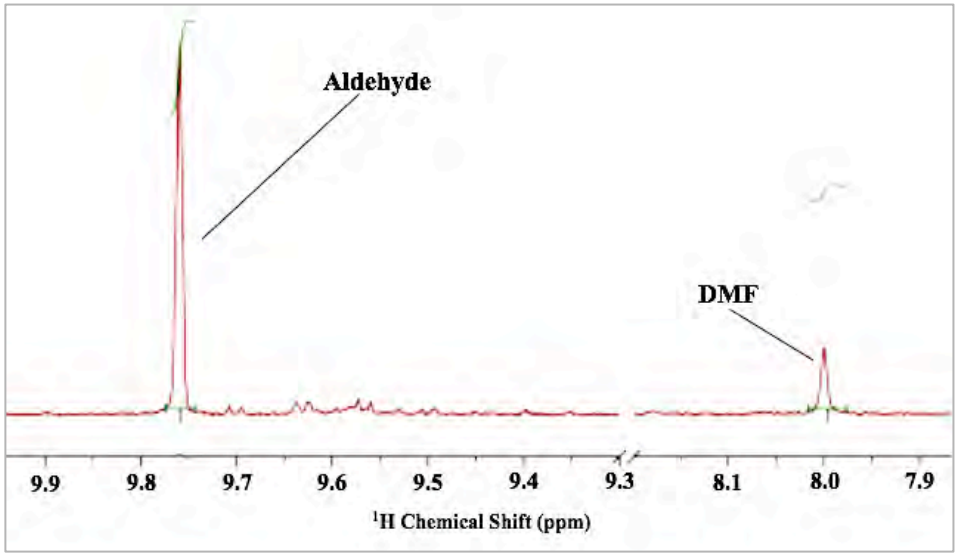

Figure 3. NMR spectrum of *Ulva armoricana* with a principal aldehyde peak at 9.76 ppm and a DMF peak at 8.03 ppm.

Multivariate analysis was carried out using Community Analysis Package 4.0 (CAP: Pisces Ltd., UK) on the normalized NMR data. Principal components analysis (PCA) was conducted on the data to generate two-dimensional plots of the first and second components. Data for each treatment was tested individually for normality and homogeneity using one-way analysis of variance (ANOVA) and comparisons after ANOVA were made using the post hoc Tukey test at 95 % significance level to identify specific differences (Zar, 1999) using R software (version 3.1.0).

2.3 Results

The effects of environmental variations and grazing were tested on *Ulva armoricana* metabolites. Results from the three different experiments were presented on ^{1}H NMR spectra and the absolute intensity for the aldehyde peak at δ_H 9.76 was reported as average ± standard deviation (SD) (see Appendix for all spectra). All the ^{1}H NMR spectra show significant variations in term of chemical signals between δ_H 0.71 to δ_H 6.65 and δ_H 9.00 to δ_H 10.50, but also the presence of other trace impurities such as residual water (δ_H 1.52 to δ_H 1.63), methanol (MeOH; δ_H 3.47 to δ_H 3.50), dichloromethane (CH_2Cl_2; δ_H 5.30 to δ_H 5.36), chloroform ($CHCl_3$; δ_H 7.27) and dimethylformamide (DMF; δ_H 2.86 to δ_H 2.94 and δ_H 7.98 to δ_H 8.03). In the aldehyde region (δ_H 9.00 to δ_H 10.50), peak signal at δ_H 9.76 appears to be the principal aldehyde peak among the treatments and the one to be most affected by the variations of the different environmental factors.

2.3.1 Effects of grazing on *Ulva* metabolites

The ^{1}H NMR spectra for the grazing treatments are illustrated in Figure 4. The aldehyde signal at δ_H 9.76 was more prominent for natural grazing compare to artificial grazing, where aldehyde peak at δ_H 9.54 and δ_H 9.40 were the most dominant. Natural grazing had the highest aldehyde values (δ_H 9.76) with an average intensity of 3.19 ± 1.919 ($x10^5$) compare to -0.05 ± 0.233 ($x10^5$) for the artificial grazing. Aldehyde peak intensity (δ_H 9.76) of the treatments was significantly affected by grazing ($p < 0.05$) and there was a significant difference in the aldehyde intensity at δ_H 9.76 between the natural grazing treatment and the artificial grazing treatment (ANOVA $p < 0.05$).

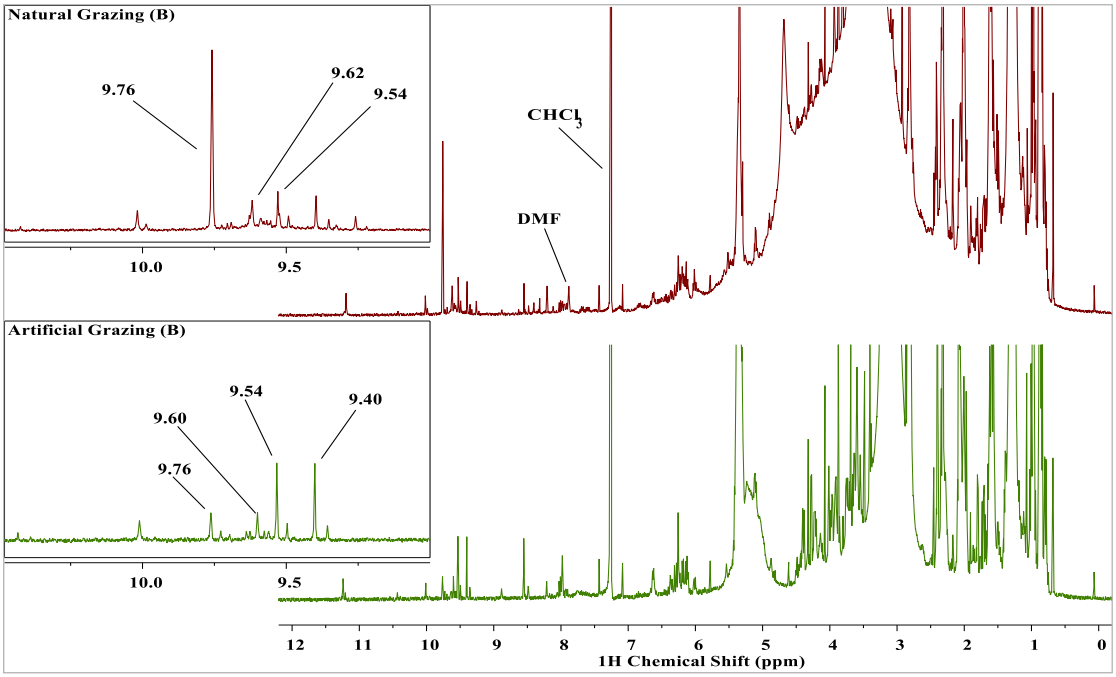

Figure 4. ^{1}H NMR spectra of *Ulva armoricana* for grazing experiments with replicate B (maroon) representing natural grazing experiment and replicate B (green) representing the artificial grazing experiment from δ_H -0.20 to δ_H 12.20 for the whole spectra and δ_H 9.00 to δ_H 10.50 for the aldehyde region.

2.3.2 Effects of nutrient and temperature variations on *Ulva* metabolites

The ^{1}H NMR spectra for effects of nutrient and temperature variations are represented by Figure 5 (low nutrient supply, 0 % ES) and Figure 6 (high nutrient supply, 100 % ES) at 10 °C and 15 °C respectively. There are several aldehyde signals present in the aldehyde region such as δ_H 9.76, δ_H 9.62 and δ_H 9.54, but the signal at δ_H 9.76 is most prominent. Results obtained for low nutrient supply (0 % ES) showed that *Ulva* grown at 10 °C produced more aldehyde (δ_H 9.76) compared to the 15 °C treatment (Table 1). A similar trend was also observed with high nutrient supply (100 % ES), with the samples at 10 °C reporting higher aldehyde values than samples at 15 °C. The aldehyde peak intensity (δ_H 9.76) of the treatments was significantly affected by nutrient concentration ($p < 0.05$). Significant differences were found in the aldehyde

intensity between samples of low and high nutrient supply at 10°C ($p < 0.05$), low and high nutrient supply at 10 °C and 15 °C ($p < 0.01$), and low and high nutrient supply at 15 °C ($p < 0.05$).

Table 1. Average aldehyde intensity for peak signal at δ_H 9.76 for the nutrient experiment at 10 °C and 15 °C.

Treatments	**Average absolute intensity of aldehyde peak at δ_H 9.76 (Average ± SD) * 10^5**
10 °C	
Low nutrient supply	2.55 ± 0.329
High nutrient supply	1.25 ± 0.306
15 °C	
Low nutrient supply	1.93 ± 0.506
High nutrient supply	0.76 ± 0.316

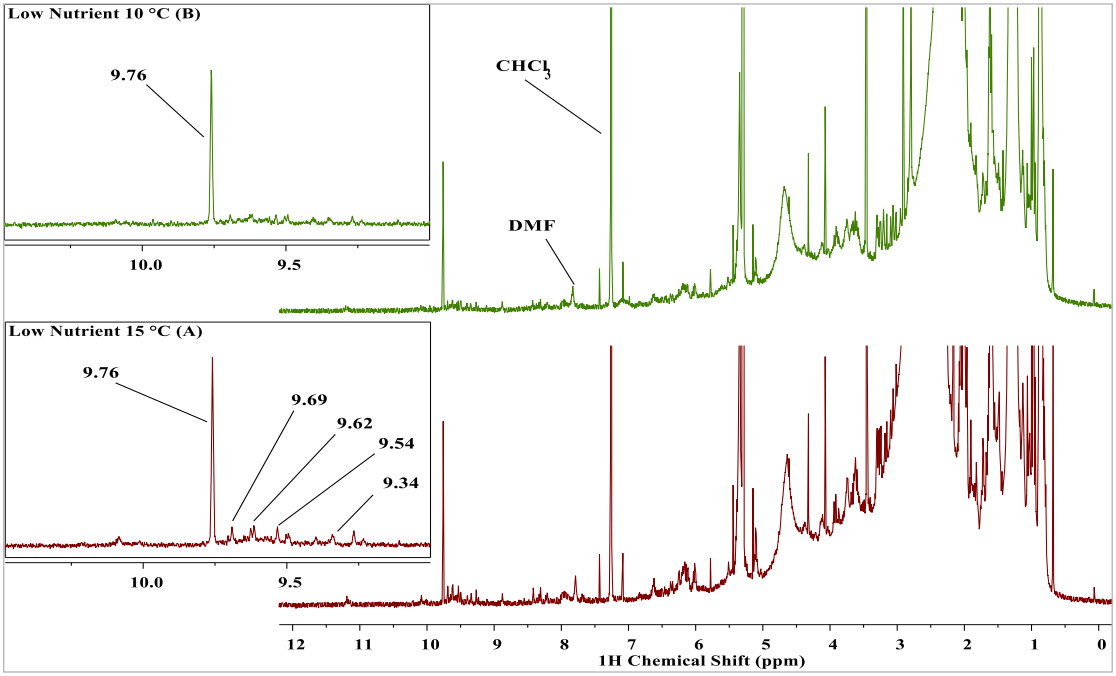

Figure 5. ^{1}H NMR spectra of *Ulva armoricana* for low nutrient supply (100% ES) treatment with replicate B (green) representing treatment at 10 °C and replicate A (maroon) for treatment at 15 °C from δ_H -0.20 to δ_H 12.20 for the whole spectra and δ_H 9.00 to δ_H 10.50 for the aldehyde region.

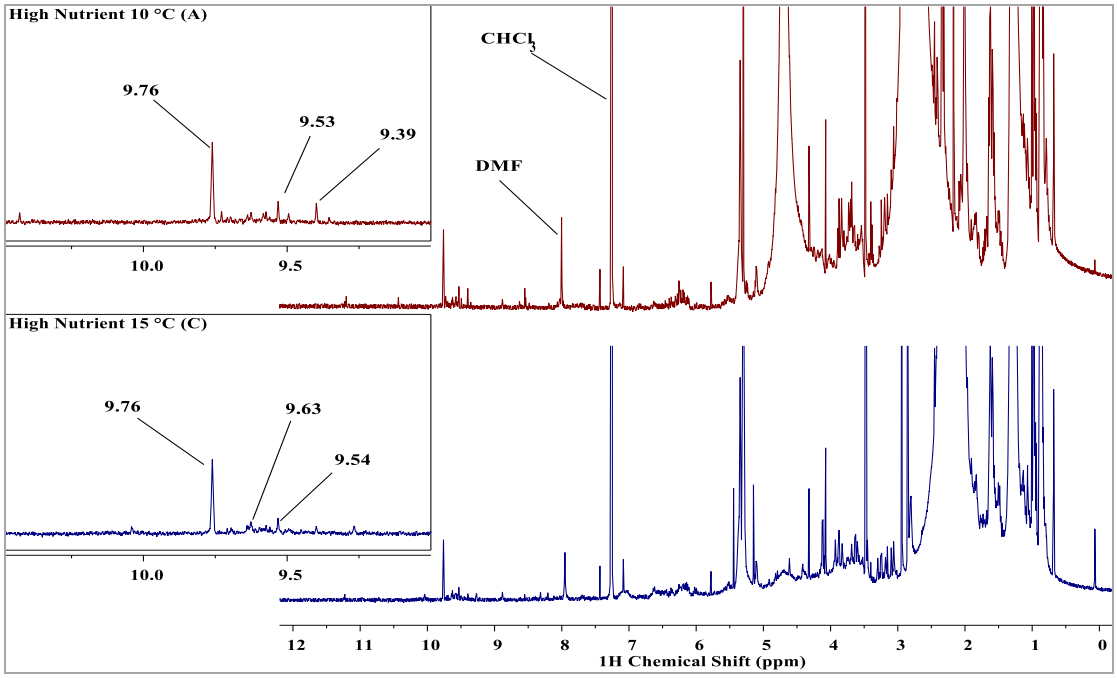

Figure 6. ^{1}H NMR spectra of *Ulva armoricana* for high low nutrient supply (100% ES) treatment with replicate A (maroon) representing treatment at 10 °C and replicate C (blue) for treatment at 15 °C from δ_H -0.20 to δ_H 12.20 for the whole spectra and δ_H 9.00 to δ_H 10.50 for the aldehyde region.

2.3.3 Effects of salinity and temperature variations on *Ulva* metabolites

The chemical profiles of *Ulva* at different salinities and temperatures between δ_H 0.70 to δ_H 4.30 were similar in composition but differed in intensities. Several aldehyde signals occurred at δ_H 9.76, δ_H 9.69, δ_H 9.56 and δ_H 9.54, but the aldehyde peak at δ_H 9.76 was of higher intensity among salinity treatments. Results from the experiment showed that all treatments at 10 °C produced higher level of aldehyde (at δ_H 9.76) compared to the treatments at 15 °C (Table 2). Salinity 25 ‰ treatment at 10 °C accounted for the highest aldehyde value (3.31 ± 0.505 x10^5; Figure 7), while salinity 10 ‰ treatment at 15 °C reported the lowest on average (1.29 ± 1.603 x10^5). No significant differences were found ($p < 0.05$) between the aldehyde peak intensity (δ_H 9.76) of the treatments.

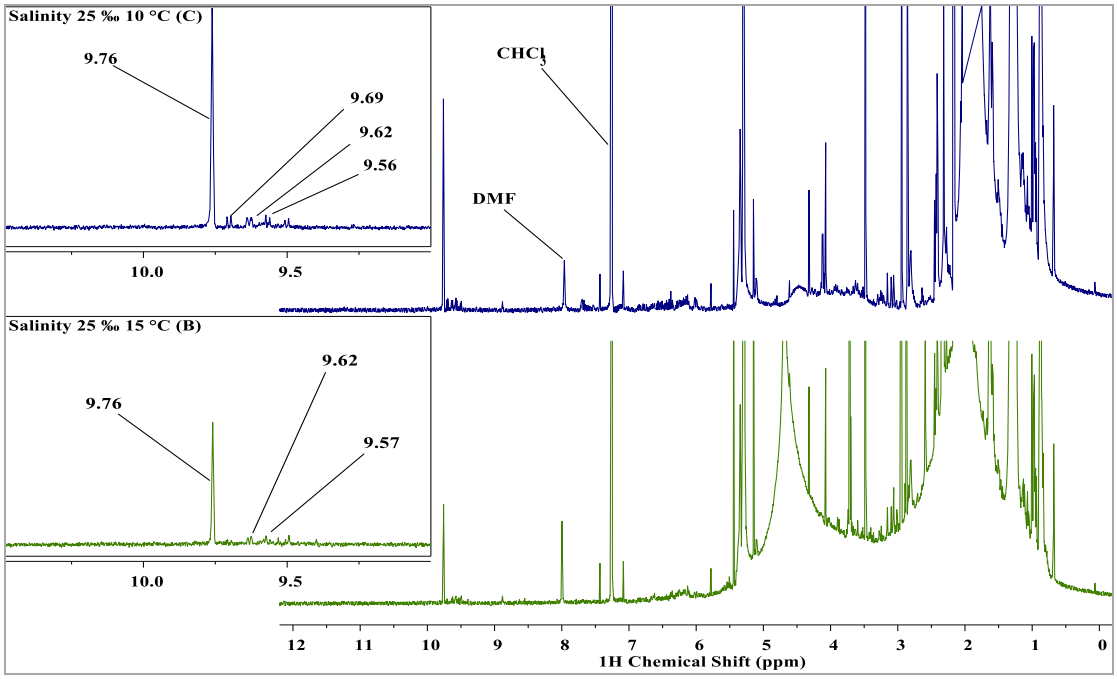

Figure 7. ^{1}H NMR spectra of *Ulva armoricana* for salinity 25 ‰ treatment with replicate C (blue) representing treatment at 10 °C and replicate B (green) for treatment at 15 °C from δ_H -0.20 to δ_H 12.20 for the whole spectra and δ_H 9.00 to δ_H 10.50 for the aldehyde region.

Table 2. Average aldehyde intensity for peak signal at δ_H 9.76 for the salinity experiment at 10 °C and 15 °C.

Treatments	**Average absolute intensity of aldehyde peak at δ_H 9.76 (Average ± SD) * 10^5**
Salinity (‰) at 10 °C	
5	1.96 ± 0.421
10	1.96 ± 1.017
20	2.29 ± 1.800
25	3.31 ± 0.505
35	2.02 ± 1.515
Salinity (‰) at 15 °C	
5	1.82 ± 1.002
10	1.29 ± 1.603
20	1.70 ± 0.981
25	2.15 ± 1.146
35	1.43 ± 0.448

2.3.4 Principal Components Analysis

Principal Components Analysis (PCA) was performed on the normalized bin values obtained from ^{1}H NMR data of three experiments for the region between δ_H -0.20 to δ_H 12.20 (A; whole spectrum) and δ_H 9.00 to δ_H 10.50 (B; aldehyde region) to compare the chemical profiles of *Ulva armoricana* in the different treatments.

2.3.4.1 Grazing experiment

The PCA plots (Figure 8) below depict the distribution of the replicates for the grazing experiments with PCA (A; *left*) representing the whole spectrum (δ_H -0.20 to δ_H 12.20) and PCA (B; *right*) the aldehyde region (δ_H 9.00 to δ_H 10.50). The first two principal components (PCs) for the whole spectrum (A) explained 76.9 % of variability present in the data reporting a high value for the total variance for PC 1 and low figure for PC 2. On the other hand, PCA for the aldehyde region (B) explained 96.8 % variability in the data set accounting PC 1 with very high variation in chemical composition while very little information for PC 2.

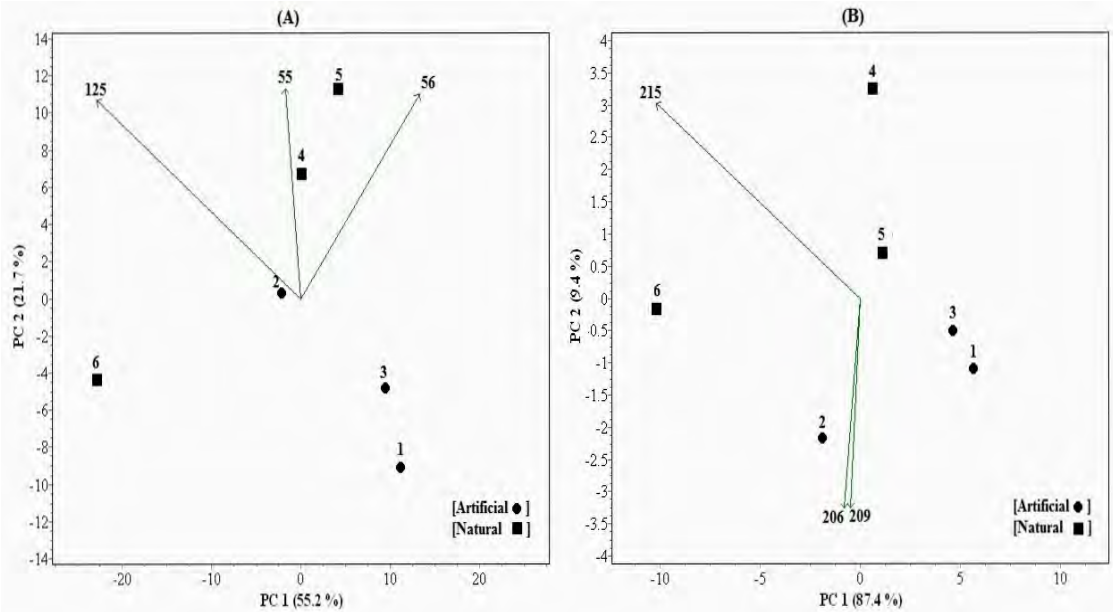

Figure 8. PCAs of normalized bins values of *Ulva armoricana* for the grazing experiments with PCA (A; *left*) representing the whole spectrum between δ_H -0.20 to δ_H 12.20 and PCA (B; *right*) representing the aldehyde region between δ_H 9.00 to δ_H 10.50.

PCA (A) shows that replicates from the artificial grazing experiment had low values for metabolites found between δ_H 2.19 to δ_H 2.70 compare to the natural grazing replicates, where these compounds were more variable and present in higher amounts.

Compounds between δ_H 2.19 to δ_H 2.30 (bin – 55) and δ_H 2.30 to δ_H 2.70 (bin – 56) corresponding to the carbonyl (HC–C=O) region, were higher in the natural grazing replicates compare artificial grazing replicates. In PCA (B), artificial grazing replicates showed to have higher aldehyde levels between δ_H 9.39 to δ_H 9.45 (bin – 206) and δ_H 9.51 to δ_H 9.55 (bin – 209), while replicates from the natural grazing experiment had higher aldehyde levels for the compounds between δ_H 9.75 to δ_H 9.79 (bin – 215).

2.3.4.2 Nutrient experiment

The PCA plots below (Figure 9) illustrate the distribution of the replicates for the four different nutrient treatments with (A; *left*) representing the whole spectra (δ_H -0.20 to δ_H 12.20) and (B; *right*) for the aldehyde region (δ_H 9.00 to δ_H 10.50). The first two principal components (PCs) for the whole spectrum (A) explained 75.8 % of variability present among the data reporting a higher value for the total variance in PC 1 and a lower value for PC 2 while for the aldehyde region (B), there was 97.3 % variability in the data set with PC 1 accounting for very high variation and PC 2 with very little information. In both PCAs, there was very little clustering, suggesting that treatments had no consistent effects on total chemical composition, or aldehyde profiles.

PCA (A) shows that, replicates from the high nutrient experiment at 15 °C had higher levels of chemical compounds from the δ_H 6.35 to δ_H 6.55 region corresponding to the aromatic (benzene-containing) compounds, compared to the high nutrient experiment at 10 °C. On the other hand, two replicates from the low nutrient experiment at 10 °C showed to have higher levels of chemical compounds between the δ_H 6.35 to δ_H 6.55

region compared to replicates of the low nutrient experiment at 15 °C. In PCA (B), replicates from high nutrient experiments were clustered and had relatively low levels of aldehydes for bin – 215 (δ_H 9.75 to δ_H 9.79) compared to two replicates from the low nutrient experiment at 10 °C and one from 15 °C.

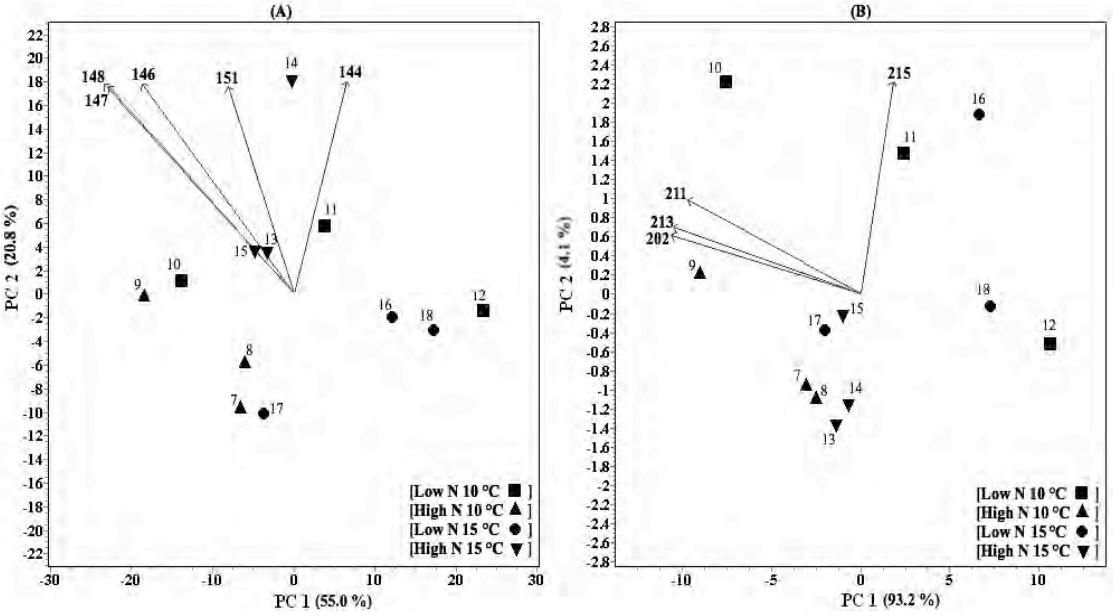

Figure 9. PCAs of normalized bins values of *Ulva armoricana* for the nutrient experiments with PCA (A; *left*) representing the whole spectrum between δ_H -0.20 to δ_H 12.20 and PCA (B; *right*) representing the aldehyde region between δ_H 9.00 to δ_H 10.50.

2.3.4.3 Salinity experiment

Figure 10 shows the PCAs for salinity experiments at 10 °C with A (*left*) representing the bin values for the whole region of the spectrum (δ_H -0.20 to δ_H 12.20) and B (*right*) indicating the bin values for the aldehyde region (δ_H 9.00 to δ_H 10.50). The first two principal components (PCs) explained 73.1 % variability present among the data in PCA (A) accounting for a high value in the total variance for PC 1 and a low value to PC 2. Similar trend was observed where PC 1 of PCA (B) which had a very high value compare to PC 2, thus explaining larger variability in PC 1. Both PCAs did

not indicate any clear patterns between the chemical profile of the seaweed, and suggested that the treatments had no consistent effect on total chemical composition and aldehyde profiles of *Ulva* for salinity variations at 10 °C.

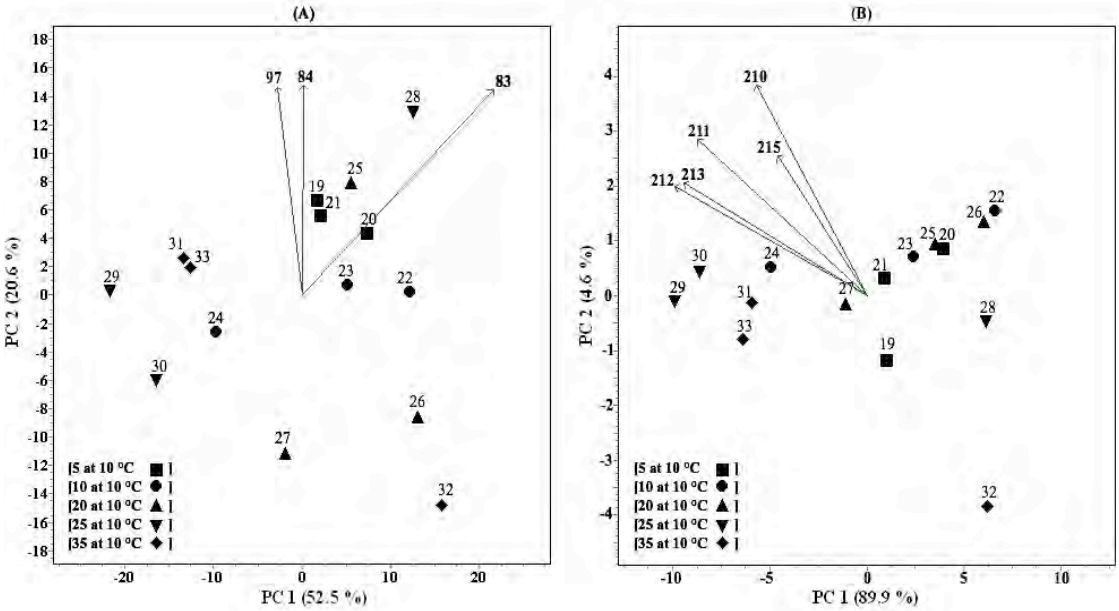

Figure 10. PCAs of normalized bins values of *Ulva armoricana* for the salinity experiments at 10 °C with PCA (A; *left*) representing the whole spectrum between δ_H -0.20 to δ_H 12.20 and PCA (B; *right*) representing the aldehyde region between δ_H 9.00 to δ_H 10.50.

Figure 11 below illustrates the PCA plots for the distribution of the replicates for the salinity experiment at 15 °C with (C; *left*) representing the bin values whole spectra (δ_H -0.20 to δ_H 12.20) and (D; *right*) the bin values for the aldehyde region (δ_H 9.00 to δ_H 10.50). The first two principal components (PCs) explained 74.1 % variability present among the data in PCA (C) accounting for a high value in the total variance for PC 1 compare to a low value for PC 2 while for the aldehyde region (D), 99.2 % of variability was present in the data which reported PC 1 with a very high value and PC 2 with a very low value. In PCA (C), replicates of the salinity treatments at 35 were clustered near bin – 80 and bin – 93, indicating that the chemical composition

between the δ_H 3.31 to δ_H 4.03 region were higher in these replicates, while other salinity treatments did not indicate any clear patterns between the chemical profile of the seaweed. Similar trend was observed for the aldehyde region in PCA (D), where variations in salinity at 15 °C did not appear to have a clear effect on the chemical composition of the *Ulva* species.

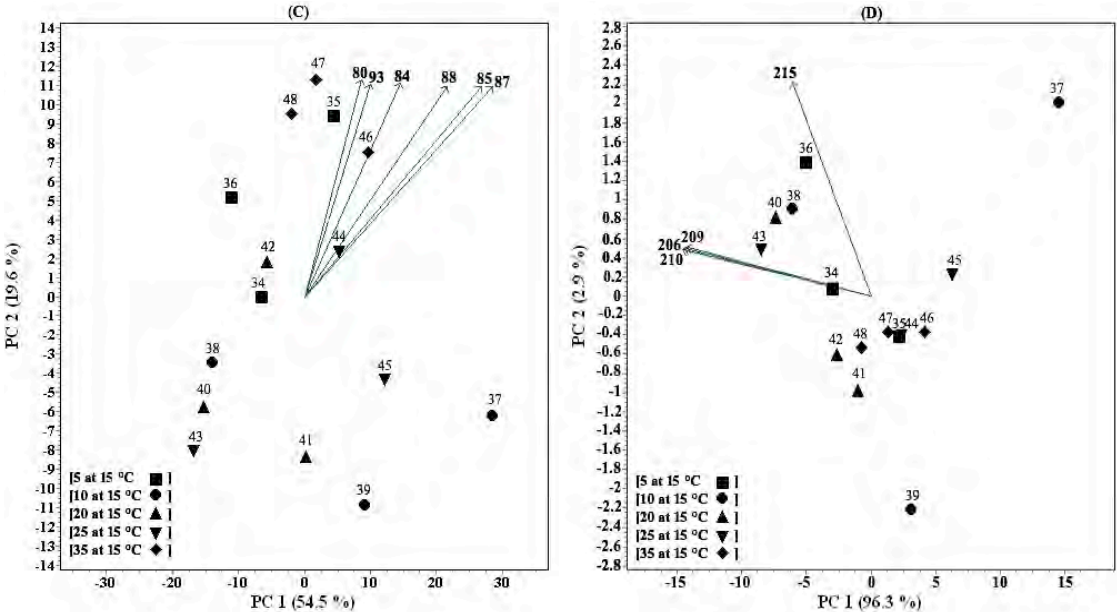

Figure 11. PCAs of normalized bins values of *Ulva armoricana* for the salinity experiments at 15 °C with PCA (C; *left*) representing the whole spectrum between δ_H -0.20 to δ_H 12.20 and PCA (D; *right*) representing the aldehyde region between δ_H 9.00 to δ_H 10.50.

2.4 Discussion and Conclusion

For more than a decade, *Ulva* cultivation on South African abalone farms has shown to be very successful and its use as feed has been increasing (Bolton *et al.,* 2009). There has been little research on the chemistry of locally grown *Ulva*. What has been done is limited to quantification of nutritional content as feed, especially crude protein levels (Shuuluka *et al.,* 2013). The present study is a preliminary work towards the elucidation of more detailed aspects of the chemistry of locally aquacultured *Ulva.* The combination of NMR techniques and multivariate statistical analysis was used to investigate what potential effects of environmental variables and grazing might have on the general chemistry of the seaweed, and also specifically on the aldehyde region (δ_H 9.00 to δ_H 10.50). Previous studies on the chemistry of *Ulva* have confirmed the presence of several aldehydes that have sensory aroma characteristics (*e.g.* Sugisawa *et al.,* 1990; Fujimura *et al.,* 1990; Akakabe *et al.,* 2003). The presence of compounds with stimulant- or attractant-like properties would be beneficial in term of knowledge for the local aquacultured *Ulva*, but most importantly for the abalone industry. Results from this study show that an aldehyde peak at δ_H 9.76 was prominent in almost all the replicates and is suggested to be the short-chain saturated aldehyde hexanal (based on the shape of the peak and chemical shift; D Beukes, *pers. comm.*), but further isolation and characterization needs to be done to confirm the accurate identity of the peak.

Aldehydes consist the main group of volatile compounds in green algae such as *Ulva* (Fujimura *et al.,* 1990; Kajiwara *et al.,* 1992). Volatile compounds belonging to both long- and short-chain aldehydes, such as hexanal, (E)-2-octenal, (E)-2-nonenal, (Z,E)-2,6-nonadienal, pentadecanal, (Z,Z)-8,11-heptadecadienal, (Z,Z,Z)-8,11,14-heptadecatrienal and (Z)-8-heptadecenal, have been identified and isolated from *Ulva*,

and they are considered to be the most important flavor components (*e.g.* Sugisawa *et al.,* 1990; Fujimura *et al.,* 1990; Akakabe *et al.,* 2003). The biosynthesis of these aldehydes occurs mainly through α-oxidation by α-oxygenase of long-chain fatty acids followed by decarboxylation (Akakabe *et al.,* 2003). (3Z)-unsaturated aldehydes such as (2E)-hexenal and (2E)-nonenal are biosynthesized from linolenic acid and arachidonic acid when seaweeds are damaged or macerated (Boonprab *et al.,* 2003a,b; Kajiwara *et al.,* 2006). Furthermore, it has also been suggested that aldehydes and ketones could originate from the degradation of carotenoids and unsaturated fatty acids (Rzama *et al.,* 1995).

In the present study, outcomes obtained from PCAs for the whole spectra for grazing and nutrient experiments showed that there were more variations occurring in the ^{1}H NMR data between δ_H 2.19 to δ_H 2.70 and δ_H 6.33 to δ_H 6.55, which represent compounds from the aromatic (benzene–containing; Ar–H) origins such as phenol, hydroxylic (R–OH) and compounds from carbonyl (HC–C=O) origins such as ketone. For the grazing experiment, the natural grazing treatment showed higher variation in the chemical profiles than the artificial grazing treatment, while for the nutrient experiment, low nutrient treatments at both 10 °C and 15 °C showed more variation among the data compared to the other nutrient treatments. Phenolic compounds such as phenol, 4–hydroxybenzoic acid, 4–hydroxyphenylacetic acid, 4–hydroxyphenyllactic acid, 4–hydroxybenzaldehyde, 3,5–dibromo–4–hydroxybenzoic acid and 2,4,6–tribromophenol originated from aromatic compound hydroxybenzoic acid, have been reported in *Ulva* species (*e.g.* Flodin and Whitfield, 1999). Recently, *in situ* nutrient enrichment experiments on brown seaweed *Fucus vesiculosus* showed that polyphenolic contents of that seaweed were higher at a low nutrient site compared to that at a high nutrient site (Yates and Pectol, 1993). Conversely, the level

of phenol compounds in the tissue of *Ulva rigida* could increase by 60 % when incubated at high nitrate concentration of 50 µM NO_3^- relative to low nitrate concentration of 0 µM NO_3^- (Cabello-Pasini *et al.,* 2011). Phenolic compounds are common in terrestrial and marine plants, they could have several biological activities such as antimicrobial and antioxidant capacities (Vijayavel and Martinez, 2010; Trigui *et al.,* 2013). On the other hand, the synthesis of carbonyl groups in *Ulva* may be produced indirectly through the improvement of photosynthesis due to nitrate enrichment (Lee and Wang, 2001).

Hyper/hypo-saline conditions are known to affect the osmotic regulation, which is crucial to maintain the homeostasis within cells in seaweeds (Eggert *et al.,* 2007; Beauchamp, 2012). Under hypersaline conditions, osmotically active substances are synthesized and accumulated in seaweeds, but these are excreted or degraded under hyposaline conditions (Kirst, 1990). The maintenance of constant cell turgor pressure in response to salinity changes by variations of osmotic potentials is a mechanism characteristic of seaweeds which is obtained by the adjustment of internal inorganic ions and organic osmolytes concentrations (Edwards *et al.,* 1987; Kirst, 1990; Liu *et al.,* 2000; Kakinuma *et al.,* 2006). *Ulva* species have been shown to be tolerant over a wide range of salinity from as low as 0 PSU to very high salinity levels (*e.g.* Taylor *et al.,* 2001). PCAs from this study for the whole region of the ^{1}H NMR show that there were considerable variations among the chemical profile in the δ_H 3.59 to δ_H 4.19 region in *Ulva* cultured under different salinities, and this region represents compounds from the alcohol (HC–OH), ester (RCOO–CH) and phenolic (AR–OH) regions. Salinity treatment of 35 ‰ at 15 °C had higher levels of chemical compounds between the δ_H 3.31 to δ_H 4.03 region compared to the other salinity treatments. Therefore, it can be suggested that the chemical compounds with NMR

values in the region δ_H 3.59 – 4.19 could be associated with compounds responsible for the osmoregulation of the seaweed, since the seaweed was subjected to range of salinity from 5 to 35 ‰.

The change in chemical components due to low and high salinity conditions may be present in *Ulva* and it could also affect photosynthesis, carbon and nitrogen mechanism, where under hypersaline conditions formation and accumulation of organic osmolyte was noted (Kakinuma *et al.,* 2006). A previous study using (^{1}H) proton Nuclear Magnetic Resonance Spectroscopy has confirmed the identification of methylated osmolytes such as glycine betaine and *β*-dimethylsulfoniopropionate between the range δ_H 3.00 – 4.00 in algae species such as *Ulva lactuca* L., *Ulva intestinalis* and *Ulva linza* (Chudek *et al.,* 1987). Organic *β*-dimethylsulfoniopropionate (DMSP) could be the main low molecular weight osmolyte present in *Ulva intestinalis* and could play a role in the osmotic adjustment process (Edwards *et al.,* 1987). *β*-dimethylsulfoniopropionate (DMSP) in the tissue content of *Ulva lactuca* responded to changes in salinity and when the salinity was increased the concentration of DMSP also rose while that of Na+ ions fell (Dickson *et al.,* 1980). However, it has been suggested that *Ulva fenestrata* uses metabolites other than DMSP for osmotic acclimation (Van Alstyne *et al.,* 2003). Other compounds such as tissue sucrose and proline could be involved in hyperosmotic adjustment in *Ulva intestinalis* (L.) Link (Edwards *et al.,* 1987).

Principal component analysis done on the aldehyde region (δ_H 9.00 to δ_H 10.50) showed that the aldehyde peak at δ_H 9.76 was dominant in almost all treatments except under artificial grazing. For the grazing experiment, results obtained from the PCA showed much of the variation occurred between δ_H 9.39 to δ_H 9.55. Aldehyde

peaks associated with those variations were from the artificial grazing treatment and they were at δ_H 9.54 and δ_H 9.40. These two peaks were also present in the natural grazing treatment and control but to at a lesser intensity compared to the artificial grazing treatment, and they could be associated with wound healing and grazer resistance in *Ulva armoricana*. When comparing the aldehyde region of the salinity 35 ‰ at 15°C treatment (non–grazing, slight difference in methods – shaken versus aerated) to that of the grazing experiments, there were some differences in term of intensity of the aldehydes with peak at δ_H 9.76 being the most prominent. On the other hand, nutrient and salinity variations did not seems to have significant effects on the aldehyde region. From treatments in these two experiments, the aldehyde peak at δ_H 9.76 was the most prominent compared to other peaks such δ_H 9.69, δ_H 9.62, δ_H 9.56, δ_H 9.54 and δ_H 9.39. For the nutrient experiment, the highest absolute aldehyde intensity at δ_H 9.76 was reported in low nutrient treatments, while for the salinity experiment, salinity treatments at 25 ‰ reported the highest absolute aldehyde intensity. In both these experiments, seaweed cultured at 10 °C produced higher aldehyde intensity than that cultured at 15 °C. Therefore, it can be suggested that *Ulva armoricana* grown at 10 °C under low nutrient condition in a medium with a salinity of 25 ‰ would produce the highest amount of aldehyde at peak δ_H 9.76.

Volatile short-chain aldehydes C-6 and C-9 such as (E)-2-nonenal, and (E)-2-hexenal, which have "*fresh green*" odor, could to play a role in the wound healing mechanism and in fungicidal activities in higher plants (Noordermeer *et al.*, 2001; Matsui *et al.*, 2006), and these aldehydes could be also present in the brown alga *Saccharina* (as *Laminaria*) *angustata* (Boonprab *et al.*, 2003a). Middle-chain aldehydes such as (E,Z)-2,4-decadienal and (E,E)-2,4-decadienal were reported in *Ulva conglobata* (Akakabe *et al.*, 2003), and a previous study on the South African abalone *Haliotis*

midae concluded that diatom derived 2,4-decadienal can act as potential chemotherapeutic agent against shell-infesting polychaetes (Simon *et al.,* 2010).

Akakabe and Kajiwara (2008) reported that long-chain aldehydes that were the main component from *Ulva pertusa* essential oil that were found to increase by mechanical wounding of the alga. The feeding behaviors of turbinid gastropod *Lunella coronata coreensis* also showed to be controlled by chemoreception compounds found in the essential oil of *Ulva pertusa,* which acted as feed attractants (Akakabe and Kajiwara (2008). Feeding preferences tests on *Littorina striata* King and Broderip using red, brown and green algae, demonstrated a preference for *Ulva rigida* and *Enteromorpha ramulosa* (currently regarded as a synonym of *Ulva clathrata* (Roth) C. Agardh) (Granado and Caballero, 1991).

This preliminary study towards the elucidation of the chemistry of local *Ulva* has made some interesting initial findings. The potential effects of environmental variables and grazing on the chemistry of *Ulva* have been investigated in this study. It was aimed to investigate the potential effects of environmental variables and grazing on the chemistry, and more specifically on the aldehyde profile of aquacultured Ulva using Nuclear Magnetic Resonance (NMR) techniques and a quantitative metabolomic approach using multivariate statistics. *Ulva armoricana* cultured at different salinities: 5, 10, 20, 25 and 35 ‰ (all ± 0.1 ‰) at 10 °C and 15 °C, showed that variations of salinity affect the metabolites corresponding to the alcohol, ester and phenolic regions of the ^{1}H NMR spectra, but the variations did not produce any pattern in the data that could be used to separate the seaweed grown at different salinities. In contrast, *Ulva* grown at different nutrient concentrations: 100 % Provasoli ES medium (high nutrient supply) and 0% Provasoli ES medium (low

nutrient supply) at 10 °C and 15 °C, affected the seaweed mostly in aromatic compounds regions. However in the grazing experiment, the greatest effect was seen on the hydroxylic and carbonyl compounds regions. For the aldehyde region, peak at δ_H 9.76 was the most prominent in almost all the treatments, and it was suggested by Assoc Prof. Denzil Beukes that the peak could be hexanal based on its shape and chemical shift, but further isolation and characterization needs to be done to confirm the exact identity of the compound. Peaks δ_H 9.54 and δ_H 9.40 were the most dominant in the artificial grazing experiment. Seaweeds cultured at 10 °C were found to have the most effect on the principal aldehyde peak (δ_H 9.76), where *Ulva* grown at 10 °C in both nutrient and salinity experiments were found to produce higher amount of aldehyde compare to nutrient and salinity experiments at 15 °C. The present work was the first step towards clarification of the chemical composition of *Ulva* and its potential change when subjected to various environmental variations. Further isolation and characterization studies need to be done in order to validate metabolites of interest and their potential applications in the South African aquaculture industry.

Recommendations for future work

Future studies should focus on:

- Isolation and characterization of the compounds of interest such as the aldehyde peak at δ_H 9.76 using a combination of Gas Chromatography–Mass Spectrometry (GC-MS).

- Screening for compounds in the *Ulva* which may have attractant effects (particularly aldehydes).

- Evaluate other compounds (aldehydes) that may have repellant properties.

There are several questions that still needs to be answered based on the outcome from this project and these are:

Does *Ulva* from aquaculture produce significant amounts of aldehyde(s) which may have attractant properties?

What is the amount of chemical (aldehydes or other chemicals such as DMSP) that *Ulva* needs to produce to act as an attractant, stimulant, arrestant or repellent?

Does laboratory synthesized attractant has the same effect as pure *Ulva* extract?

Can outcome(s) from this research be applied to abalone, sea urchin or fish aquaculture to increase grazing properties of feed?

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Appendices

I. Whole ^{1}H NMR Spectra (Results)

1) Grazing experiments

a) Natural grazing

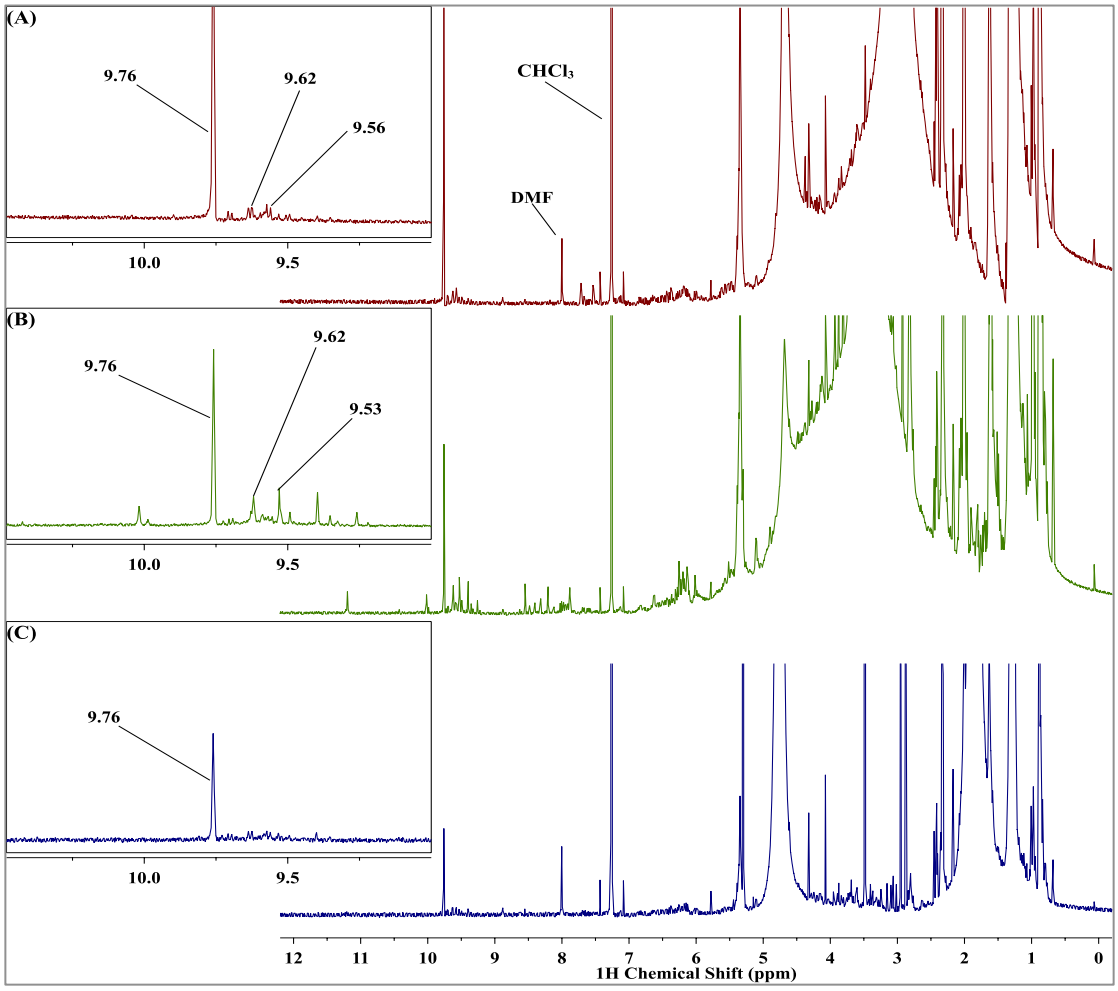

Figure A 1. ^{1}H NMR spectra of *Ulva armoricana* for natural grazing experiment with replicates: A (maroon), B (green) and C (blue) from δ_H -0.20 to δ_H 12.20 for the whole spectra and from δ_H 9.00 to δ_H 10.50 for the aldehyde region.

b) Artificial grazing

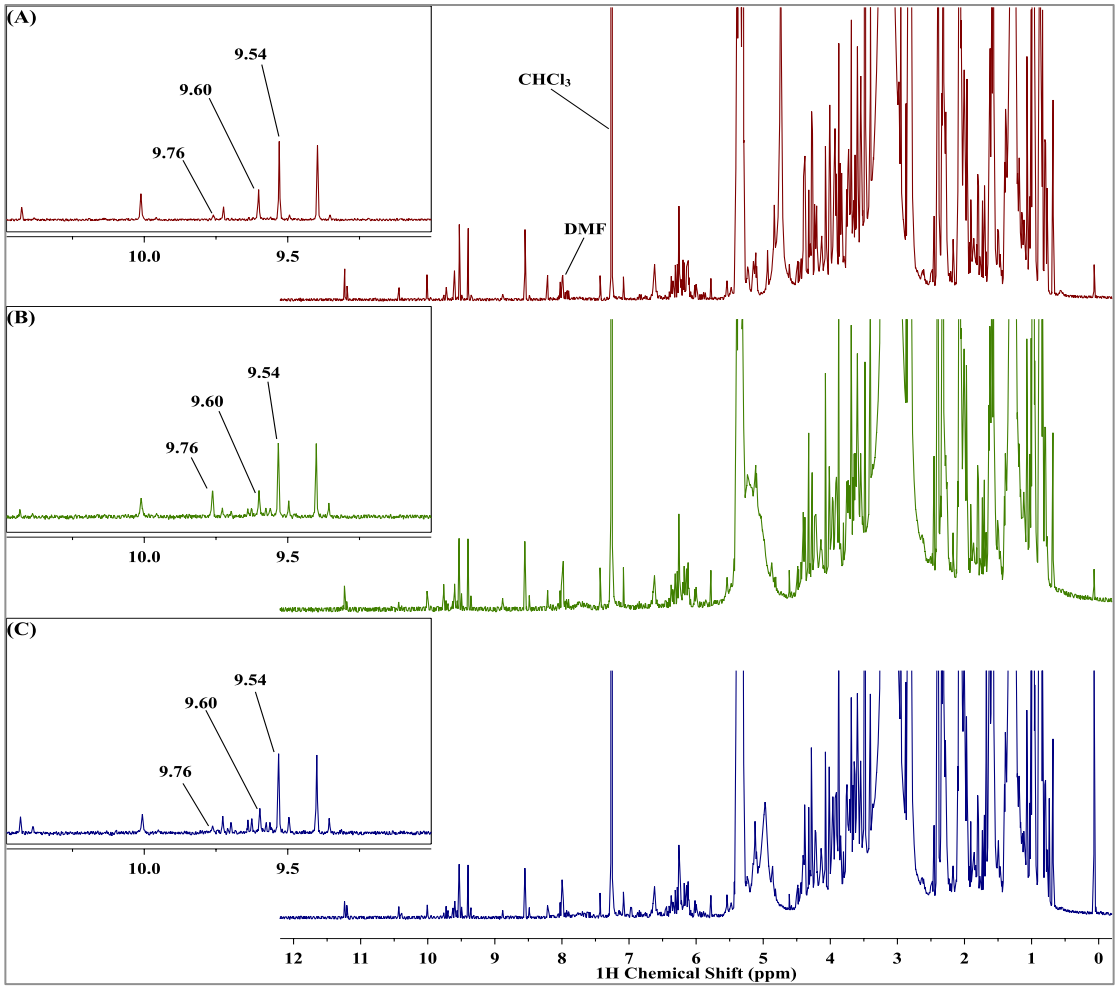

Figure A 2. ^{1}H NMR spectra of *Ulva armoricana* for artificial grazing experiment with replicates: A (maroon), B (green) and C (blue) from δ_H -0.20 to δ_H 12.20 for the whole spectra and from δ_H 9.00 to δ_H 10.50 for the aldehyde region.

2) Nutrient experiment

Treatments at 10 °C

a) Low nutrient supply (0% ES)

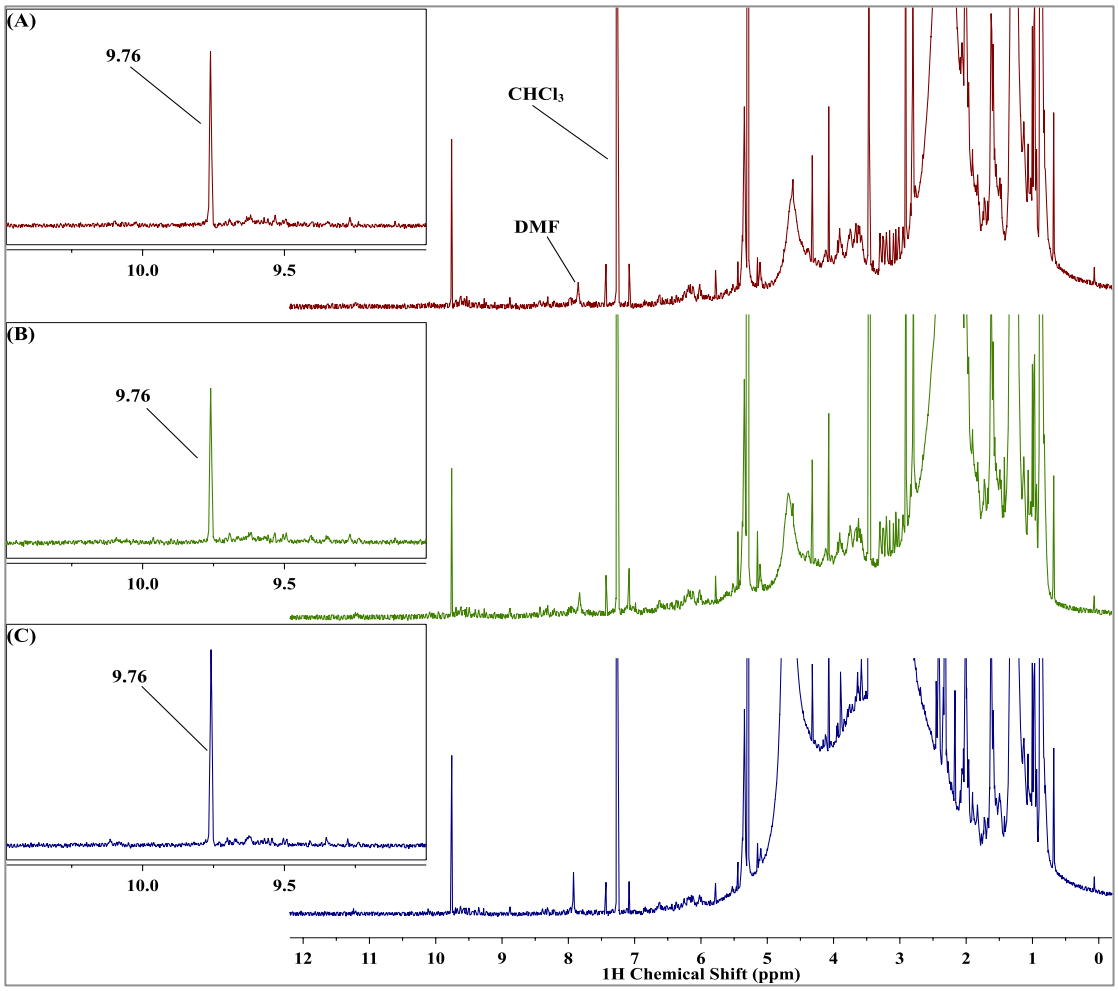

Figure A 3. ^{1}H NMR spectra of *Ulva armoricana* for low nutrient supply (0% ES) at 10 °C experiment with replicates: A (maroon), B (green) and C (blue) from δ_H -0.20 to δ_H 12.20 for the whole spectra and from δ_H 9.00 to δ_H 10.50 for the aldehyde region.

b) High nutrient supply (100% ES)

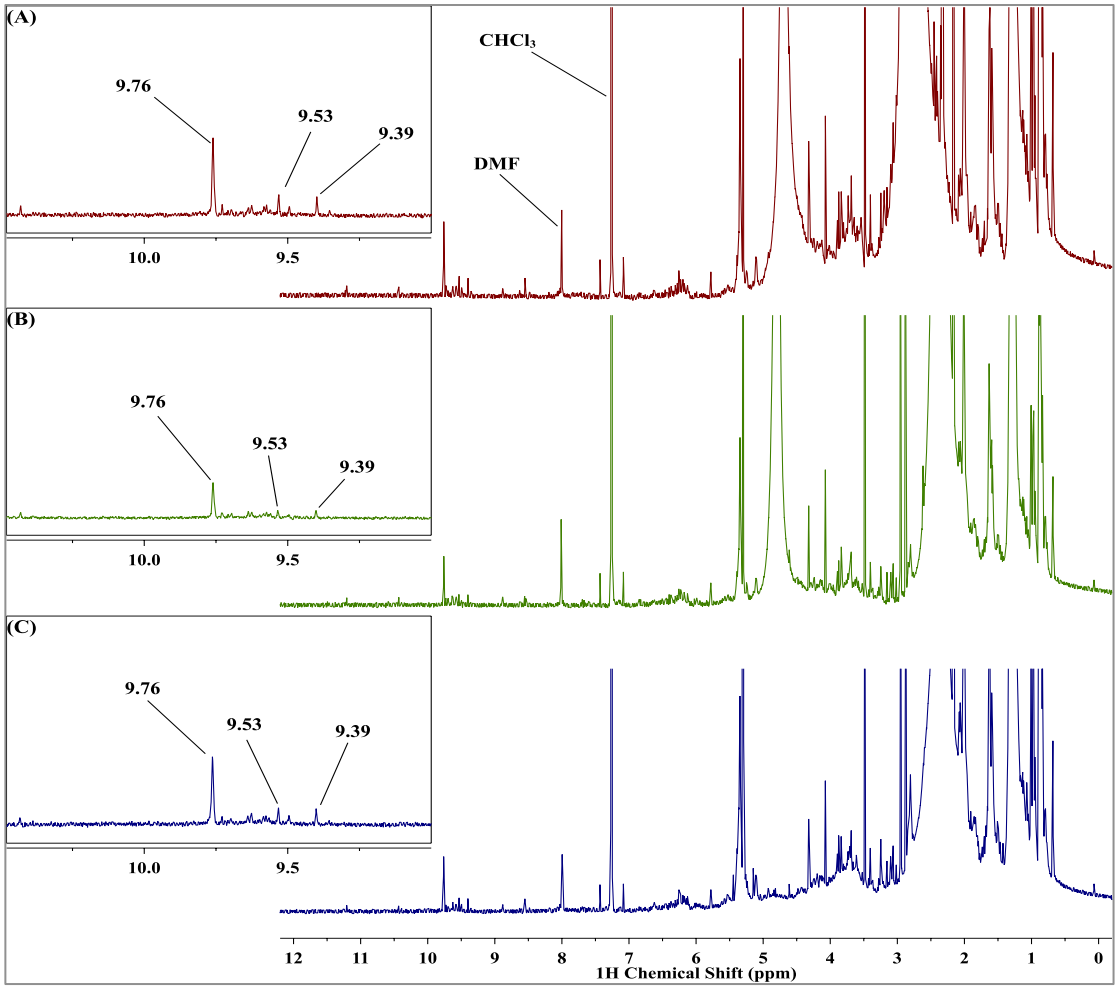

Figure A 4. ^{1}H NMR spectra of *Ulva armoricana* for high nutrient supply (100% ES) at 10 °C experiment with replicates: A (maroon), B (green) and C (blue) from δ_H -0.20 to δ_H 12.20 for the whole spectra and from δ_H 9.00 to δ_H 10.50 for the aldehyde region.

Treatments at 15 °C

a) Low nutrient supply (0% ES)

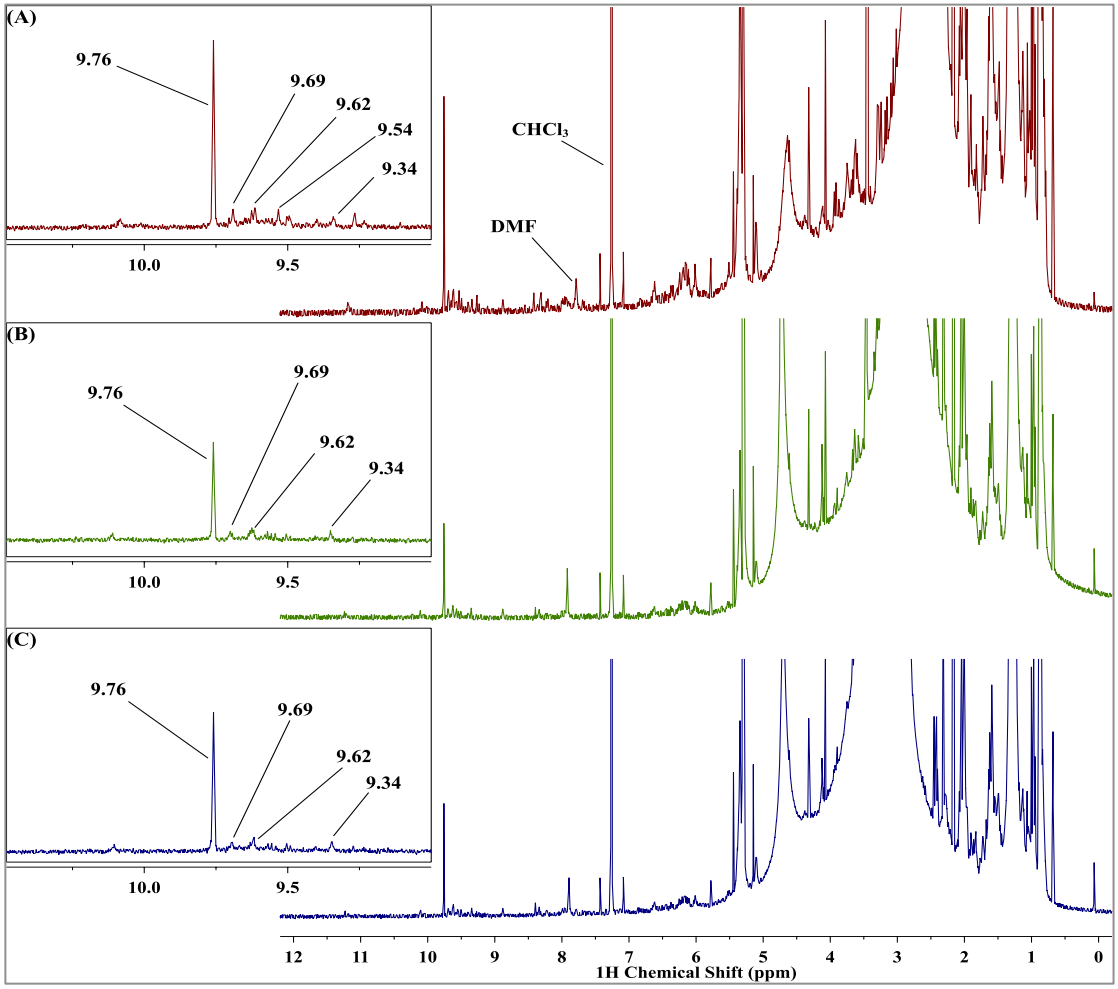

Figure A 5. ^{1}H NMR spectra of *Ulva armoricana* for low nutrient supply (0% ES) at 15 °C experiment with replicates: A (maroon), B (green) and C (blue) from δ_H -0.20 to δ_H 12.20 for the whole spectra and from δ_H 9.00 to δ_H 10.50 for the aldehyde region.

b) High nutrient supply (100% ES)

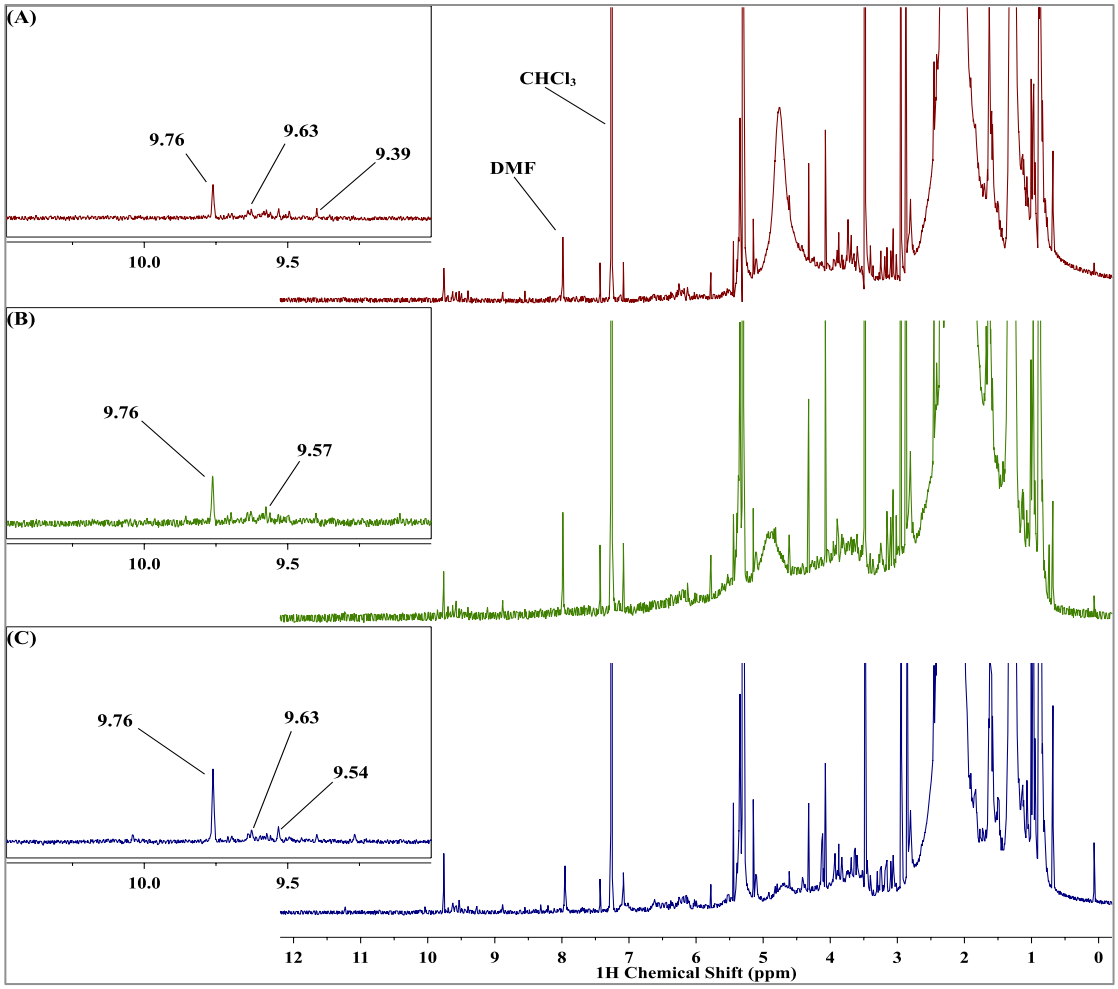

Figure A 6. ^{1}H NMR spectra of *Ulva armoricana* for high nutrient supply (100% ES) at 15 °C experiment with replicates: A (maroon), B (green) and C (blue) from δ_H -0.20 to δ_H 12.20 for the whole spectra and from δ_H 9.00 to δ_H 10.50 for the aldehyde region.

3) Salinity experiments

Treatments at 10 °C

a) Salinity 5 ‰ treatment

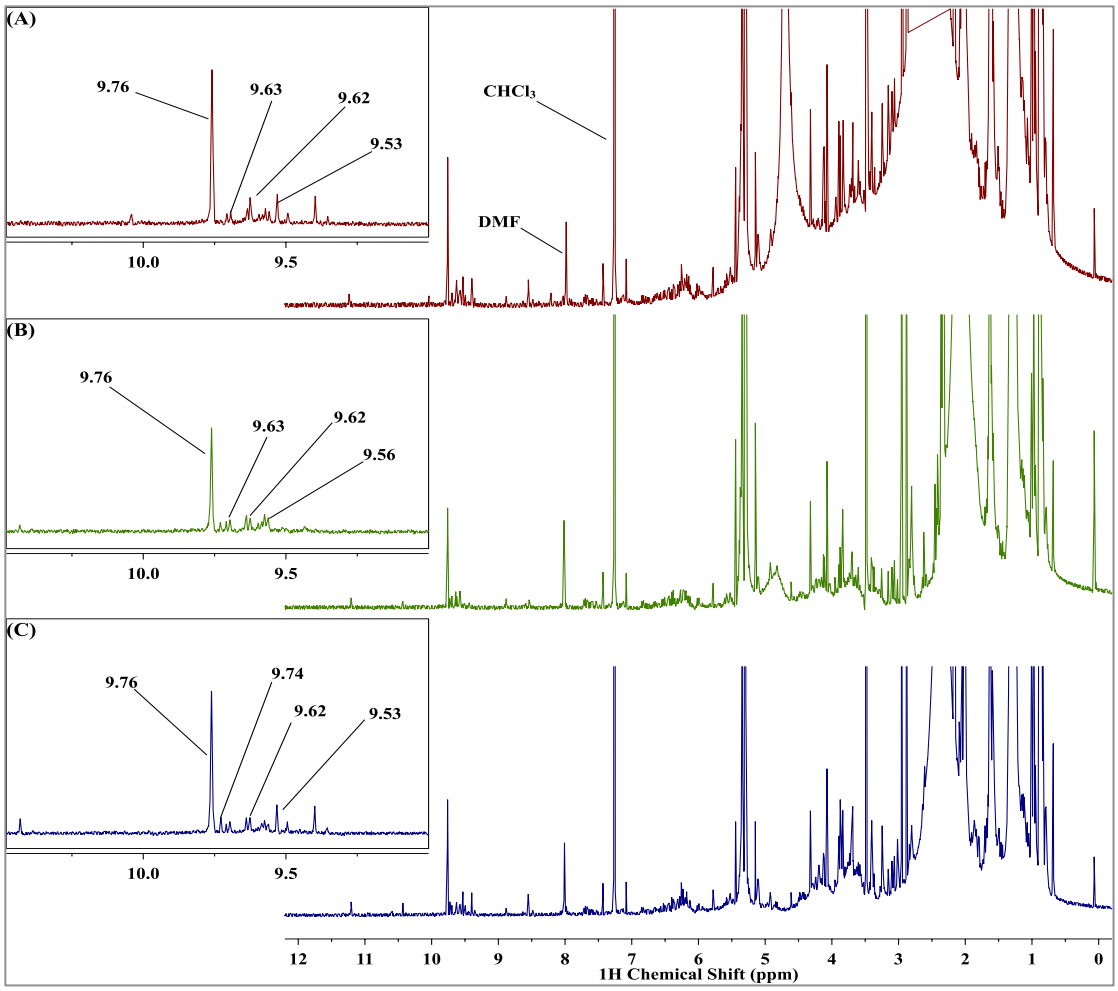

Figure A 7. ^{1}H NMR spectra of *Ulva armoricana* for salinity 5 ‰ at 10 °C experiment with replicates: A (maroon), B (green) and C (blue) from δ_H -0.20 to δ_H 12.20 for the whole spectra and from δ_H 9.00 to δ_H 10.50 for the aldehyde region.

b) Salinity 10 ‰ treatment

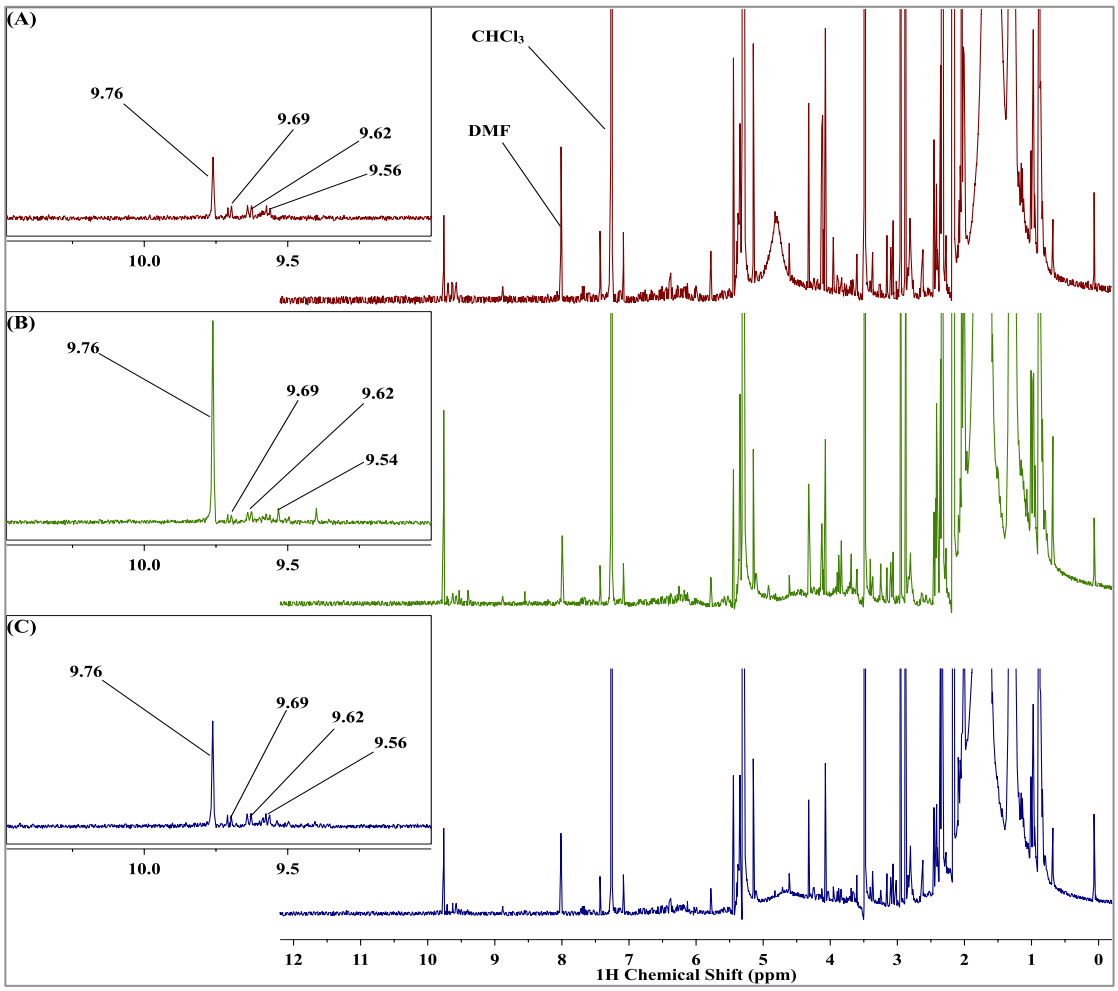

Figure A 8. ^{1}H NMR spectra of *Ulva armoricana* for salinity 10 ‰ at 10 °C experiment with replicates: A (maroon), B (green) and C (blue) from δ_H -0.20 to δ_H 12.20 for the whole spectra and from δ_H 9.00 to δ_H 10.50 for the aldehyde region.

c) Salinity 20 ‰ treatment

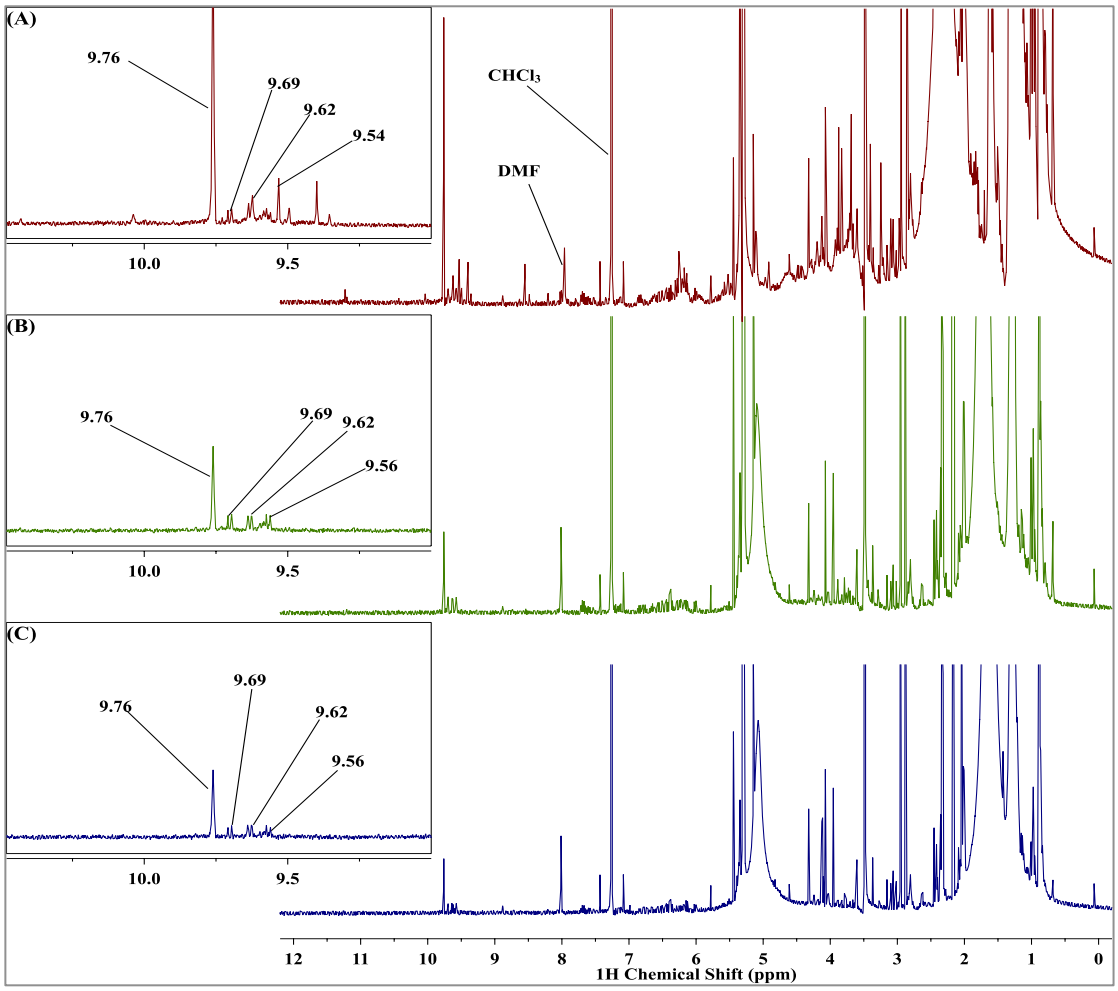

Figure A 9. ^{1}H NMR spectra of *Ulva armoricana* for salinity 20 ‰ at 10 °C experiment with replicates: A (maroon), B (green) and C (blue) from δ_H -0.20 to δ_H 12.20 for the whole spectra and from δ_H 9.00 to δ_H 10.50 for the aldehyde region.

d) Salinity 25 ‰ treatment

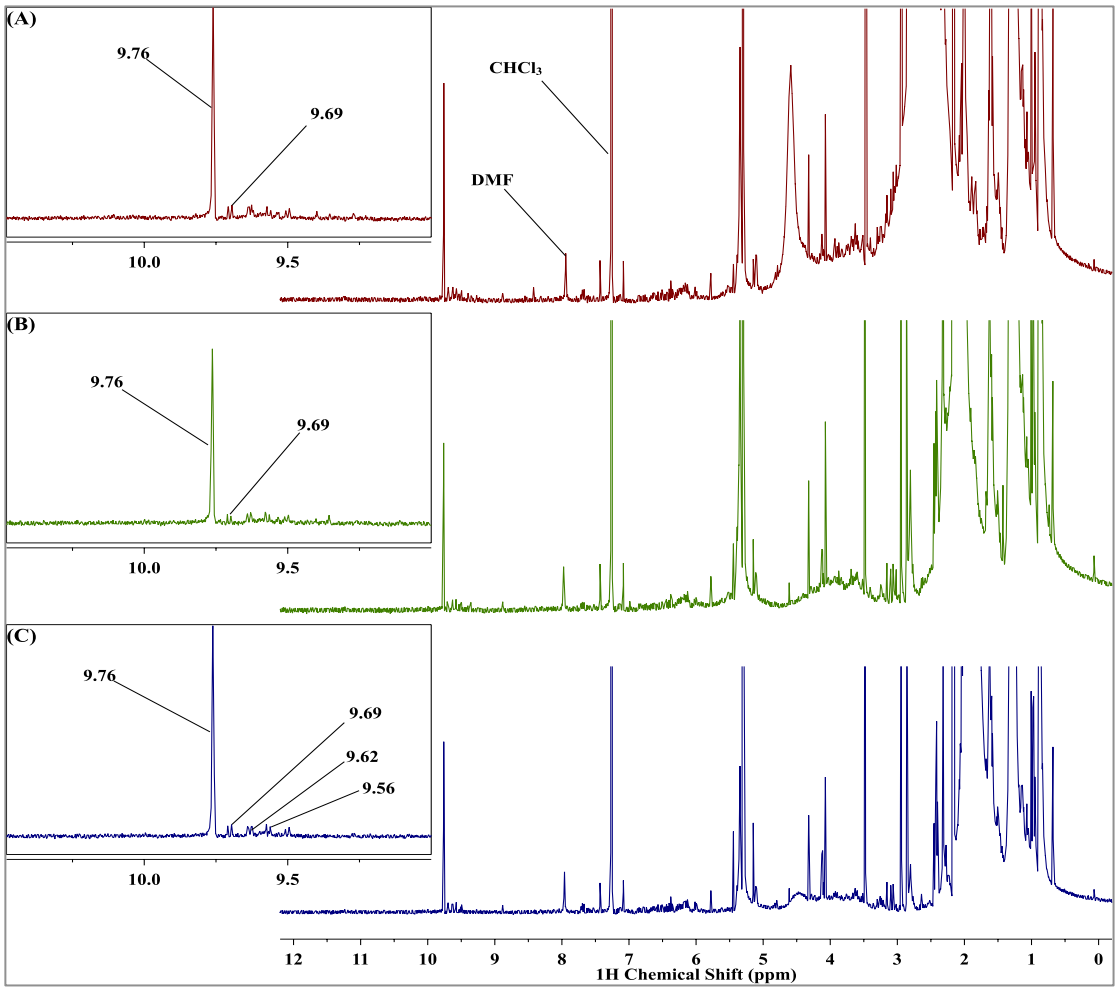

Figure A 10. ^{1}H NMR spectra of *Ulva armoricana* for salinity 25 ‰ at 10 °C experiment with replicates: A (maroon), B (green) and C (blue) from δ_H -0.20 to δ_H 12.20 for the whole spectra and from δ_H 9.00 to δ_H 10.50 for the aldehyde region.

e) Salinity 35 ‰ treatment

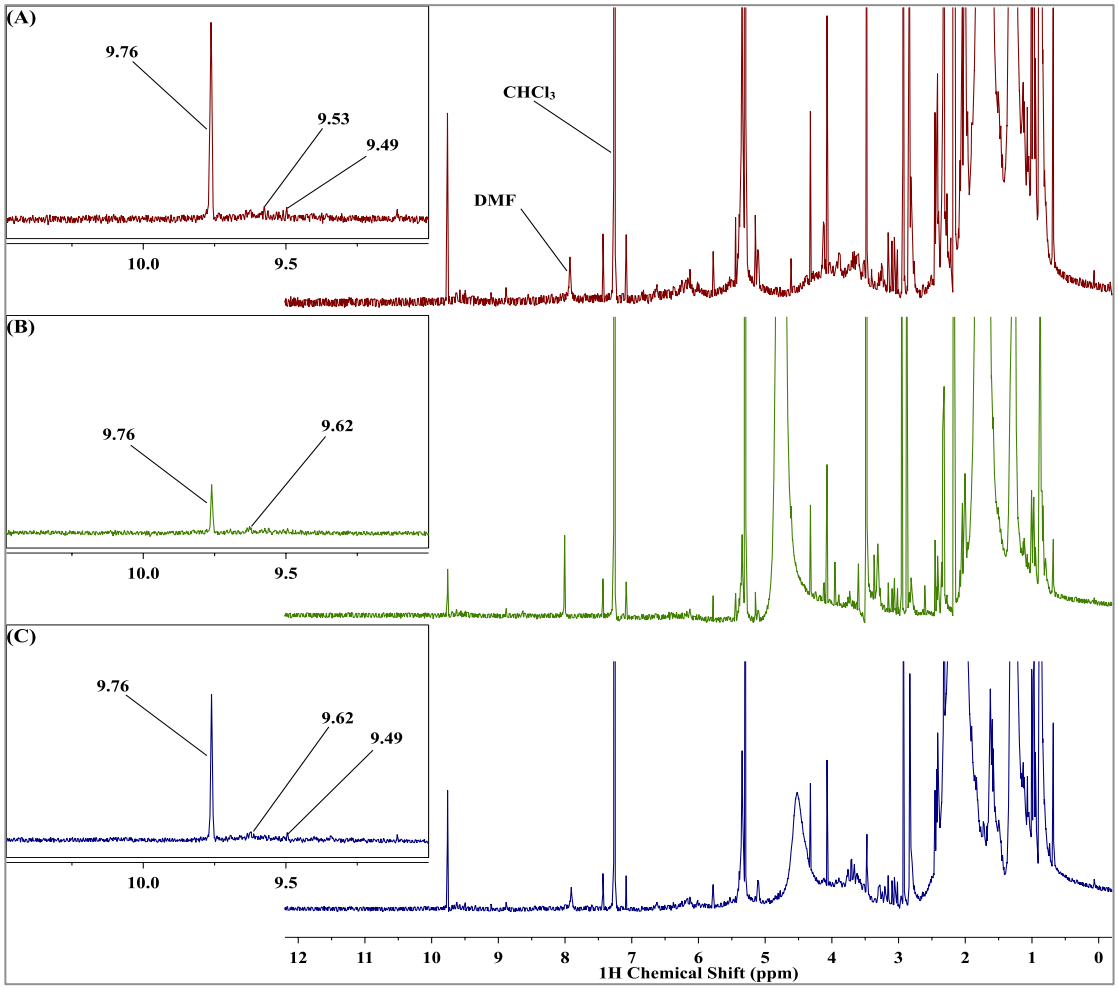

Figure A 11. ^{1}H NMR spectra of *Ulva armoricana* for salinity 35 ‰ at 10 °C experiment with replicates: A (maroon), B (green) and C (blue) from δ_H -0.20 to δ_H 12.20 for the whole spectra and from δ_H 9.00 to δ_H 10.50 for the aldehyde region.

Treatments at 15 °C

a) Salinity 5 ‰ treatment

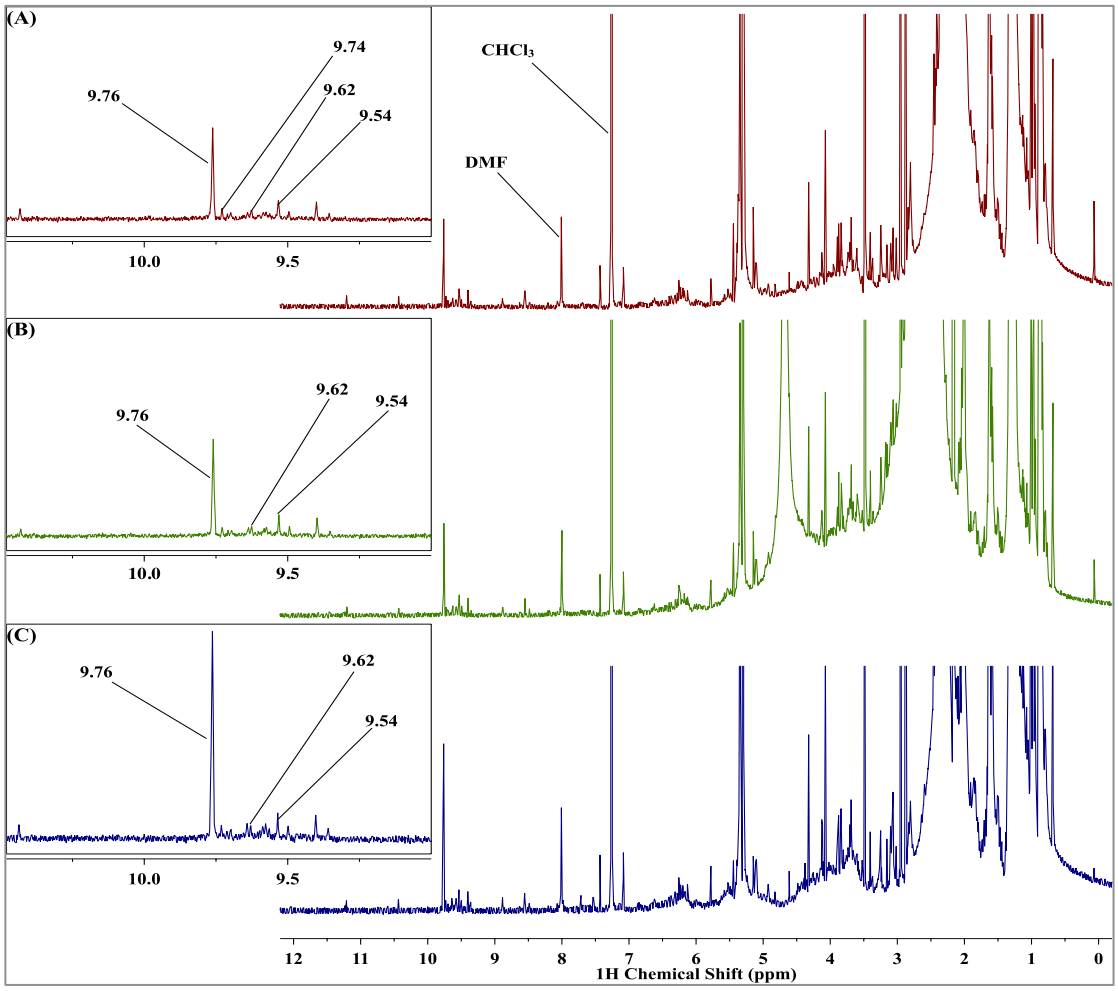

Figure A 12. ^{1}H NMR spectra of *Ulva armoricana* for salinity 5 ‰ at 15 °C experiment with replicates: A (maroon), B (green) and C (blue) from δ_H -0.20 to δ_H 12.20 for the whole spectra and from δ_H 9.00 to δ_H 10.50 for the aldehyde region.

b) Salinity 10 ‰ treatment

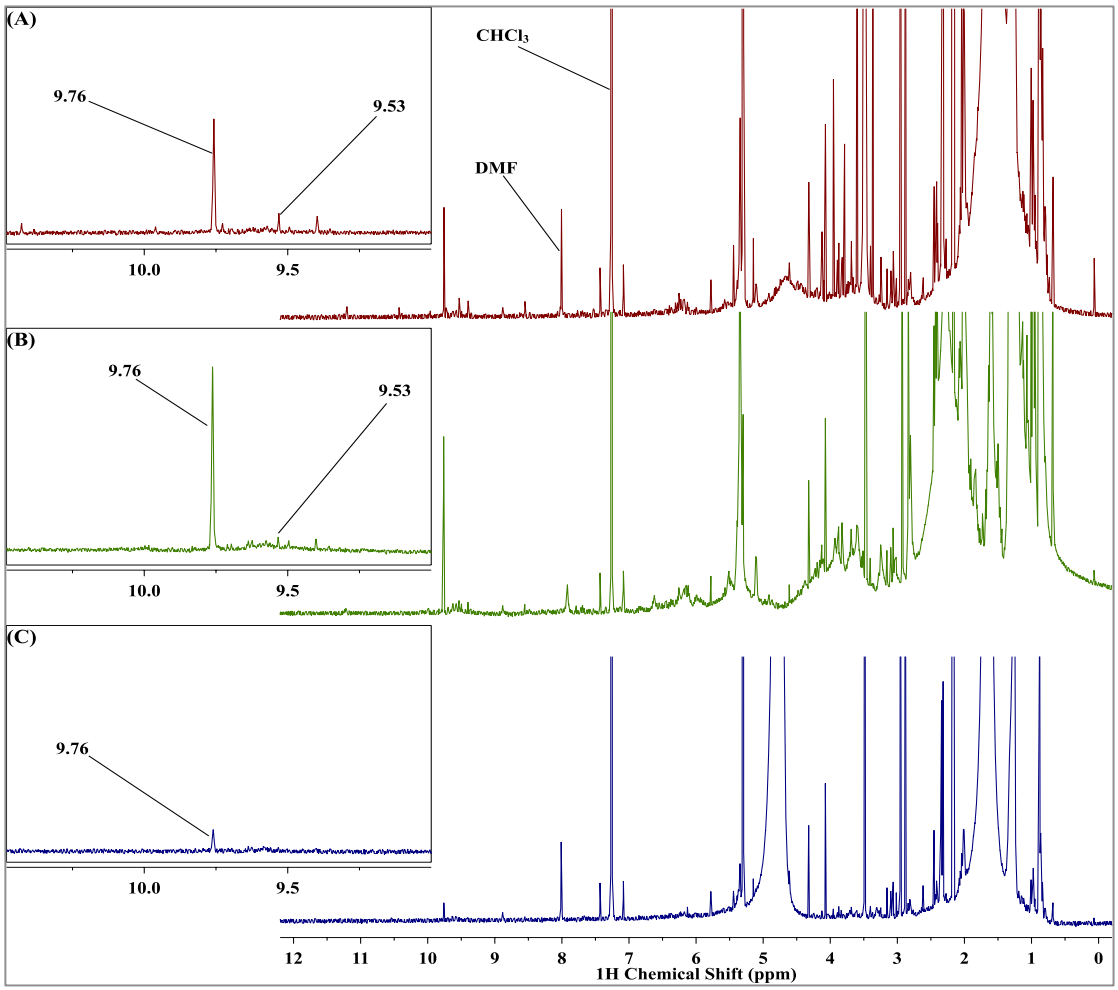

Figure A 13. ^{1}H NMR spectra of *Ulva armoricana* for salinity 10 ‰ at 15 °C experiment with replicates: A (maroon), B (green) and C (blue) from δ_H -0.20 to δ_H 12.20 for the whole spectra and from δ_H 9.00 to δ_H 10.50 for the aldehyde region.

c) Salinity 20 ‰ treatment

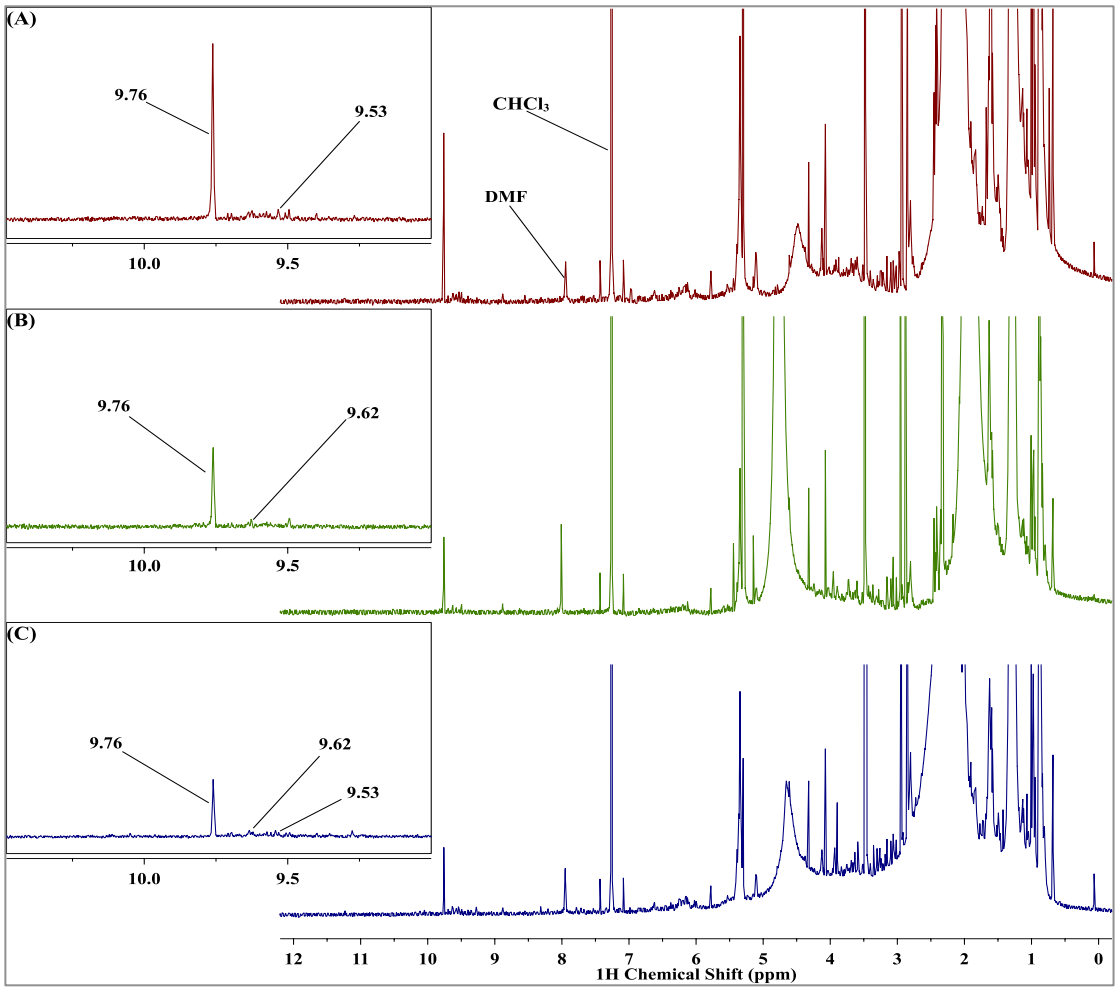

Figure A 14. ^{1}H NMR spectra of *Ulva armoricana* for salinity 20 ‰ at 15 °C experiment with replicates: A (maroon), B (green) and C (blue) from δ_H -0.20 to δ_H 12.20 for the whole spectra and from δ_H 9.00 to δ_H 10.50 for the aldehyde region.

d) Salinity 25 ‰ treatment

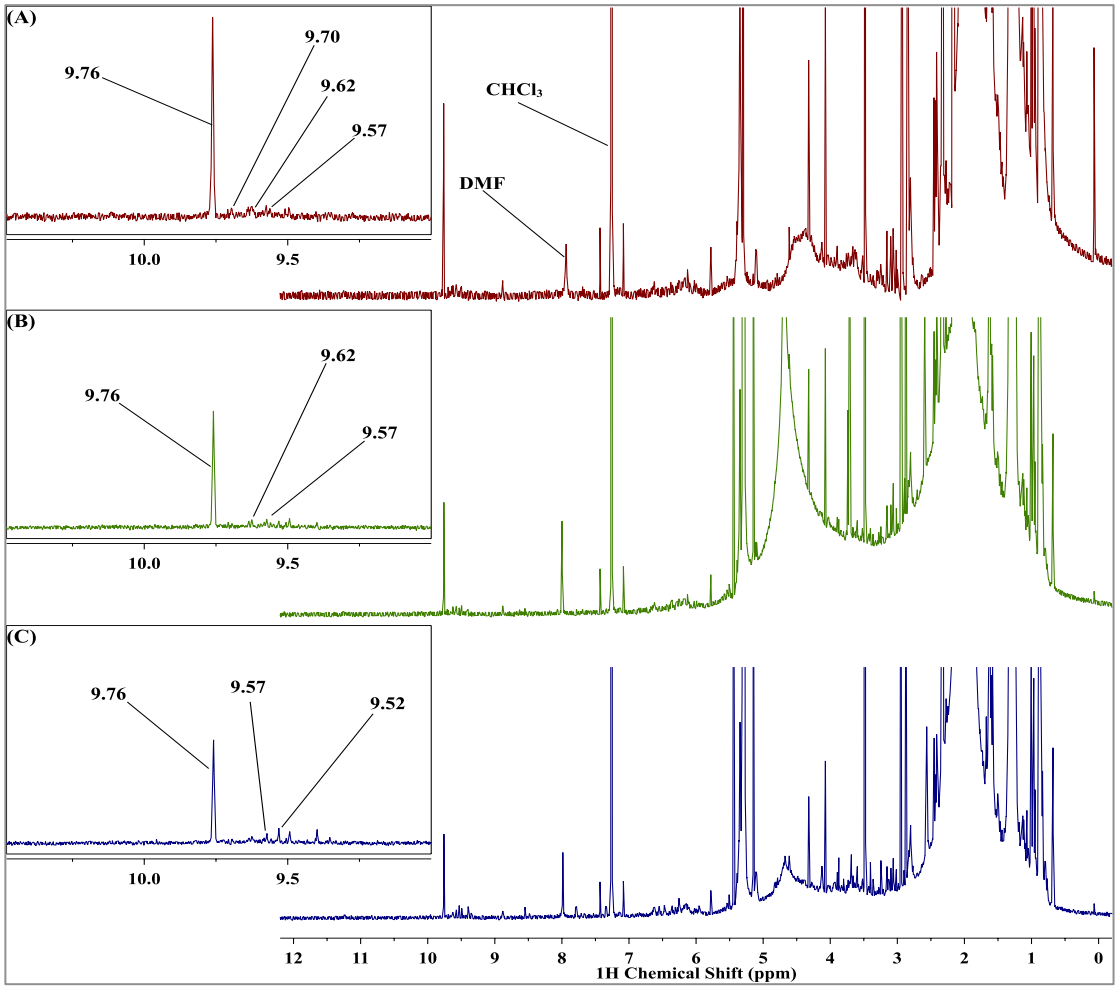

Figure A 15. ^{1}H NMR spectra of *Ulva armoricana* for salinity 25 ‰ at 15 °C experiment with replicates: A (maroon), B (green) and C (blue) from δ_H -0.20 to δ_H 12.20 for the whole spectra and from δ_H 9.00 to δ_H 10.50 for the aldehyde region.

e) Salinity 35 ‰ treatment

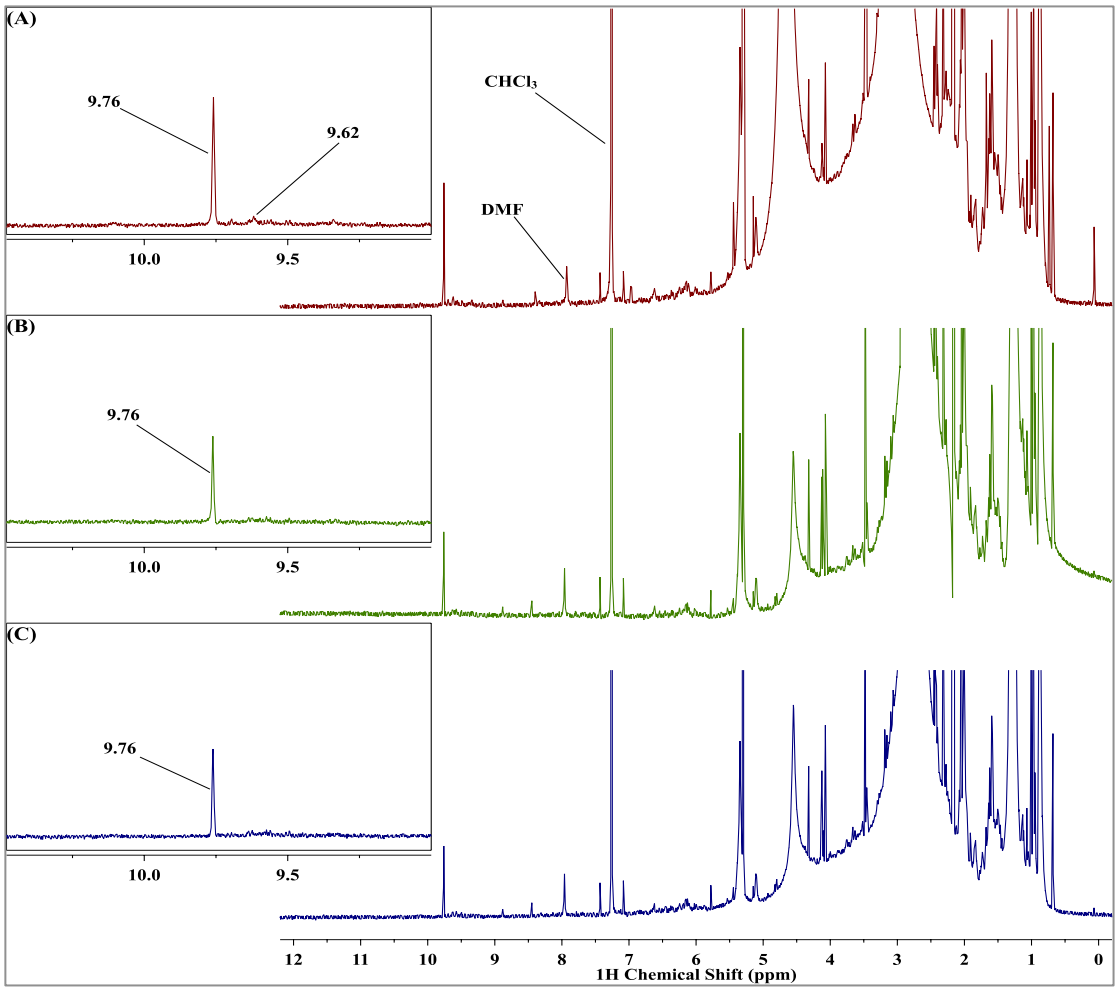

Figure A 16. ^{1}H NMR spectra of *Ulva armoricana* for salinity 35 ‰ at 15 °C experiment with replicates: A (maroon), B (green) and C (blue) from δ_H -0.20 to δ_H 12.20 for the whole spectra and from δ_H 9.00 to δ_H 10.50 for the aldehyde region.

II. Normalized ^{1}H NMR spectra

1) Grazing experiment

a) Natural grazing

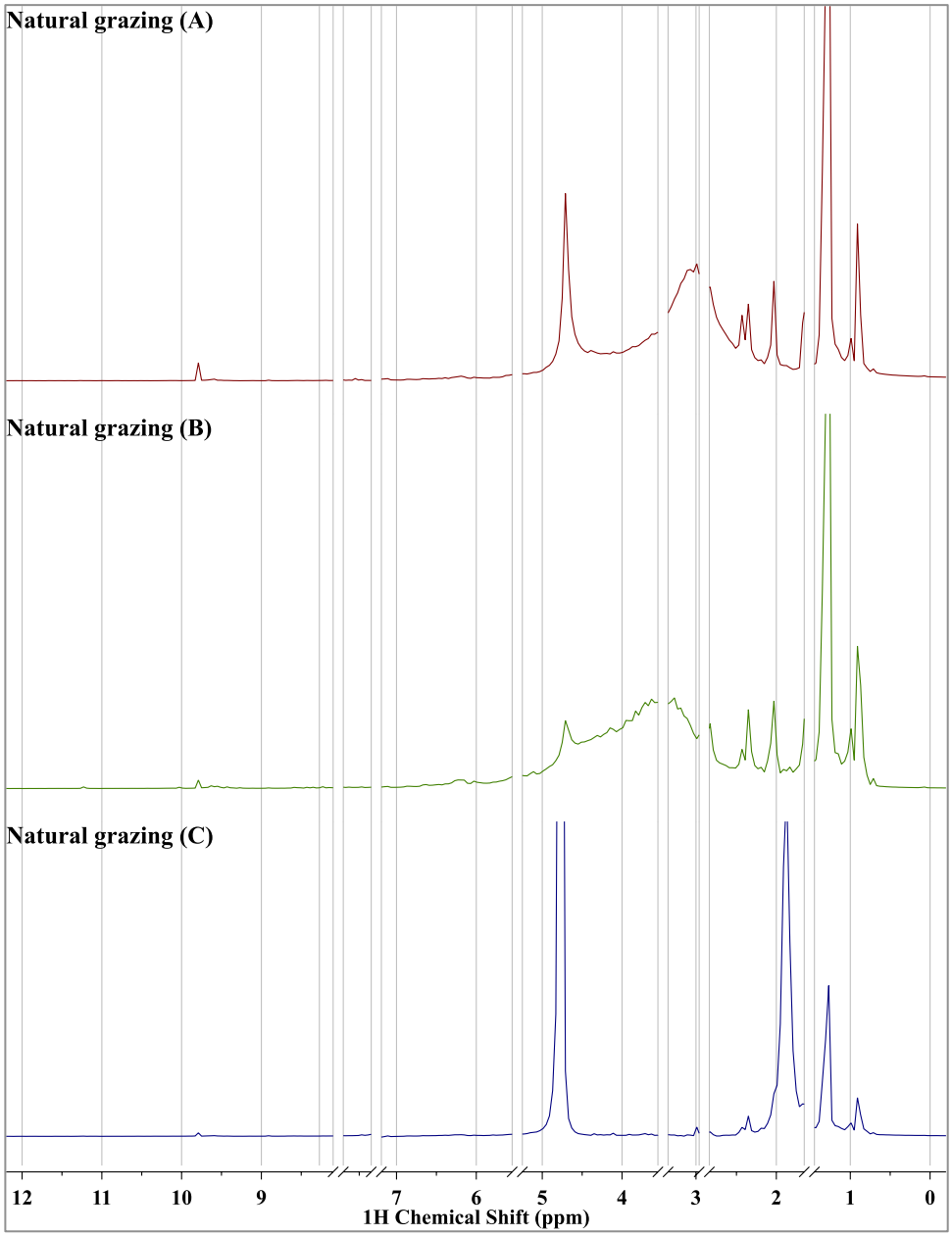

Figure A 17. Normalized ^{1}H NMR spectra of natural grazing experiments with replicate A (maroon), B (green) and C (blue) from δ_H -0.20 to δ_H 12.20 for the whole spectra and δ_H 9.00 to δ_H 10.50 for the aldehyde region.

b) Artificial grazing

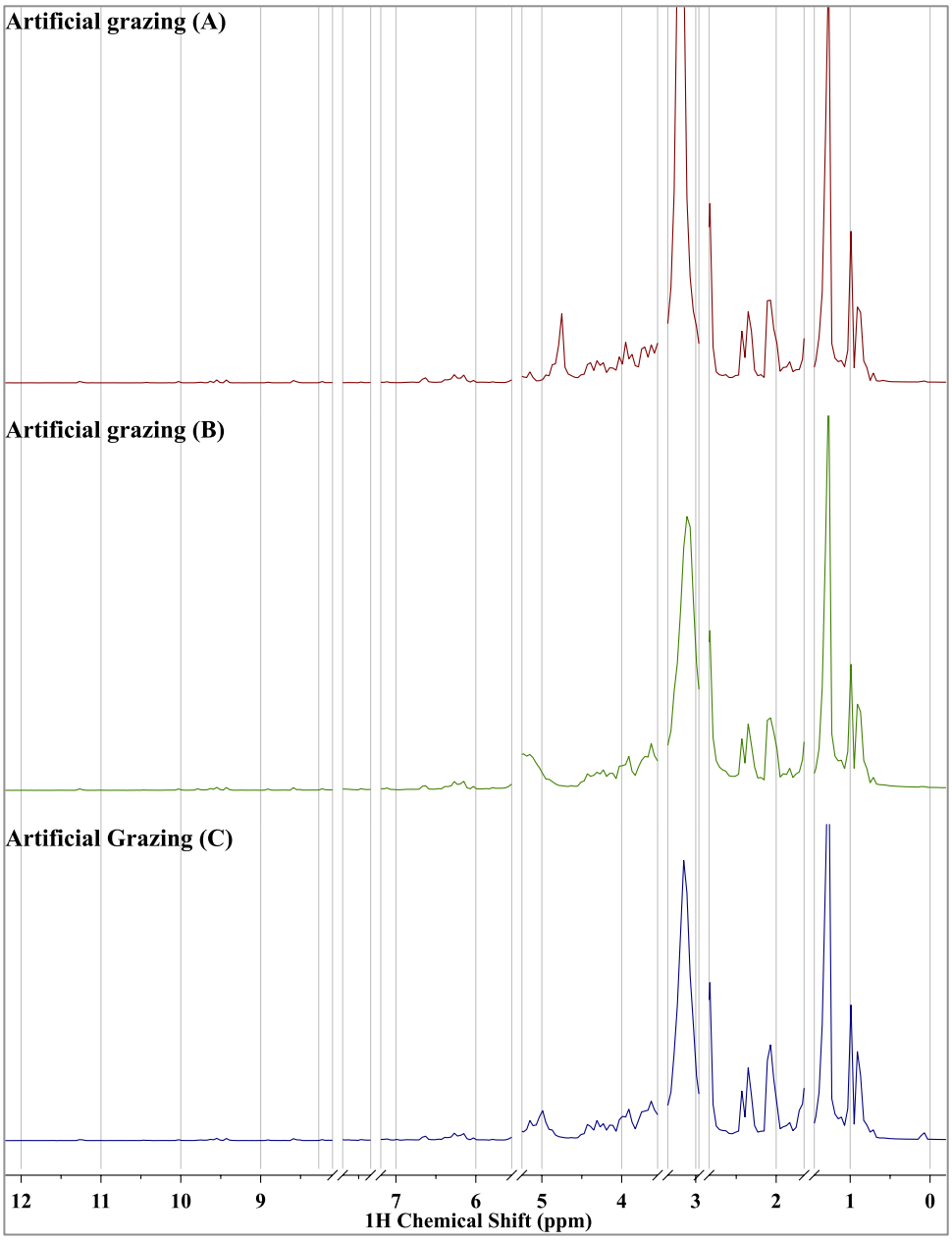

Figure A 18. Normalized ^{1}H NMR spectra of artificial grazing experiment with replicate A (maroon), B (green) and C (blue) from δ_H -0.20 to δ_H 12.20 for the whole spectra and δ_H 9.00 to δ_H 10.50 for the aldehyde region

2) Nutrient Experiment

Treatments at 10 °C

a) Low nutrient supply (0% ES)

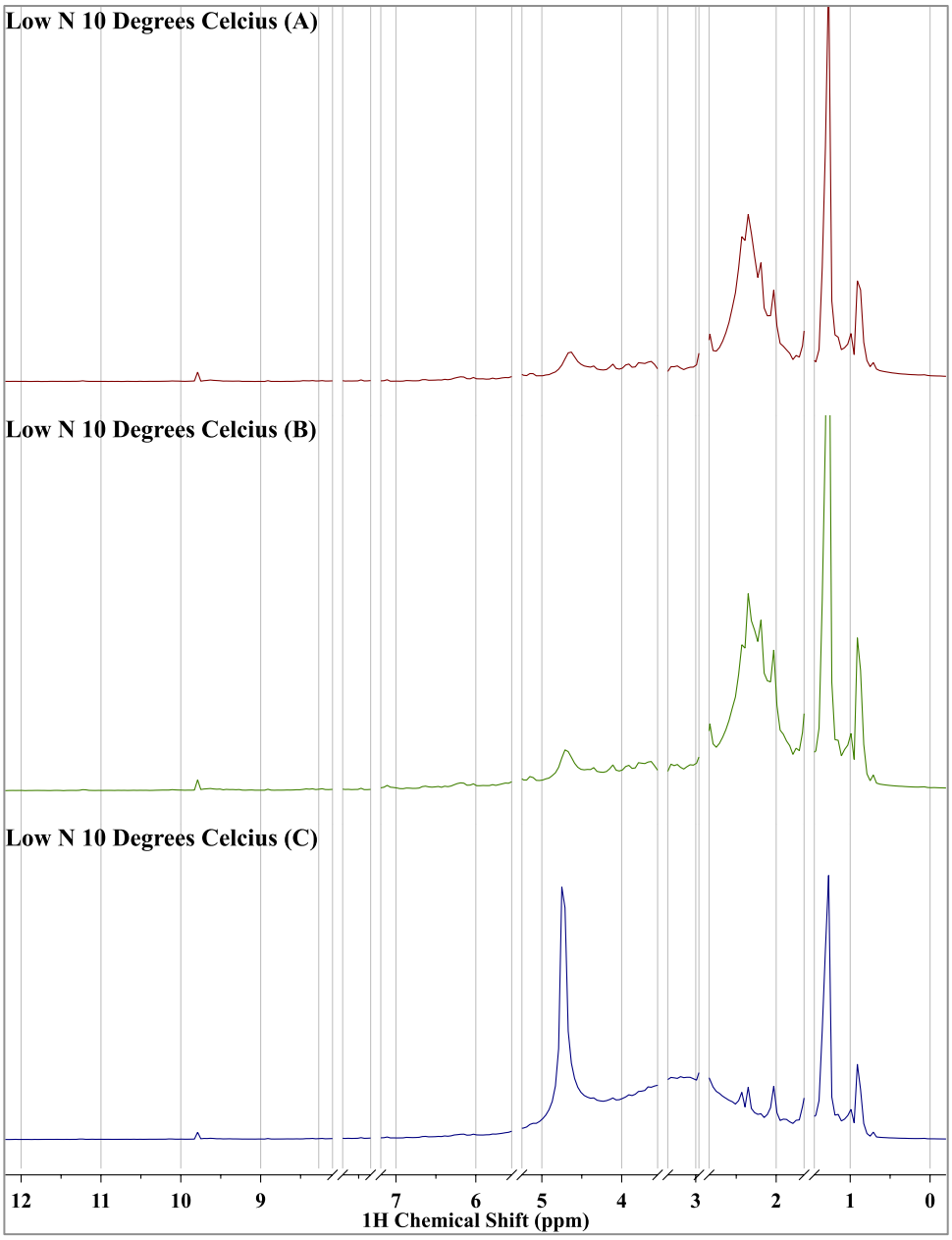

Figure A 19. Normalized ^{1}H NMR spectra of low nutrient supply (0% ES) at 10 °C experiment with replicates: A (maroon), B (green) and C (blue) from δ_H -0.20 to δ_H 12.20 for the whole spectra and from δ_H 9.00 to δ_H 10.50 for the aldehyde region.

b) High nutrient supply (100% ES)

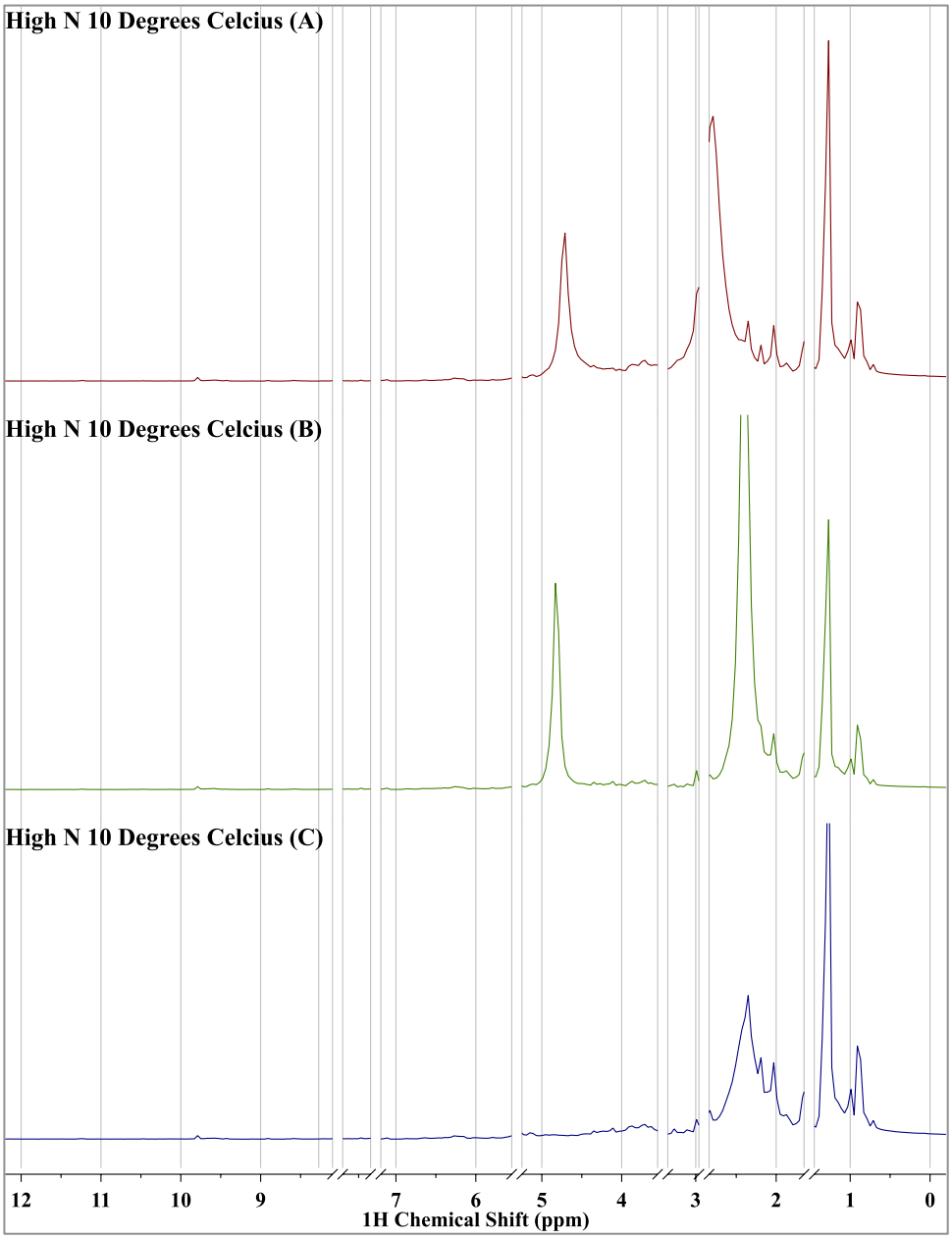

Figure A 20. Normalized ^{1}H NMR spectra of high nutrient supply (100% ES) at 10 °C experiment with replicates: A (maroon), B (green) and C (blue) from δ_H -0.20 to δ_H 12.20 for the whole spectra and from δ_H 9.00 to δ_H 10.50 for the aldehyde region.

Treatments at 15 °C

a) Low nutrient supply (0% ES)

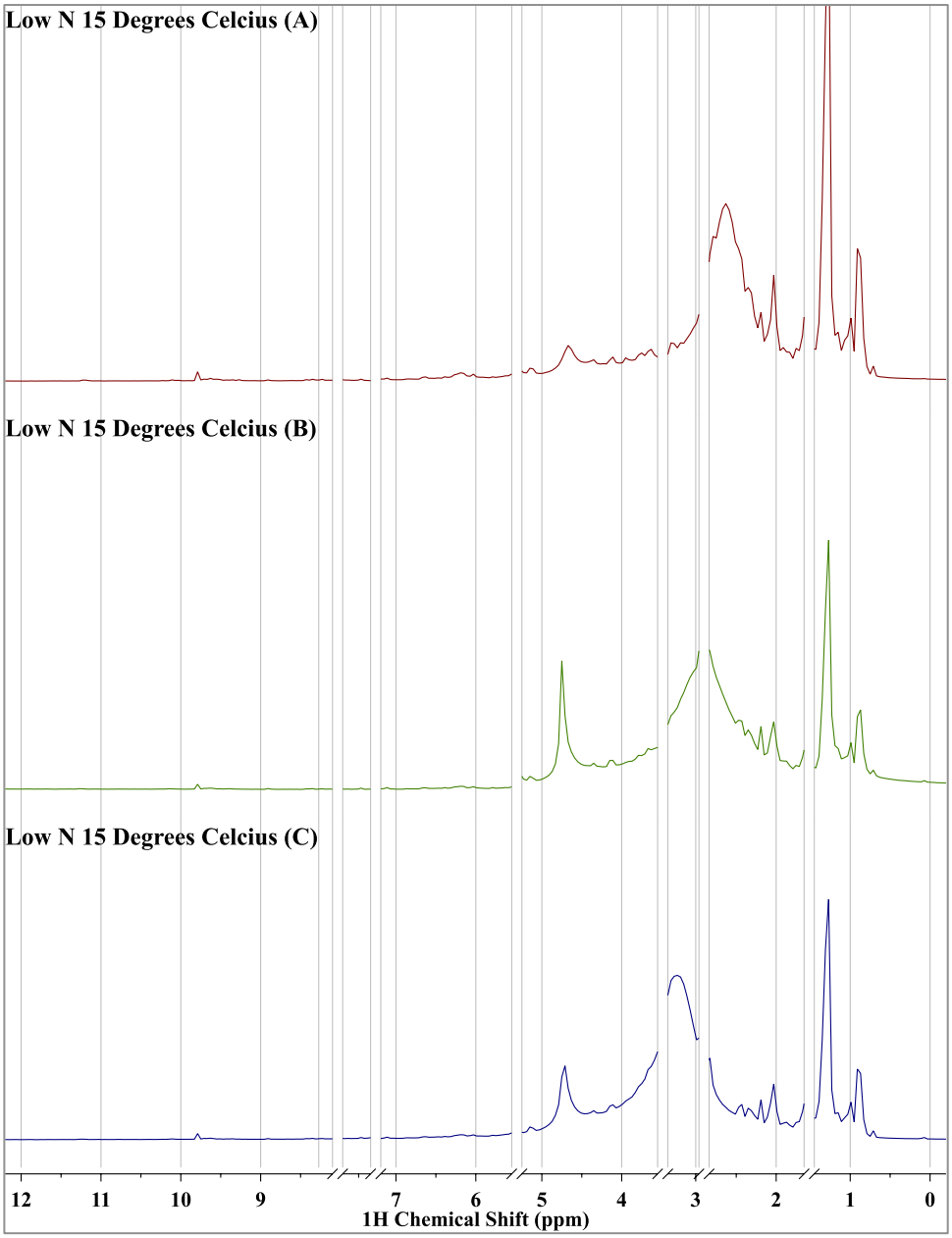

Figure A 21. Normalized ^{1}H NMR spectra of the low nutrient supply (0% ES) at 15 °C experiment with replicates: A (maroon), B (green) and C (blue) from δ_H -0.20 to δ_H 12.20 for the whole spectra and from δ_H 9.00 to δ_H 10.50 for the aldehyde region.

b) High nutrient supply (100% ES)

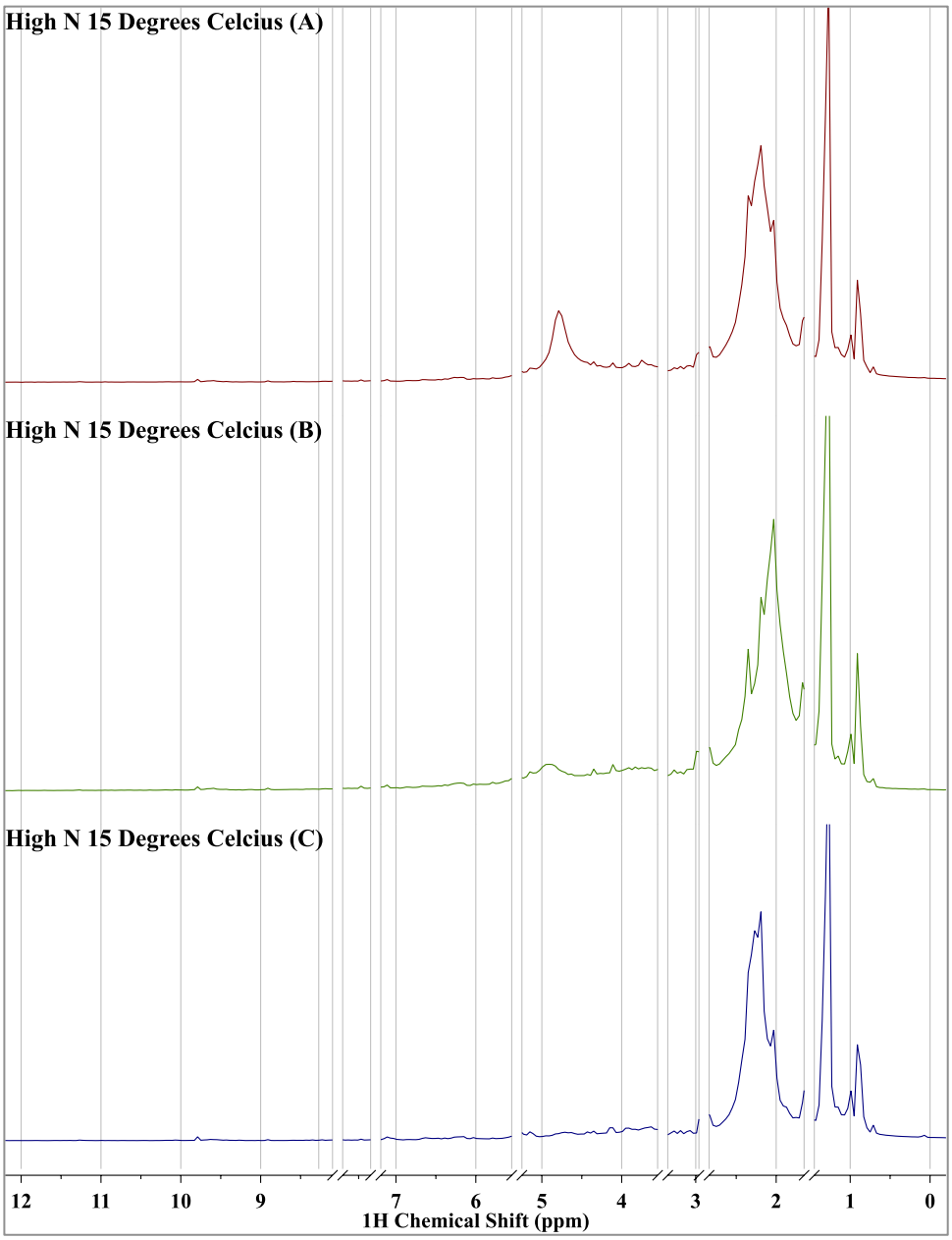

Figure A 22. Normalized ^{1}H NMR spectra of the high nutrient supply (100% ES) at 15 °C experiment with replicates: A (maroon), B (green) and C (blue) from δ_H -0.20 to δ_H 12.20 for the whole spectra and from δ_H 9.00 to δ_H 10.50 for the aldehyde region.

3) Salinity Experiment

Treatments at 10 °C

a) Salinity 5 ‰ treatment

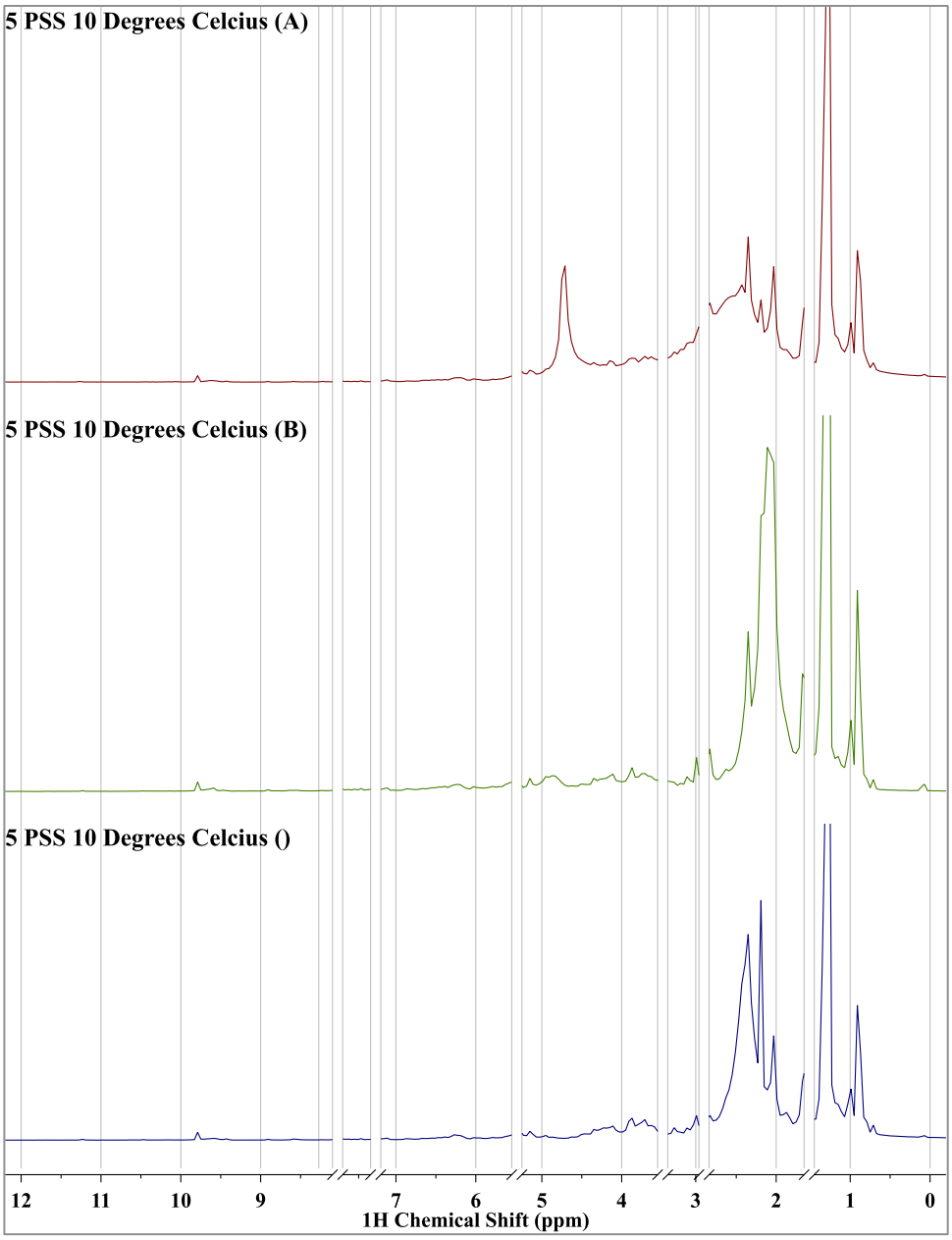

Figure A 23. Normalized ^{1}H NMR spectra of the salinity 5 ‰ at 10 °C experiment with replicates: A (maroon), B (green) and C (blue) from δ_H -0.20 to δ_H 12.20 for the whole spectra and from δ_H 9.00 to δ_H 10.50 for the aldehyde region.

b) Salinity 10 ‰ treatment

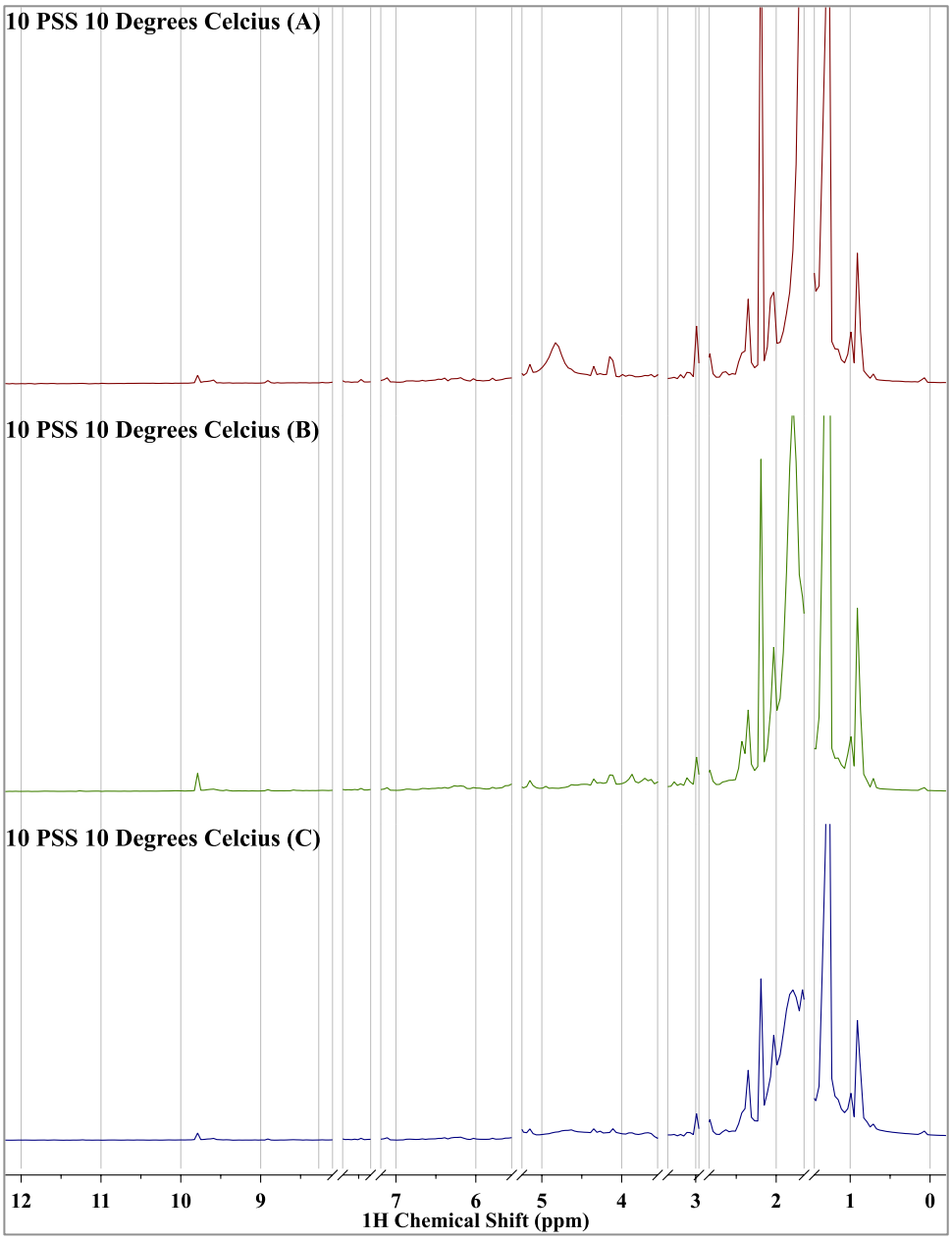

Figure A 24. Normalized ^{1}H NMR spectra of the salinity 10 ‰ at 10 °C experiment with replicates: A (maroon), B (green) and C (blue) from δ_H -0.20 to δ_H 12.20 for the whole spectra and from δ_H 9.00 to δ_H 10.50 for the aldehyde region.

c) Salinity 20 ‰ treatment

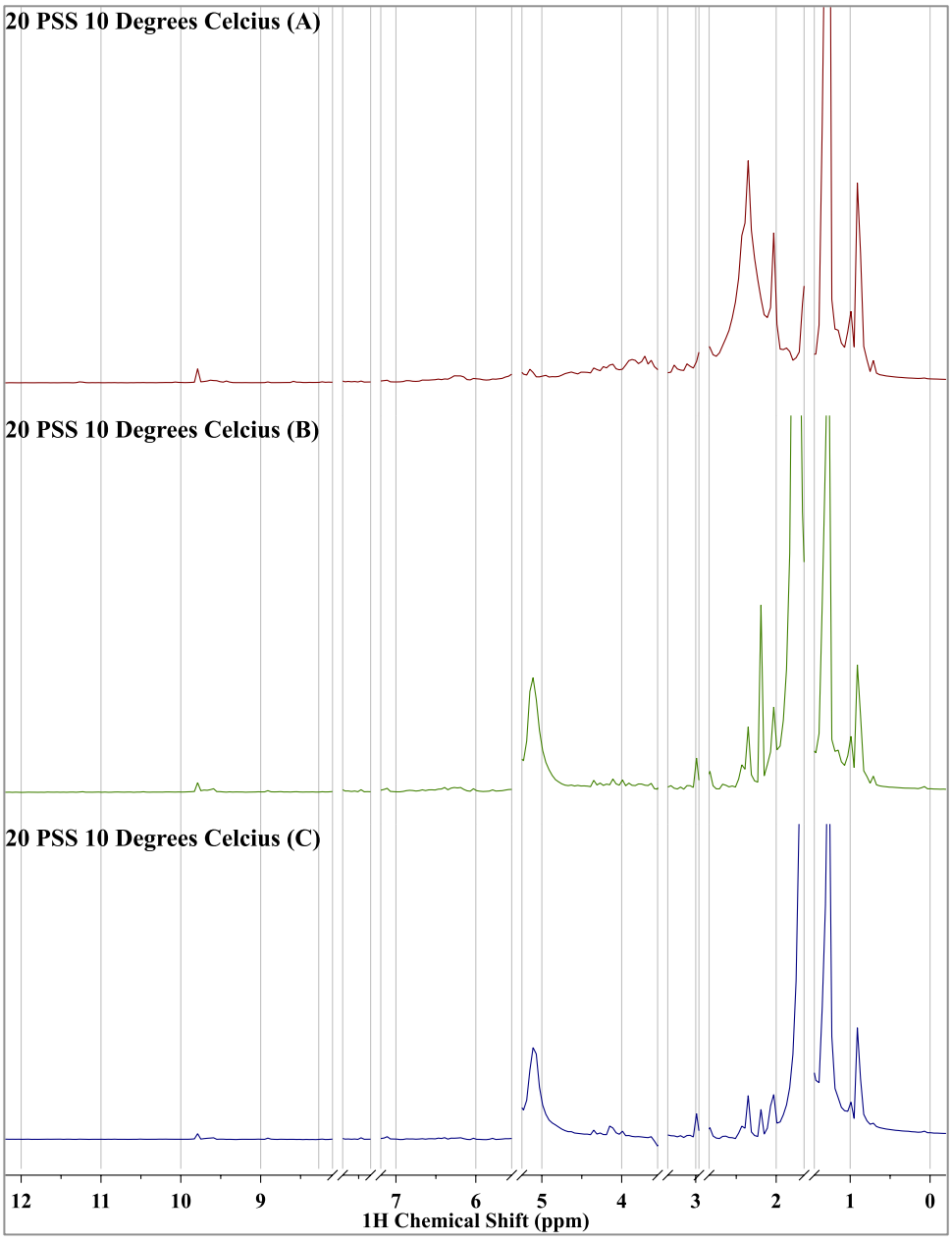

Figure A 25. Normalized ^{1}H NMR spectra of the salinity 20 ‰ at 10 °C experiment with replicates: A (maroon), B (green) and C (blue) from δ_H -0.20 to δ_H 12.20 for the whole spectra and from δ_H 9.00 to δ_H 10.50 for the aldehyde region.

d) Salinity 25 ‰ treatment

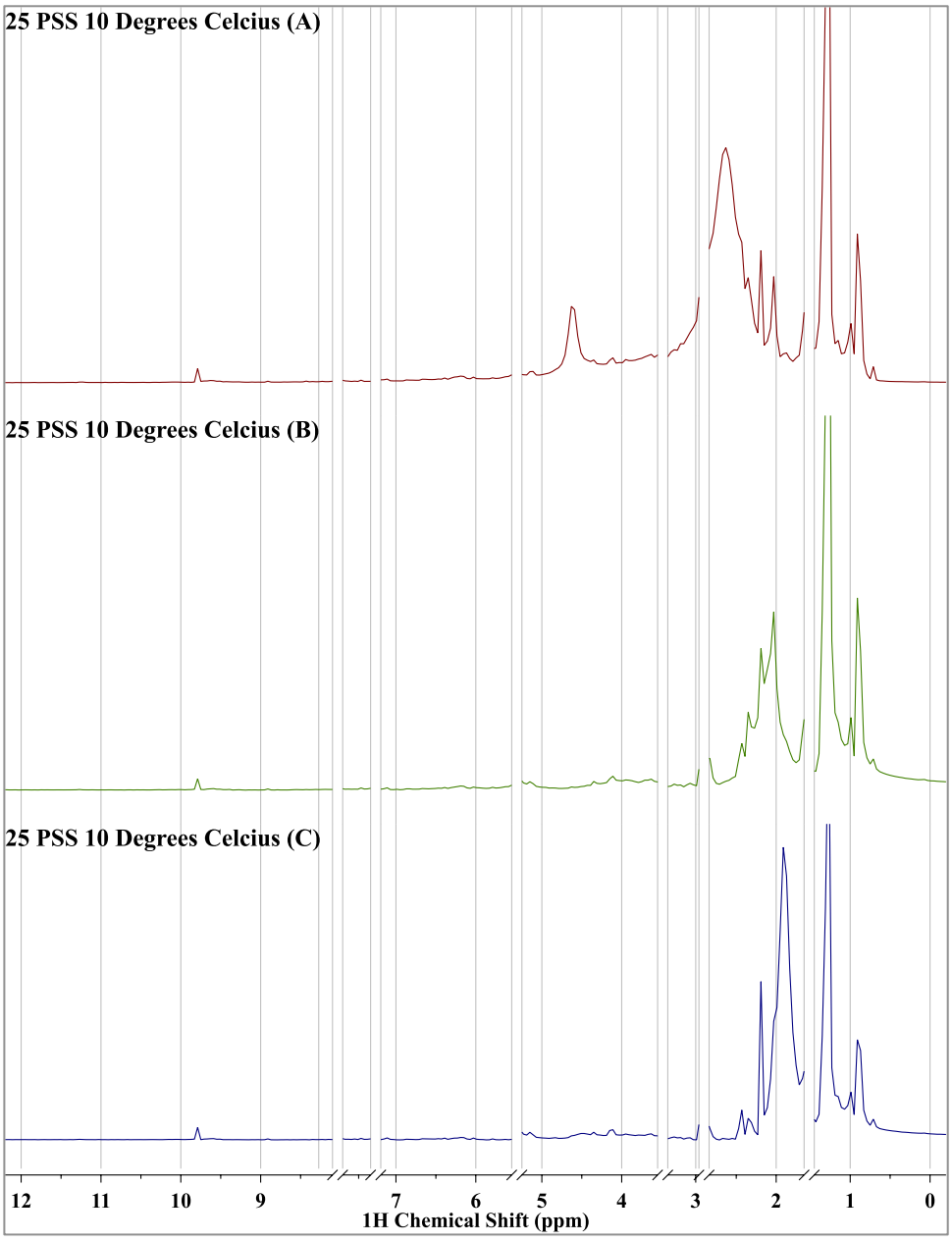

Figure A 26. Normalized ^{1}H NMR spectra of the salinity 25 ‰ at 10 °C experiment with replicates: A (maroon), B (green) and C (blue) from δ_H -0.20 to 12.20 for the whole spectra and from δ_H 9.00 to δ_H 10.50 for the aldehyde region.

e) Salinity 35 ‰ treatment

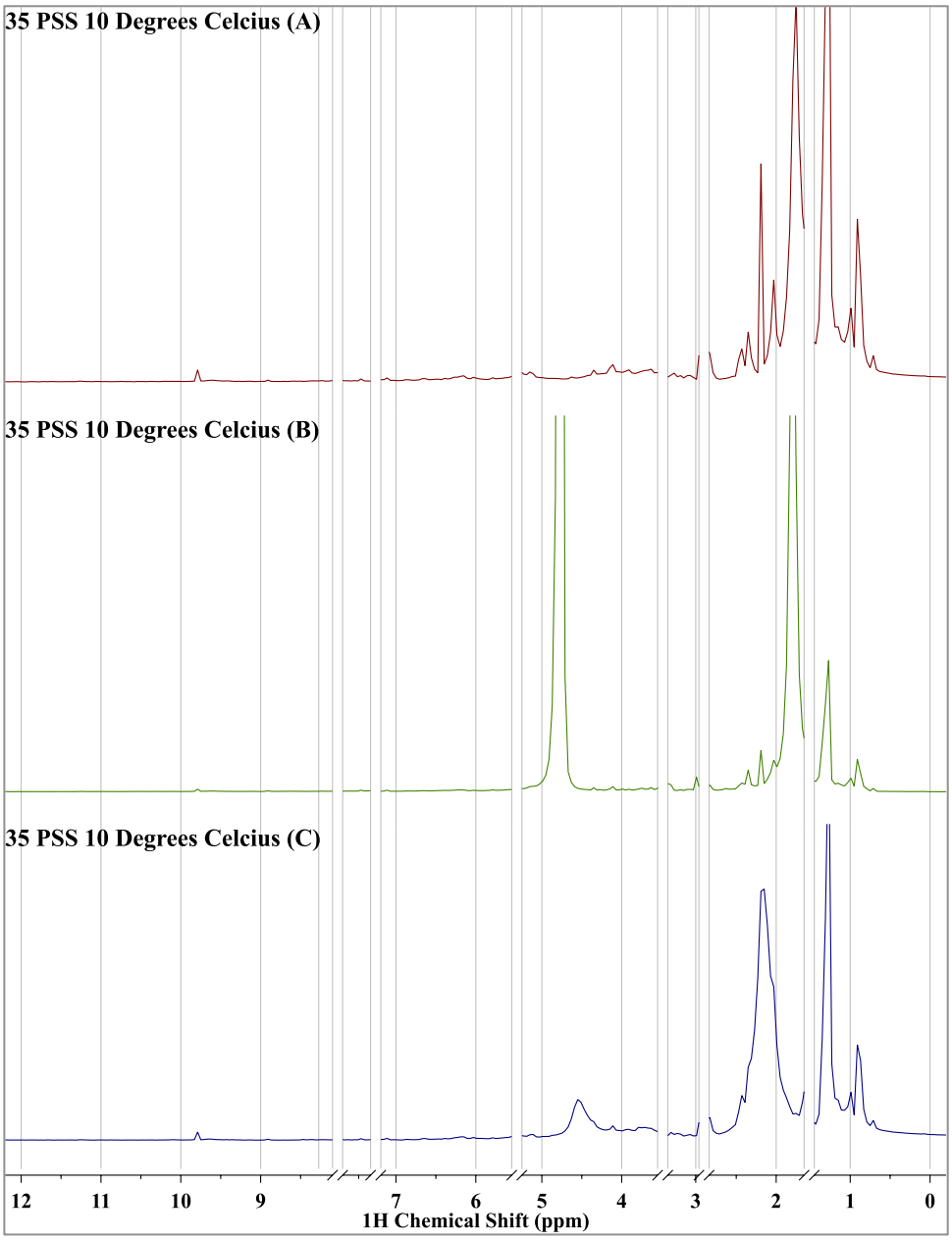

Figure A 27. Normalized ^{1}H NMR spectra of the salinity 35 ‰ at 10 °C experiment with replicates: A (maroon), B (green) and C (blue) from δ_H -0.20 to 12.20 for the whole spectra and from δ_H 9.00 to δ_H 10.50 for the aldehyde region.

Treatments at 10 °C

a) Salinity 5 ‰ treatment

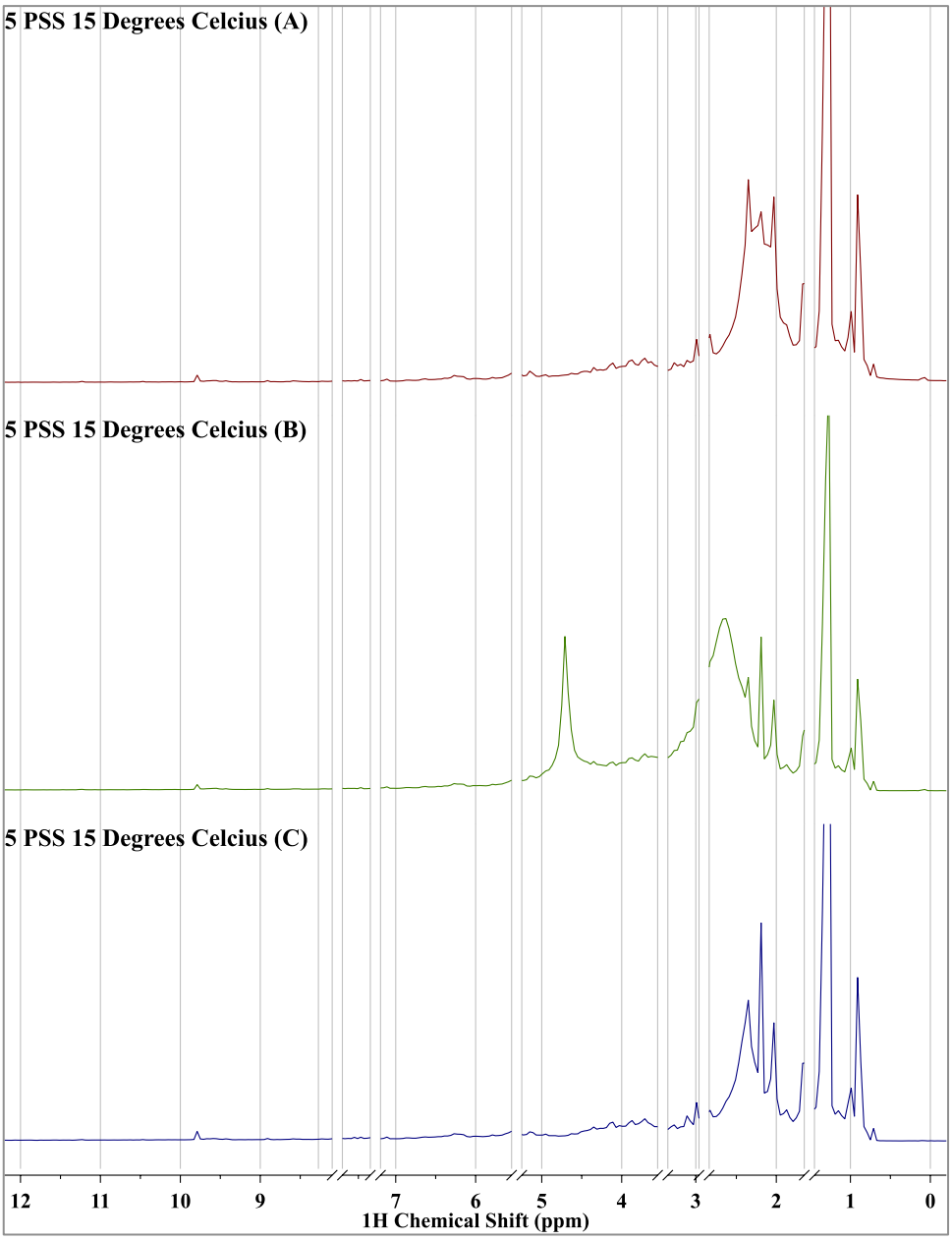

Figure A 28. Normalized ^{1}H NMR spectra of the salinity 5 ‰ at 15 °C experiment with replicates: A (maroon), B (green) and C (blue) from δ_H -0.20 to 12.20 for the whole spectra and from δ_H 9.00 to δ_H 10.50 for the aldehyde region.

b) Salinity 10 ‰ treatment

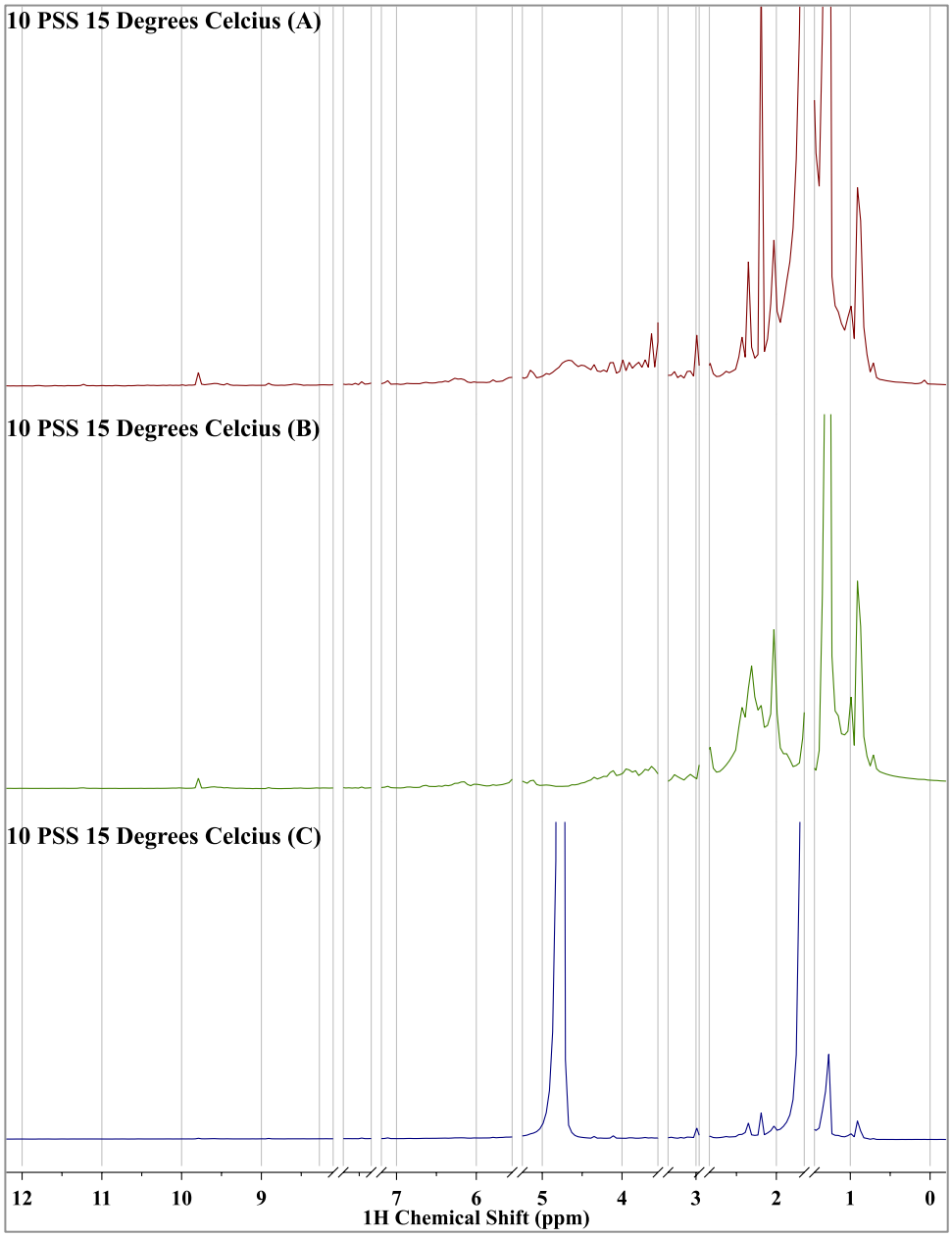

Figure A 29. Normalized ^{1}H NMR spectra of the salinity 10 ‰ at 15 °C experiment with replicates: A (maroon), B (green) and C (blue) from δ_H -0.20 to 12.20 for the whole spectra and from δ_H 9.00 to δ_H 10.50 for the aldehyde region.

c) Salinity 20 ‰ treatment

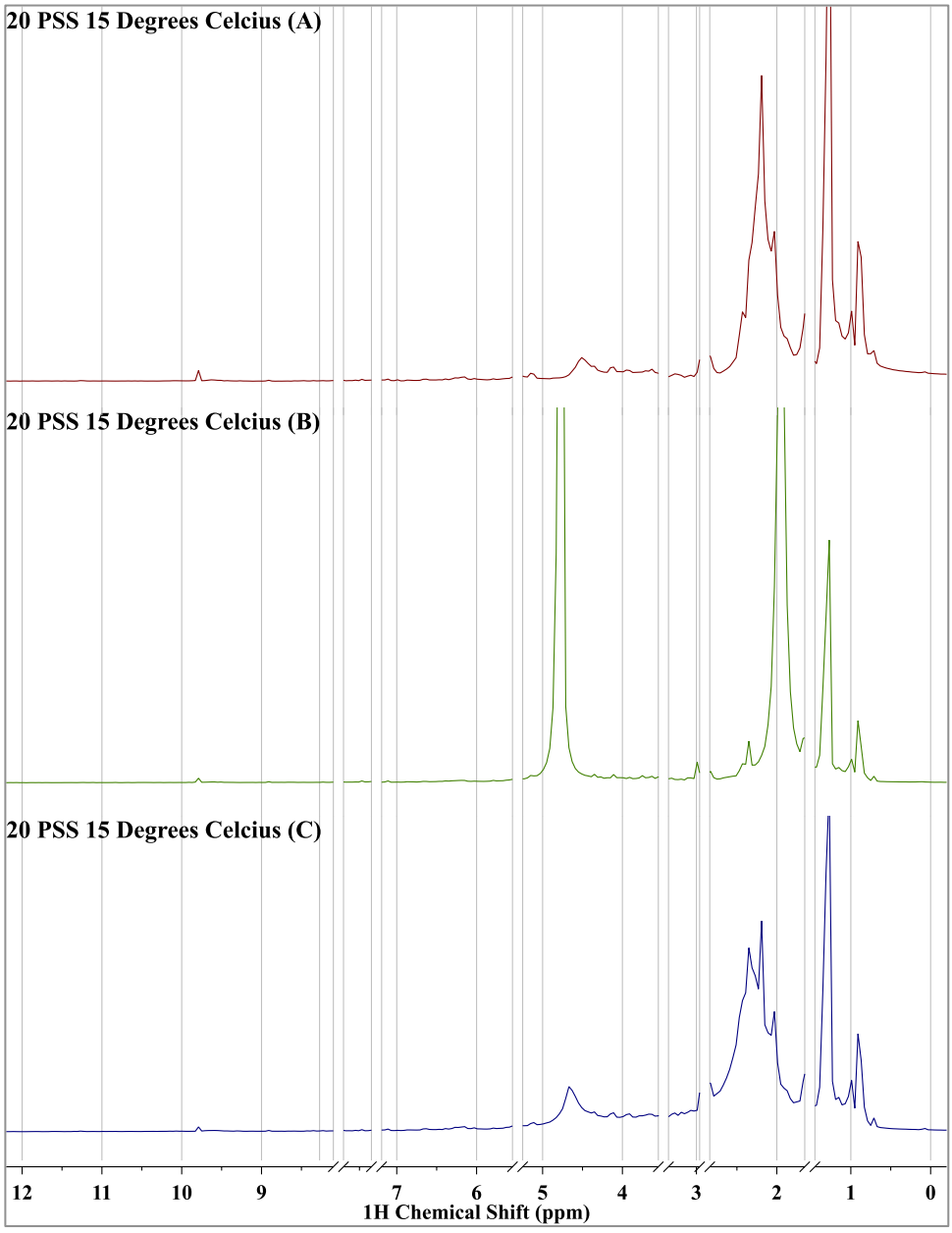

Figure A 30. Normalized ^{1}H NMR spectra of the salinity 20 ‰ at 15 °C experiment with replicates: A (maroon), B (green) and C (blue) from δ_H -0.20 to 12.20 for the whole spectra and from δ_H 9.00 to δ_H 10.50 for the aldehyde region.

d) Salinity 25 ‰ treatment

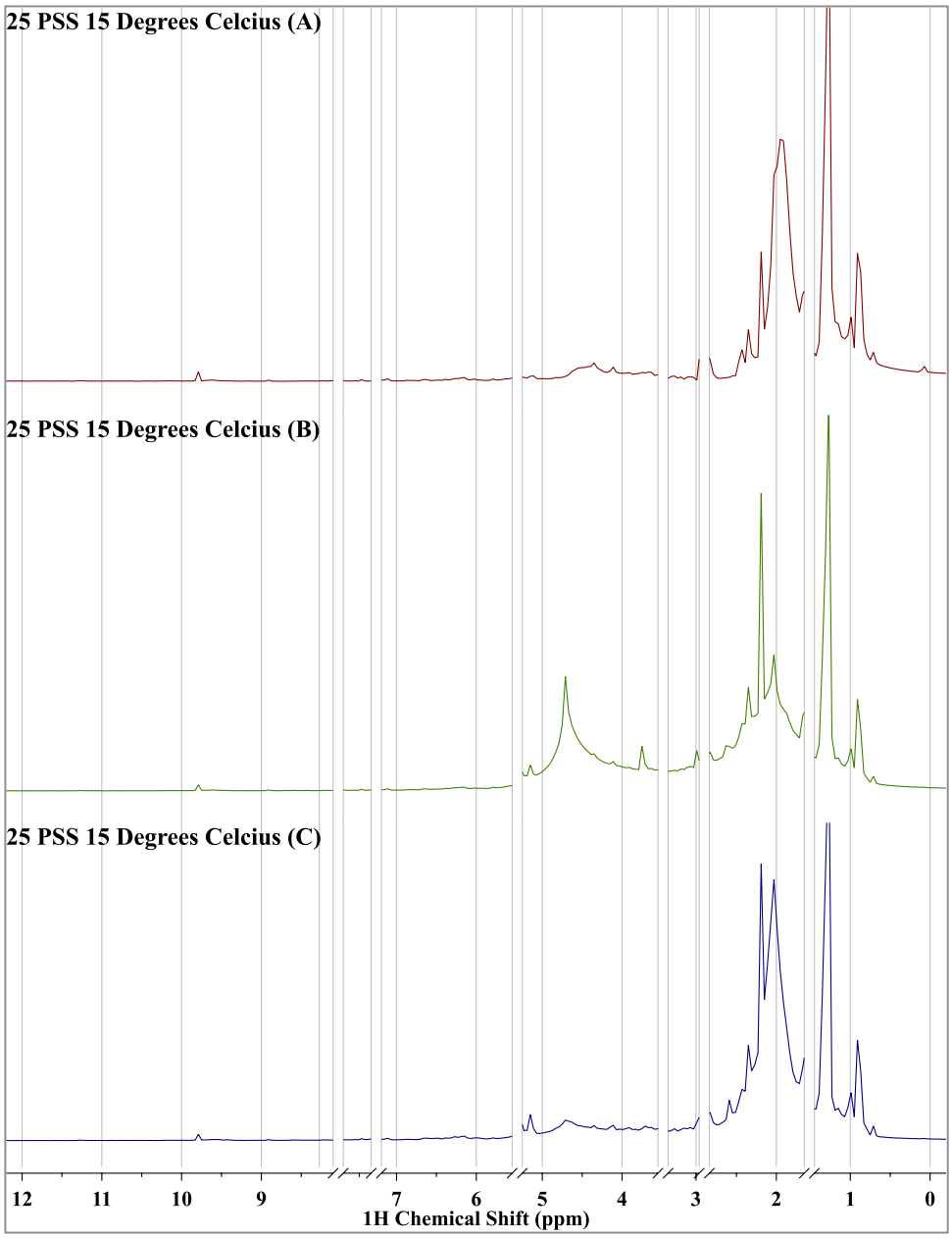

Figure A 31. Normalized ^{1}H NMR spectra of the salinity 25 ‰ at 15 °C experiment with replicates: A (maroon), B (green) and C (blue) from δ_H -0.20 to 12.20 for the whole spectra and from δ_H 9.00 to δ_H 10.50 for the aldehyde region.

e) Salinity 35 ‰ treatment

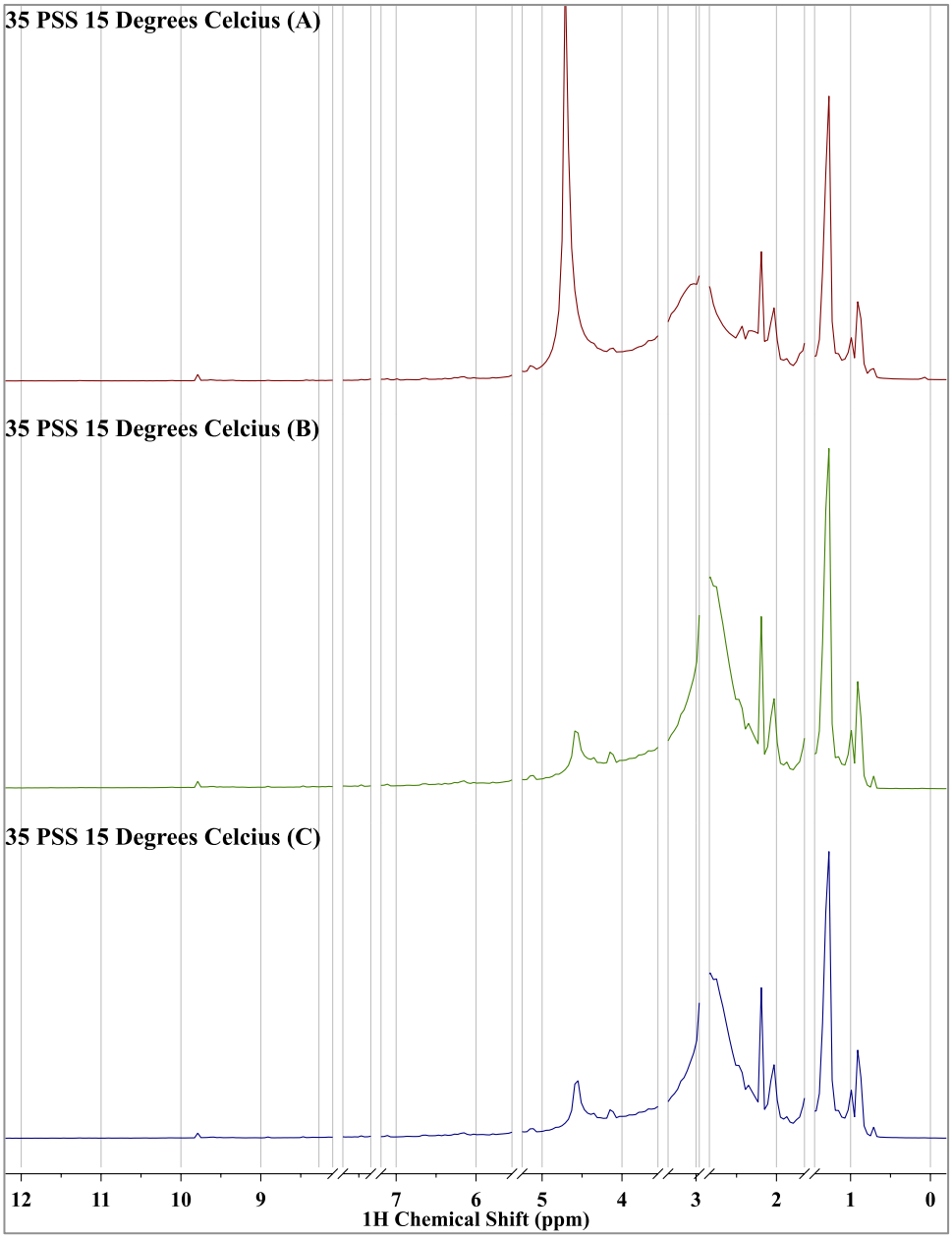

Figure A 32. Normalized ^{1}H NMR spectra of the salinity 35 ‰ at 15 °C experiment with replicates: A (maroon), B (green) and C (blue) from δ_H -0.20 to 12.20 for the whole spectra and from δ_H 9.00 to δ_H 10.50 for the aldehyde region.