Using the $^{13}C/^{12}C$ Ratio of Bacteria-Specific Fatty Acids to Determine the Lability of Terrestrial and Marine Organic Matter in Coastal Sediments

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Abstract

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Frédéric Leone
Concordia University 2021

The bottom waters of the St. Lawrence Estuary and Gulf are currently characterized by low dissolved oxygen levels (hypoxia), which influence the health of this ecosystem. The progression of hypoxia since the beginning of the century is due in part to an increase in the flux of organic matter (OM) and inorganic nutrients discharged in this aquatic system by the St. Lawrence River. The increase in nutrient abundance leads to higher primary productivity and concentrations of dissolved and particulate OM in the water column, which in turn results in a higher consumption rate of oxygen during the degradation of OM by microorganisms. To further our understanding of the carbon cycle in the St. Lawrence system, the biological lability of terrestrially-derived and marine OM was indirectly estimated through the analysis of the $^{13}C/^{12}C$ ratio of bacteria-specific fatty acid (*iso* C15:0 and *anteiso* C15:0) using gas chromatography coupled to an isotope ratio mass-spectrometer (GC-IRMS). Ubiquitous bacteria strains responsible for the degradation of OM were cultivated in marine broth enriched in ^{13}C with ^{13}C-sodium acetate to assess the relationship and isotopic fractionation between the ^{13}C signature of the food source and that of the bacteria. Using this calibration and the isotopic signature of the terrestrial and marine OM end-members, it was possible to determine the proportions of each type of OM being degraded at the different sampling stations along the St. Lawrence Estuary and Gulf continuum. Better constraining of the role of bacteria in terrestrial and marine OM degradation within the St. Lawrence Estuary and Gulf allows for better understanding of the causes driving deep water hypoxia and, eventually, will allow better remediation efforts to improve the health of this important ecosystem. Finally, as part of a small side project, the method used to decarbonate samples with HCl vapour in preparation for elemental analysis (EA) was investigated using a ^{13}C-labelled short-chain organic acid to assess the extent to which the acid is lost through volatilization upon its protonation.

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Contribution of authors

Chapter II corresponds to a small side project that emerged from the main research project. It was written by Frédéric Leone and reviewed by Anic Imfeld and Yves Gélinas. The data analysis was performed by Frédéric Leone. The experiment was designed by Frédéric Leone, Anic Imfeld and Yves Gélinas. All the necessary laboratory manipulations were carried out by Frédéric Leone and Anic Imfeld. The manuscript is currently under revision in *Limnology and Oceanography: Methods.*

Chapter III documents the main project of this master's thesis. It was written by Frédéric Leone and reviewed by Yves Gélinas. The sediment samples were collected by members of our laboratory during a sampling mission onboard the *RV Coriolis* in June 2018. The sediments lipid extractions and separations (3 lipid fractions) were completed by Anic Imfeld as part of her PhD project. Dr. Brandon Findlay provided all the material for the bacterial cultivation. Bahar Pakseresht (MSc student from Dr. Findlay's lab) helped with the bacterial cultures. All the subsequent laboratory manipulation and data analysis were done by Frédéric Leone. The manuscript will be submitted for publication to *Marine Chemistry.*

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List of Abbreviations

OC: Organic Carbon
OM: Organic Matter
SLEG: St. Lawrence Estuary and Gulf
SLR: St. Lawrence River
USLE: Upper St. Lawrence Estuary
LSLE: Lower St. Lawrence Estuary
Stn: Station
CDOM: Chromophoric Dissolved Organic Matter
VPDB: Vienne Pee Dee Belemnite
IRMS: Isotope-Ratio-Mass-Spectrometer
EA: Elemental Analyzer
HCl: Hydrochloric Acid
CSIA: Compound-Specific Isotope Analysis
m/z: Mass-to-charge Ratio
GC: Gas Chromatography
FAME: Fatty Acid Methyl Ester
C15:0: Pentadecanoic Acid
Acetyl CoA: Acetyl Coenzyme A
RuBisCO: Ribulose-1,5-bisphosphate carboxylase-oxygenase

Chapter I: General Introduction

Estuaries link freshwater systems and the open ocean. They are particularly interesting research sites since they exist in a continuum between the physico-chemical characteristics of freshwaters and those of saltwaters (e.g., salinity, temperature, dissolved oxygen). Estuaries are also a deposition centre for organic matter originating from two endmembers, marine and terrestrial, with sediments containing varying proportions of the two along the continuum. These transition zones harbour unique fauna and flora and are areas where primary production is at its highest. The migration, breeding, and feeding of certain species often depend on the quality of estuarine environments. One example for the St. Lawrence Estuary (Quebec, Canada) is the endangered belugas population that rely on the estuary for their reproduction and survival (Government of Canada, 2019).

The St. Lawrence and Estuary and Gulf (SLEG), one of the largest estuary-gulf system in the world, is no exception to these characteristics. The St. Lawrence River (SLR) that discharges into the estuary originates from the North American Great Lakes and drains the heavily populated and industrialized St. Lawrence Valley. The river broadens and reaches maximum turbidity just east of Québec City, marking the inland limit of the Upper St. Lawrence Estuary (USLE). The average depth of the estuary increases rapidly from about 150 m west of the Saguenay Fjord to more than 350 m, corresponding to the eastern limit of the USLE and the start of the Lower St. Lawrence Estuary (LSLE), where dissolved oxygen concentrations in the deep waters are the lowest. The town of Ste-Anne-des-Monts, on the south shore of the LSLE marks the western limit of the Gulf of St. Lawrence (GSL). The estuary and gulf system extends over 1050 km from Québec City to the Cabot Strait. With its unique properties, the estuary is home to many species such as birds (e.g., Harlequin ducks), marine mammals (e.g., belugas, blue whale), fish (e.g., rainbow smelt), and flora (Chalut & Brêthes, 2015). The estuary is also essential for the economy of Quebec and Canada as it represents a major maritime route for the transport of goods by ships to and from cities in the Canadian provinces of Quebec and Ontario, as well as the American Midwest (as far as Lake Superior). The fishing industries also contribute significantly to the Canadian economy, particularly in the maritime provinces on the east coast and Quebec (Trépanier, 2017). While the SLEG is an important source of biodiversity and revenues, it is also important culturally and socially. The shores of the SLEG are a very popular destination during summer vacation. Many vacationers use the waterway for sea kayak, leisure boating, beaches, diving, sightseeing, and many more.

For the above reasons, it is primordial to keep the SLEG as pristine as possible. It is a fragile environment that is under threat from global climate change as well as direct and indirect impacts from anthropogenic activities through the river discharge (Diaz & Rosenberg, 2008). Ocean circulation is currently changing at a rate never seen in geological times, and its temperature is increasing due to climate change. The increase of anthropogenic discharge of organic matter and nutrients (nitrogen, phosphorus) is correlated with the appearance of a permanent hypoxic zone in the bottom waters of the LSLE (Gilbert et al., 2005a). Hypoxia (depletion of oxygen) is an extremely serious threat that leads to massive consequences on the ecosystem. Hypoxia is caused in large parts by the consumption of dissolved oxygen ($O_{2(d)}$) by marine bacteria during organic

matter degradation, which highlights the importance of understanding the organic matter cycling in the SLEG.

1.1 Coastal Sediment – St. Lawrence Estuary and Gulf

1.1.1. Carbon Cycle in the Coastal Sediment

The St. Lawrence system is characterized by a salinity gradient from freshwater at Quebec City to saltwater in the Gulf. The estuary receives its water both from the ocean and the river, forming a 3-layer stratified system with limited mixing between layers. The river brings warmer and lighter water rich in nutrients and terrestrial dissolved and particulate organic matter, creating a surface water layer that slowly gets mixed with the deep salty waters. A cold intermediate layer forms between the surface water and the deep water, at depths varying between about 30 and 100 meters. It is mainly composed of freshwater from the melting of the winter's ice and water coming from the Strait of Belle Isle. The cold deep water feeds into the gulf and estuary from two different water masses from the Atlantic Ocean. The Labrador Current water is cold and oxygen-rich, while the North Atlantic Current water is slightly warmer and depleted in oxygen (Jutras, et al., 2020a). This stratification allows little mixing of dissolved oxygen and dissolved organic and inorganic compounds. The sedimentary organic matter composition varies along the SLEG continuum as the proportion of terrestrial and marine inputs vary depending on the distance from the St. Lawrence River mouth.

Marine organic matter is produced by phytoplankton and algae via photosynthesis. Phytoplankton uptake dissolved carbon dioxide and bicarbonate, which is the most available form of inorganic carbon in the ocean (Figure 1). The carbonate equilibrium (eq. 1) depicts how atmospheric CO_2 reacts with water to form carbonic acid which in turn dissociates into bicarbonate/carbonate and hydrogen ions (Killops & Killops, 2005).

Carbonate equilibrium:

$$CO_{2(d)} + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^- \rightleftharpoons 2\ H^+ + CO_3^{2-} \qquad \text{(eq 1.)}$$

Organic matter in the SLEG sediments account for about 1.5 to 2.0 % of the total sediment mass and originates in different proportions from two major sources. Terrestrial organic matter is delivered to the estuary and gulf through freshwater discharge from the major rivers, whereas marine organic matter is synthesized in the surface waters of the estuary and gulf, followed by the sedimentation of a small fraction of this primary production to the sediment-water interface (Goñi et al., 2003). Terrestrial organic matter is mostly composed of large molecules that are derived from land plants (agricultural plants, forests) or leached from soils.

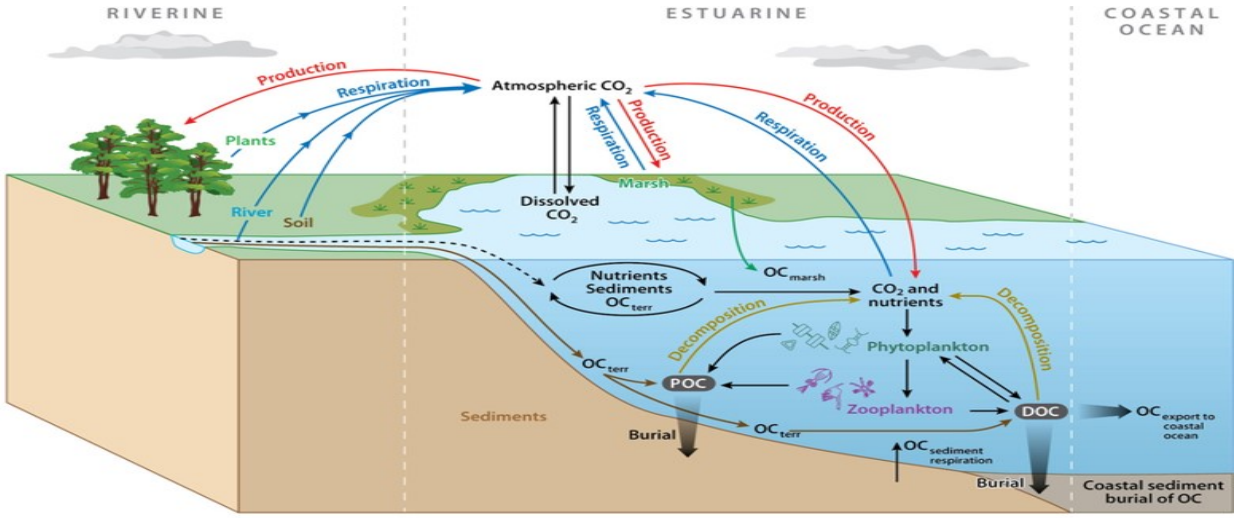


Figure 1. Simplified depiction of the carbon cycle in coastal ocean setting showing OC sources and sink (from Canuel et al., 2012)

Terrestrial plants synthesise refractory organic molecules that are unique such as lignin, condensed tannins and cellulose (Hedges et al., 1997). For example, lignin is a large molecule with many aromatic moieties that are arranged randomly, thus making it harder to degrade by bacteria due to the lack of specific enzymes able to degrade such compounds with complex structures (Brown & Chang, 2014). On the contrary, marine OM is mostly derived from autotrophic phytoplankton that uses carbonate (HCO_3^-) and atmospheric CO_2 and the energy of the sun to produce it. Marine OM is mostly composed of smaller molecules such as proteins/peptides, carbohydrates, and lipids that are easier to degrade, thus making it more labile than terrestrially-derived organic molecules (Wakeham et al., 1997). While the OM introduced in the water column is in large part degraded in the water column by marine bacteria, a small and varying fraction ultimately reaches the sea floor and accumulates in the sediment. Since the SLEG consists in a continuum between in-shore areas that are mostly influenced by terrestrial OM inputs and off-shore areas where marine OM inputs predominate, the surface sediment at the different sampling stations visited for this work contain different proportions of OM from these two predominant sources (Figure 2). Noteworthy, riverine freshwaters contribute to the estuarine and gulf sedimentary organic matter pool in two ways, first through direct injection of particulate and dissolved terrestrial OM, and second through nutrient fertilization of the estuarine surface waters, thus stimulating primary production of marine-like organic matter. This OM from all sources raining down the water column is degraded by bacteria that thrive in the bottom waters and sediments, with the bacterial biomass contributing to the total organic matter pool in different proportions in all compartments and at all sites.

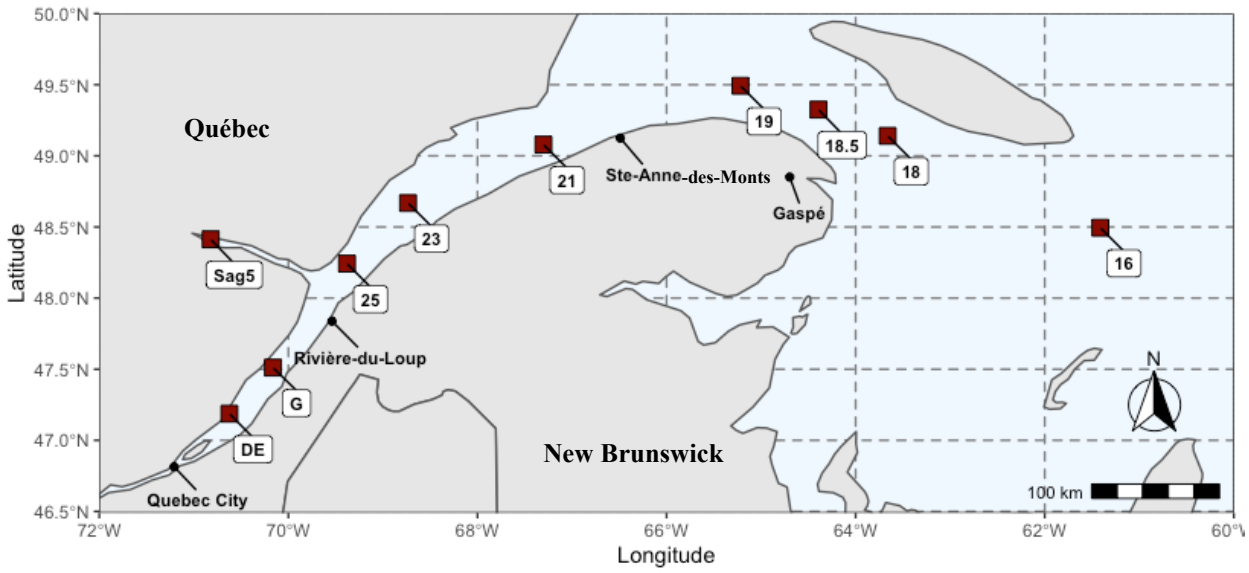


Figure 2. Sampling stations of the St. Lawrence Estuary and Gulf.

1.1.2. Degradation of Organic Matter

OM raining down the water column is either degraded in the water column and in the surface sediments, or it accumulates and is reserved in the sediment (organic matter burial). Bacteria and Archaea are the main organisms mediating the degradation of particulate and/or dissolved organic matter (Carlson et al., 2004; Wetzel, 1984). High molecular weight organic compounds are first hydrolyzed to the smaller units (e.g. protein to amino acids, polysaccharides to oligosaccharides, complex lipids to fatty acids) by specific enzymes produced by the bacterial or Archaea cells. Microorganisms can then transport the resulting low molecular weight organic compounds into their cells. This organic matter respiration process can be represented by a highly simplified equation (see Eq. 2) in which "CH_2O" depicts OM and is not meant to be a particular compound. Indeed, a ^{13}C-NMR study showed using this depiction, or the similar and widely used Redfield Ratio (Redfield, 1934) for the composition of plankton $(CH_2O)_{106}(NH_3)_{16}(H_3PO_4)$ is not accurate (Hedges et al., 2002), because it is too oxygen-rich compared to fresh planktonic organic matter that is mainly composed of proteins, with minor contributions of carbohydrates and lipids.

$$CH_2O + O_2 \rightarrow CO_2 + H_2O \qquad (eq.\,2)$$

Organic matter can also be degraded through ultraviolet irradiation from the sun. This process is known as photodegradation in which chromophoric dissolved organic matter (CDOM) is transformed into smaller molecules and CO_2 (Cory & Kling, 2018). The smaller organic compounds produced by photodegradation can then be further processed by bacteria, leading to more extensive degradation. A shift in solar irradiation or an increase of terrestrial CDOM leached into a body of water can thus lead to increased degradation (either via bacterial or photo-induced degradation), thus making more nutrients available for primary production (Zhang et al., 2013).

1.1.3. Eutrophication

The nutrients nitrogen and phosphorus are normally the limiting factor of primary production in a body of water (Paerl, 2009). However, due to human activity such as industrialization, discharge of wastewaters from water treatment plants, and agricultural fertilizer used for crops, these limiting nutrients are being discharged more and more in bodies of water hence, making them readily available for primary production. This increase in nutrients causes larger phytoplankton blooms to occur, thus increasing primary production by phytoplankton in a body of water and leading to eutrophic conditions (Diaz & Rosenberg, 2008). Marine OM has multiple fates in a water column. It can be directly consumed by zooplankton in the surface waters, or it can be degraded back to CO_2 by bacteria throughout the water column and surface sediments. Only a small fraction of the OM produced in surface waters eventually reaches the seafloor where it can be preserved for thousands of years when sorbed with minerals or metal ions such as iron (Barber et al., 2017; Lalonde et al., 2012) . These high fluxes of OM create an opportunity for aquatic life (e.g., algae, benthic organisms, fishes, etc.) to strive as there is more food readily available. It is important to note that microorganisms also thrive under these conditions of high primary productivity. Aerobic microorganisms consume dissolved oxygen as an electron acceptor when oxidizing organic matter, which makes dissolved oxygen scarce in eutrophic waters. Owing to the limited diffusion of dissolved oxygen between the stratified water layers of the SLE and its high consumption by bacteria, its availability becomes limiting to aerobic organisms, a condition known as hypoxia.

1.1.4. Hypoxia

Hypoxia is a condition in a body of water where the level of oxygen is limited. In the literature, hypoxia is defined as having a concentration of oxygen lower than 62.5 mM or 2 mg/L (Gilbert et al., 2005a). This phenomenon can occur in lakes and coastal waters due to human activities and poor ecological legislation regarding fertilization (increasing nutrient concentration) in lakes and along the coast (Figure 3). Even though anthropogenic sources of nutrients and an increase in OM in a water system can eventually lead to hypoxia, it can also be promoted by global warming. One of the best examples is the bottom water of the lower St. Lawrence Estuary (between Rivière-du-Loup and Sainte-Anne-des-Monts). Hypoxic conditions have been detected since the 1960s due to industrialization and the increase of population in the St. Lawrence Valley (Gilbert et al., 2005a). Eutrophication was then believed to be the main factor causing hypoxia as this period corresponds to a maximum in the direct discharge of organic matter rich waters (e.g., untreated sewage water and process waters from the pulp and paper industry, among others). However, the level of oxygen in the LSLE has kept decreasing despite the regulations and improvements to the waste waters treatment infrastructure introduced in the 1970s and 1980s. It has become increasingly clear that eutrophication, while still to this day an important contributing factor, was not the only cause of hypoxia. In the estuary, the cold and salty bottom waters flowing inland are denser than the warmer and less salty surface waters that flow towards the sea. This difference in

density causes water stratification and little mixing occurs between layers. Therefore, little dissolved oxygen diffusion between the oxygen saturated surface waters and the O_2-depleted deep waters of the estuary is occurring. Moreover, because the solubility of oxygen is lower in warmer waters, changes in ocean circulation have progressively been contributing to hypoxia in recent years as the proportion of deep waters feeding the bottom waters of the estuary now favours the warmer oxygen-depleted North Atlantic Current Waters over the colder, O_2-rich deep Labrador Sea waters; this global warming-driven phenomenon is now believed to be the main driver of hypoxia in the estuary, accounting for about 75% of the decrease in dissolved O_2 (Jutras, et al., 2020a).

Even if today's hypoxia is due in large part to global warming and changing oceanic circulation, the high quantities of nutrients and OM derived from anthropogenic sources are still a significant contributing factor. In the case of the SLEG, it is still unknown whether the main driver of hypoxia, aside from the increase in bottom water temperature (ocean warming), is terrestrial OM directly discharged by the river, or increased phytoplankton productivity caused by the discharge of nutrients (fertilization) from the river (Gilbert et al., 2005a). It is thus important to be able to quantify the proportion from these two sources of OM in the sediment, and whether they are consumed to the same extent by bacteria in the water column and sediment of the SLEG.

Stable carbon isotopic techniques allow distinguishing between these two sources and facilitate the study of the cycling of carbon in the SLEG. Discriminating between the different sources of OM that are incorporated into the surface sediment via burial/deposition ultimately leads to a better understanding of the onset of hypoxia.

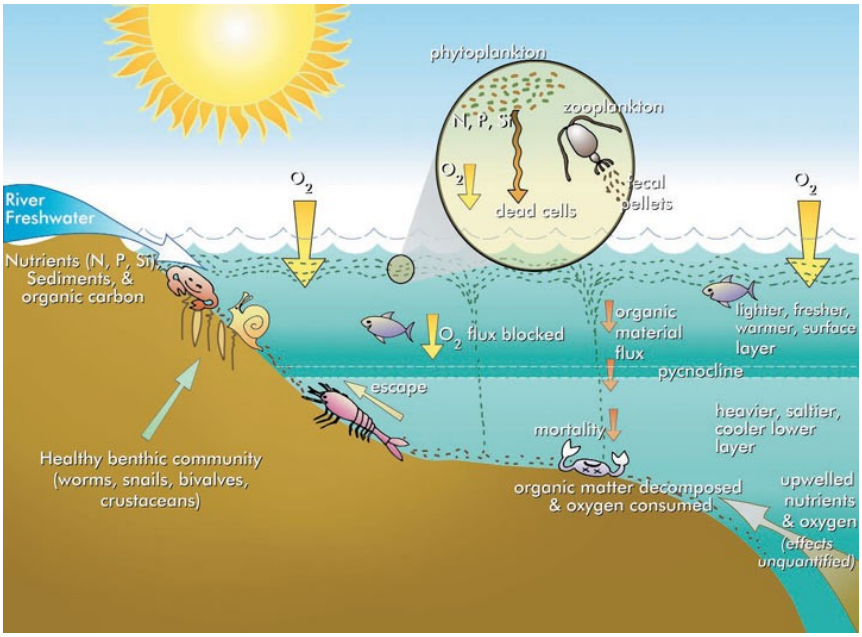


Figure 3. Depiction of eutrophication and hypoxia in coastal waters (United States Environmental Protection Agency, n.d.)

1.2. Using Stable Carbon Isotopes to Study the Cycle of Organic Carbon

1.2.1. Stable Carbon Isotopes and the Difference Between Bulk and Compound-specific Stable Isotope Ratio Measurements

In biogeochemistry and oceanic geoscience, the use of isotope analysis is a powerful tool as it can give many insights on phenomenon that occur naturally. For example, radioactive isotopes (e.g., ^{14}C, ^{210}Pb, ^{137}Cs, ^{235}U) are used to date sediment (Arias-Ortiz et al., 2018; Nian et al., 2018), allowing scientists to understand the past and characterize the past environment of the Earth thousands or millions of years ago. In this project, we have exploited the power of stable isotopes. Stable isotopes do not decay and are found on Earth in different abundances. Stable isotopes can be separated into two categories: the light and heavy isotopes, with the reference being the most abundant isotope, which is normally the lighter version of an element, such as ^{1}H, ^{12}C, ^{14}N, and ^{16}O. However, the heavy isotope can also be relatively abundant. For example, for ^{13}C and ^{15}N the abundance is 1.11% and 0.36%, respectively (Fry, 2006). Using stable isotopes allows scientists to track specific elements throughout natural cycles (e.g., carbon or nitrogen cycle). In this project, stable carbon isotope ratios were used to determine the sources of organic carbon in the SLEG.

Using specific mass spectrometry instrumentation, the ratio of the heavy stable carbon isotope over that of the light stable carbon isotope ($^{13}C/^{12}C$) can be calculated. Differences in the ratio between different samples (e.g., leaves, sediment, etc.) are due to isotopic fractionation. Fractionation results from the difference in behavior of the light isotopes and heavy isotopes owing to their respective mass (Fry, 2006). For example, the enzyme ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBisCO) preferentially uses $^{12}CO_2$ rather than $^{13}CO_2$ (lighter isotope over the heaviest) as its substrate when synthesizing simple sugars. This process is called the kinetic isotope effect, in which lighter isotopes are favoured as they require less energy for the reaction to occur (Fry, 2006). RuBisCO fractionates the atmospheric carbon dioxide ($\delta^{13}C$: -8 ‰) to a value of about -28 ‰ on average in C3 plants. Thus, terrestrial plant-derived organic matter that accumulates in a body of water has an isotopic signature of about -28 ‰. In the surface ocean, there an equilibrium exchange between the atmospheric carbon dioxide (about -8 ‰) and the predominant carbonate species, bicarbonate ion (HCO_3^-; about 1 ‰). Phytoplankton cells use this inorganic carbon as building blocks for OM synthesis again through the RuBisCo pathway, resulting in a kinetic fractionation of about 20 ‰. Hence, marine-derived OM has an isotopic signature around -21 to 19 ‰ (Ehleringer, 1991; Maberly et al., 1992). Such natural fractionation processes are imprinted on the stable isotope signatures of terrestrial and marine OM, allowing to discriminate between these two sources of organic carbon.

Stable isotope signatures are expressed using the delta notation (δ), or per mil. The simplest form of the delta equation is shown in Equation 3. The absolute international standard used as the reference in stable carbon isotope analysis is Vienna Pee Dee Belemnite (VPDB), which has a ratio ($^{13}C/^{12}C$) of 0.0112372. By convention and as the ultimate reference for stable carbon isotope analysis, this ratio corresponds to a $\delta^{13}C$ signature of 0 ‰. Positive $\delta^{13}C$ signatures are said to be

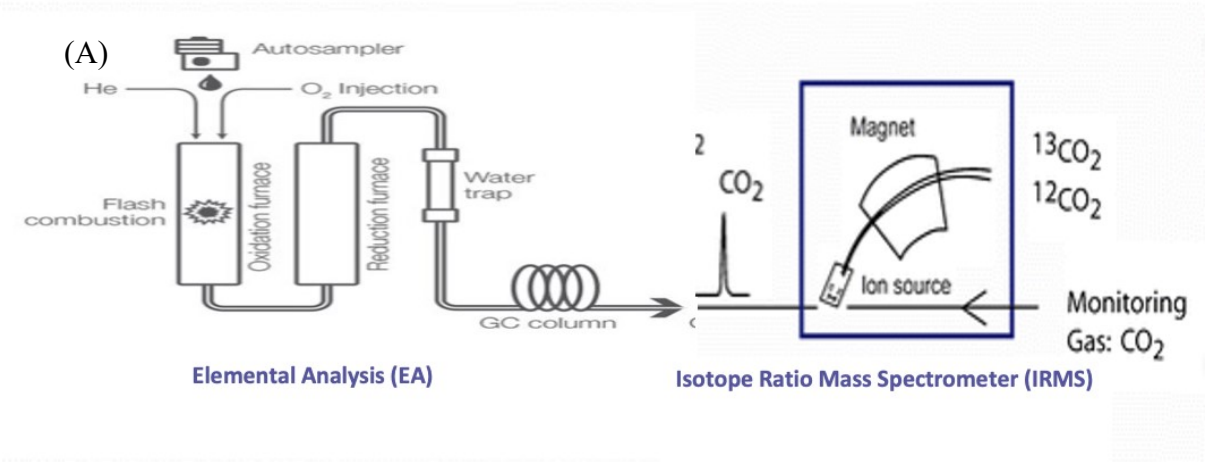
enriched (in ^{13}C) with respect to VPDB, while negative $\delta^{13}C$ signatures are considered depleted with respect to the VPDB standard.

$$\delta^{13}C = \left(\frac{\frac{^{13}C}{^{12}C_{\text{sample}}}}{\frac{^{13}C}{^{12}C_{\text{standard}}}} - 1 \right) * 1000 \qquad \text{(eq 3.)}$$

In this project, two types of $\delta^{13}C$ signatures have been acquired. Bulk $\delta^{13}C$ signatures, measured by combustion analysis, corresponding to the overall signature of whole, non-fractionated samples (e.g., sediment or bacterial broth), whereas compound-specific $\delta^{13}C$ signatures, measured on chemically and chromatographically separated compounds, correspond to the $\delta^{13}C$ signature of pure compounds isolated from a sample.

1.2.2. Instrumentation

Both bulk and compound-specific $\delta^{13}C$ analyses are carried out on an isotope ratio-mass spectrometer (IRMS). The difference between the two methods is in the separation method before the IRMS analysis. For bulk $\delta^{13}C$ analysis, an Elemental Analyzer (EA) is coupled to the IRMS (Figure 4A). A small amount, corresponding to a range of 20-100 µg of organic carbon, of the whole sample (solid or liquid) is introduced in the EA. The sample is then flash-combusted and the resulting CO_2 gas is carried to the IRMS for analysis. When performing an EA-IRMS analysis for organic carbon only, the inorganic carbon that can be present in a sample must first be removed to get an accurate $\delta^{13}C$ measurement. Fumigation with vapour-phase hydrochloric acid (HCl) prior to analysis is typically used to remove the inorganic carbon component. The importance of fumigation and certain words of caution regarding this method are addressed in Chapter II of this thesis.



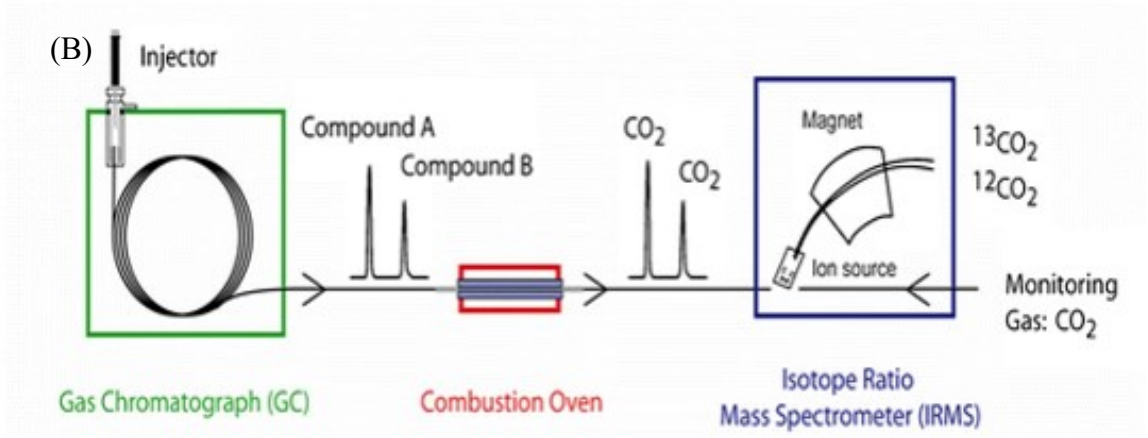


Figure 4. (A) Elemental Analyzer coupled to an Isotope Ratio Mass Spectrometer (EA-IRMS) (B) Gas-chromatograph coupled to an Isotope Ratio Mass Spectrometer (GC-IRMS ; Elsner et al., 2012; Kelly et al., 2018).

For compound-specific isotope analysis (CSIA), individual compounds must first be extracted from the sample, purified, and then separated by gas chromatography (GC). This introduces another difficulty for CSIA, as good baseline separation between each compound of a sample is essential in order to obtain accurate compound-specific $\delta^{13}C$ signatures. Therefore, optimization of GC parameters is necessary to obtain the best results possible. Once the compounds are separated, they are individually combusted to CO_2, and each peak of CO_2, corresponding to a single compound, is analyzed for its $^{13}C/^{12}C$ ratio by the IRMS (Figure 4B).

After the combustion, the different isotopologues of CO_2 are separated by a magnetic sector mass spectrometer according to their mass-to-charge ratios (m/z = 44, 45, and 46). The IRMS is equipped with three Faraday cups in which the different isotopologues are collected, corresponding to $^{12}C^{16}O_2$ for mass 44, $^{13}C^{16}O_2$, and $^{12}C^{17}O^{16}O$ for mass 45, and finally $^{12}C^{18}O^{16}O$, $^{13}C^{17}O^{16}O$, and $^{12}C^{17}O_2$ for mass 46 (NOAA, n.d.).

It is of the utmost importance to calibrate and correct the raw data generated by the IRMS to get the most accurate results. For the EA-IRMS, the standards beta-alanine and sucrose, both with a known $\delta^{13}C$ signature, are used to build the calibration curve of measured *vs.* known signatures at different CO_2 concentrations. Intermittent standard samples are also inserted between series of 6 samples to improve precision and correct for instrumentation drift. With the calibration and the intermittent standards, raw $\delta^{13}C$ signatures can be corrected for the drift that sometimes occurs during the analysis of long series of samples. For the GC-IRMS analyses, calibration is also carried out using a mix of certified compounds such as the Schimmelman F8-mix for fatty acid methyl esters (FAME) analysis. Since stable carbon isotope analyses are looking at very small differences between ratios (in the fourth or fifth decimals), it is crucial to anchor the measurements with very high precision and accuracy using standards with known $\delta^{13}C$ signatures.

1.3. Using Biomarkers to Study the Cycle of Organic Carbon

In biogeochemistry, the use of biomarkers is significant as they can be used to trace the origins of OM and track the biochemical transformation pathways that control its fate in the environment (Derrien et al., 2017). Biomarkers "can be unambiguously linked with biological precursor compounds, owing to the preservation of their basic skeleton" (Killops & Killops, 2005). To be useful, it is important for biomarkers to be specific to their source(s) and that their original structure is preserved in the environment compartment where they accumulate (Derrien et al., 2017; Killops & Killops, 2005). Some of the most routinely used biomarkers in biogeochemistry are lignin oxidation products, carbohydrates, amino acids, and a "lipid" component that comprise of hydrocarbons, neutral (sterols, triglycerides), and polar (phospholipids) lipids. For example, the lignin phenols vegetation index can be used in studies to estimate the inputs of terrestrially-derived OM in freshwater and to identify the type of plants contributing to these inputs using the concentration and molecular ratios of a series of specific lignin oxidation phenols (Hedges & Ertel, 1982). Moreover, by combining the analysis of biomarkers with the determination of their stable carbon isotope signatures, it is possible to gain a greater understanding on the local impacts of climate change on aquatic systems, the origin of OM, and even reconstruct the environmental conditions in which plants have grown (Tareq et al., 2011). For this project, a specific family of lipids was exploited as biomarkers.

1.3.1. Fatty Acids as Biomarkers

Lipids are often used in biogeochemistry for the identification of the origin of OM. Lipids are found in all organisms and their most common role is in the formation of cellular membranes. They also serve for energy storage within the cells. The amount and even the chain length of the hydrophobic tail of lipids are highly dependent on the environment in which the organism thrives (Killops & Killops, 2005). For example, the amount of light received by a plant can regulate its lipid content. Microorganisms that live under harsher conditions (such as cold waters) adjust their membrane fluidity by changing the arrangement (*iso* vs. *anteiso*) of its cellular lipid membranes (Killops & Killops, 2005). Fatty acids are commonly used in trophic studies (e.g., food web tracing), because the carbon atoms of a "food" source are used to synthesize fatty acids in organisms, and thus retain their stable isotope signature. Additionally, fatty acids are powerful biomarkers as they meet the main criteria of not being degraded and conserving the same structure throughout the food web (Derrien et al., 2017; Killops & Killops, 2005). Therefore, the analysis of fatty acids provides insights on the diet of organisms (Budge et al., 2008). The process in which a fatty acid is built is called *de novo* synthesis, which is done through the Acetyl-CoA cycle (Ruess & Chamberlain, 2010). Since marine and terrestrially derived OM have specific stable carbon isotope signatures and some microorganisms feed on organic compounds, it is possible to combine the results from fatty acid biomarkers and the stable carbon isotope signatures by CSIA to understand the lability of marine and terrestrial OM. In other words, it is possible to investigate

which type of OM is being preferentially consumed or degraded by microorganisms and in what proportion.

1.3.2. Straight *vs*. Branched Fatty Acids

Many types of fatty acids (saturated, unsaturated, polyunsaturated) can be synthesized by organisms. However, for the scope of this thesis only branched and straight-chain fatty are discussed further. One of the most ubiquitous fatty acids found in organisms and plants is palmitic acid (C16:0) (Killops & Killops, 2005). Since palmitic acid is not specific, it cannot be used as a bacteria-specific fatty acid in this project as it can be derived from different living organisms. Another characteristic of this class of lipids is that straight-chain fatty acids with an even-numbered chain are more abundant than odd-numbered fatty acids owing to their synthesis pathway that uses acetate, a two carbon atom compound, as their building block (Killops & Killops, 2005). Branched-fatty acids are normally in the form of *iso* and *anteiso* and can be found in many organisms such as phytoplankton, fungi, and bacteria. These organisms use branched fatty acids in their membranes to adapt to changes in environmental conditions. The presence of branched fatty acids leads to an increase in membrane fluidity. Furthermore, the published work reports that an *anteiso* structure lowers the melting point of the fatty acid, and thus its abundance is increased over that of the *iso* form in cold temperature environments (Kaneda, 1991; Zhu et al., 2005). In this thesis, *iso*- and *anteiso*-pentadecanoic acid (*iso* C15:0 and *anteiso C15*:0) have been used as these fatty acids are specific to bacteria (Derrien et al., 2017; Killops & Killops, 2005).

1.3.3. Fatty Acid Synthesis

The synthesis of branched fatty acids and straight-chain fatty acids have different pathways and involve different enzymes during the reactions (Figure 5). However, the concept is similar in that both synthesis pathways start with a smaller precursor molecule and undergo a chain elongation until the desired chain length. The precursor of straight-chain fatty acids is acetyl coenzyme A (acetyl-CoA), which is used in many biological pathways. Organic matter is being degraded into acetyl-CoA and can be used for the synthesis of straight-chain fatty acids (Berg et al., 2015). As mentioned above, fatty acids with even-numbered chain length are the more abundant than their odd-numbered counterparts due to the abundance of their precursors and the fact that they require fewer synthesis steps. Thus, the chance of observing isotopic fractionation is lower when fewer enzymes are needed for a reaction to occur. On the contrary, branched fatty acids are synthesized from other precursors that are also common in an organism. Both *iso* and *anteiso* are derived from an amino acid (Figure 5A). The amino acid leucine is required for the synthesis of odd-numbered *iso* fatty acid and isoleucine for odd-numbered *anteiso* fatty acids (Vlaeminck et al., 2006; Zhu et al., 2005). Many enzymes are necessary for the synthesis of the amino acid precursors for *iso/anteiso* and as well for the subsequent fatty acid synthesis. Thus,

branched fatty acids involve a greater number of steps and thus they are more likely to be affected by enzyme-driven stable isotope fractionation.

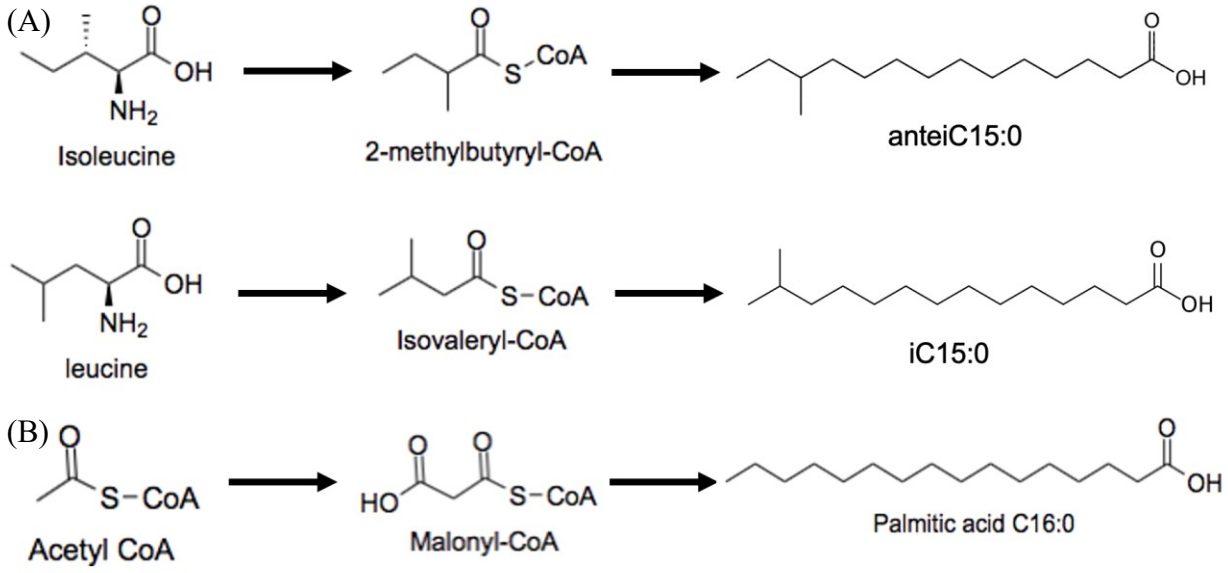


Figure 5. (A) Branched fatty acid synthesis; (B) Straight-chain fatty acid synthesis.

1.4. Objectives

Using compound-specific stable carbon isotope analysis coupled to the power of fatty acid biomarkers that are bacteria-specific, it is possible to determine the origins of OM (marine *vs.* terrestrial) that bacteria preferentially degrade and use in the synthesis of their fatty acids. Such determination provides an indirect assessment of the relative biological lability of marine and terrestrial OM that is being degraded by bacteria. The SLEG represents a perfect natural laboratory for this type of work as it consists in a continuum of stations between terrestrially influenced end-member close to land and an almost purely marine end-member in the gulf, with stations characterized by different proportions of terrestrial and marine OM inputs between the two. Because of the differences in their composition, marine OM is generally considered more labile than terrestrial organic matter. This hypothesis was tested in this work by comparing the bulk stable carbon isotope signature of the whole sediment at each station (which provide an estimate of the proportions of terrestrial and marine organic matter) with that of the bacteria-specific fatty acids to assess their preference for OM from either source. These results will provide an insight on the difference in reactivity of the two sources of OM, and thus on their respective role in the maintaining hypoxic conditions in the bottom waters of the St. Lawrence Estuary. Through a better understanding of the carbon cycle in the St. Lawrence system with respect to the degradation of OM by bacteria, and thus to consumption of dissolved oxygen in the deep waters, remediation actions can be better designed in order to reverse the century-old trend of decreasing dissolved oxygen in the bottom waters of the SLEG.

1.5. Thesis Arrangement

This thesis comprises four chapters, which include two manuscripts (the first one under review, and the second to be submitted for publication). The first chapter (Chapter I) is dedicated to the general introduction and contextualisation of the main topics covered in the thesis.

Chapter II (*Using ^{13}C Enriched Acetate in Isotope Labelling Incubation Experiments: A Note of Caution*) corresponds to a publication submitted to the journal *Limnology and Oceanography: Methods*. It is a short publication focussing on potential problems that may arise when using a well-known fumigation method to remove inorganic carbon from samples in preparation for their analysis for organic carbon and stable isotope signature. The results show the potential loss of low molecular weight organic compounds during fumigation. The implications of these findings is also discussed.

Chapter III (*Stable Carbon Isotope Signature of Bacteria-Specific Fatty Acid to Assess the Reactivity of Terrestrial and Marine Organic Matter in Sediment of the St. Lawrence Estuary and Gulf*) reports the findings for the main project of this master's thesis. It is also in the form of a manuscript that will soon be submitted to the journal *Marine Chemistry.* It investigates the lability of terrestrial and marine OM in the St. Lawrence Estuary and Gulf towards its degradation by endogenous bacteria. First, as a proof of concept, a ^{13}C enrichment on a bacterial culture was made to calibrate the relationship between the carbon stable isotope signature of bacteria-specific fatty acids and their food source. Then, this calibration was applied to a series of samples collected along the SLEG.

Chapter IV summarizes the main findings of the project, the limitations and explores future works that can be done.

Chapter II: Using ^{13}C Enriched Acetate in Isotope Labelling Incubation Experiments: A Note of Caution

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2.1. Abstract

Vapour-phase fumigation with HCl is routinely used to remove inorganic carbon in preparation for the measurement of the concentration and $\partial^{13}C$ signature of organic carbon in a sample using elemental analysis coupled to an isotope ratio mass spectrometer. Acidification of the sample to be analyzed can lead to the loss of low molecular weight conjugate bases as volatile organic acids during the acidification and/or the drying steps following fumigation. Such loss could be important in incubation experiments where ^{13}C-enriched acetate is used to trace reactions pathways or metabolites in a cultivation medium or a mesocosm for example. In this work, we enriched a carbonate-free freshwater sediment with 1-^{13}C sodium acetate by 5, 10 and 20 ‰ relative to the $\partial^{13}C$ signature of the organic carbon in the natural sediment, and then tested the effects of HCl fumigation, drying at 50°C and drying at room temperature, alone or in combination. We found that fumigation and drying at 50°C, alone or in combination, both lead to the loss of the majority of the ^{13}C-enriched acetate through protonation of the conjugate base and volatilization.

2.2. Introduction

Isotopically labelled compounds such as ^{13}C-glucose, ^{13}C-carbonate , ^{13}C-formate or ^{13}C-acetate are commonly used in biological, biochemical and biogeochemical studies, often as part of incubation experiments, to assess sources, pathways and processes affecting natural compounds (Baggott et al., 2007; Rattray et al., 2009; Rinkel and Dickschat, 2015). Owing to their unique stable isotope signature, the reactions in which they are involved, and the metabolites derived from these reactions can be followed efficiently using high resolution liquid-chromatography mass spectrometry for chromatographically separated compounds, or IRMS by measuring the stable carbon isotope signature of a bulk sample such as soil or sediment, a whole organism, or individual molecules. For example, labelled glucose has been used to track biosynthesis pathways and metabolism in cells or an organism (Antoniewicz, 2018). 1-^{13}C sodium acetate can be added to different types of soils during their incubation and used to monitor the activity of fungi or bacteria by looking at the incorporation of ^{13}C into their specific phospholipid fatty acids (Arao, 1999). Labelled compounds can also be used in community structure studies to track the trophic levels of different organisms in a given ecosystem (e.g., Bertram et al., 2013; Bühring et al., 2006). Small labelled compound such as ^{13}C-acetate and^{13}C-bicarbonate can also be used to study the physiology of anaerobic methanotrophic archaea in order to determine their methanogenic capability (Bertram et al., 2013).

Isotopically labelled compounds can be used as pure ^{13}C-labelled substrates, as in the above studies, or as a mixture of the ^{13}C-labelled and non-labelled compound to obtain a substrate with a ∂^{13}C signature in the same range as naturally produced compounds (typically -40 to -10‰ for natural organic matter and biochemicals from living organisms), but with a ∂^{13}C signature more enriched or more depleted in ^{13}C by a maximum of ±25 ‰ compared to their natural counterparts. These differences in stable isotope signatures are statistically significant and measurable with high precision and accuracy using IRMS, which allows to efficiently quantify the processes being studied. This was done for example in a study on the degradation of sedimentary organic matter where natural organic matter with a ∂^{13}C signature about -41‰ was added as a spike to sediment with a mean organic matter ∂^{13}C signature of -25‰ in a long-term incubation experiment (Barber et al., 2014).

Stable carbon isotope ratios on bulk samples are obtained using an EA-IRMS. This instrument measures the average stable carbon isotope ratio (^{13}C/^{12}C) of all carbon atoms present in the sample whether it is organic or inorganic. When assessing the bulk ∂^{13}C signature of organic carbon (OC) in complex matrices such as soil or sediments, any inorganic carbon present as carbonates must be removed as CO_2 under acidic conditions. One of the most common approaches to remove inorganic carbon in a sample is the acid fumigation method in which samples are exposed to HCl vapour to convert carbonates to CO_2 (Hedges & Stern, 1984), as used in some studies with samples containing short chain alkanes, fatty acids and spiked with small ^{13}C-labelled compounds (e.g., ^{13}C-sodium acetate) (Harris et al., 2001; Komada et al., 2008). This method is

preferred over liquid-phase HCl rinse as it removes all carbonates with an excellent OC recovery (Komada et al., 2008).

This technical note stems from another study carried out in our lab in which labelled-^{13}C sodium acetate was used to enrich a bacterial cultivation medium in ^{13}C to assess the relationship between the ∂^{13}C signature of different bacterial biomolecules and that of their source of carbon. Unexpected analytical results were obtained when analyzing the ∂^{13}C signature of the enriched media using an EA-IRMS to confirm the enrichment level. Since the only sample preparation steps involved prior to EA-IRMS analysis were weighing and acid fumigation to remove carbonates present in the media, we designed a very simple experiment to investigate the cause for these unexpected results. This article highlights the precautions that must be taken during an acid fumigation of samples containing low molecular weight compounds for the removal of inorganic carbon.

2.3. Materials and Procedures

2.3.1. Sample Collection and Preparation

The carbonate-free sediment used in this work was collected in 2008 from the man-made Cabonga Reservoir in the Quebec, Canada, boreal forest (47° 19' 57" N and 76° 34' 37" W). A 30-cm core was sampled at one of the deeper locations in the reservoir, sliced at a resolution of 1 cm, freeze-dried, homogenized with a pestle and mortar, dry-sieved and then separated in four aliquots of 1.5 g in preparation for the spiking experiment. The four sediment aliquots (OC of 1.39 ± 0.10 %) were spiked with 1-^{13}C sodium acetate (99% purity; Sigma-Aldrich) to obtain samples with stable carbon isotope signature (δ^{13}C) enriched by +0, +5, +10, +20 ‰ from the original signature of the sediment OC (-27.52 ± 0.26 ‰). The exact mass of 1-^{13}C sodium acetate necessary for each enrichment level was calculated using isotopic mass balance, where the mass of OC (m) and the relative stable isotope abundance (F) of the whole sample (Σ) comprising of the spike compound (x) and the sediment (s) were taken into account (eq. 1) (Hayes, 2004).

$$(m_x + m_s)F_\Sigma = m_xF_x + m_sF_s \qquad \text{(Eq 4.)}$$

The masses of 1-^{13}C sodium acetate needed to enrich the sediment to the desired levels were very small (24 to 95 µg) compared to the total mass of sediment (1.5 g). Once each sample was prepared, ultrapure water was added to the samples and shaken overnight at room temperature to ensure complete homogenization. The samples were then lyophilized and homogenized prior to weighing in preparation for EA-IRMS.

2.3.2. Sample Treatments and Analysis

The four freeze-dried sediment samples were pre-weighted in quadruplicate (n=4) in clean pressed silver capsules 8x5 mm (Elemental Microanalysis) for each treatment (n=5; Table 1) and enrichment level (n=4), for a total of 80 samples. Samples from the first treatment were not fumigated nor dried. The silver capsules were folded immediately and wrapped in clean 8x5 mm tin capsules (Elemental Microanalysis). All vapor-phase fumigation steps were made using 12N concentrated hydrochloric acid (ACP Chemical) following the method of Hedges and Stern (Hedges & Stern, 1984). Samples from the second treatment were fumigated overnight but they were not dried before weighing them for EA-IRMS analysis. Samples from the third treatment were fumigated overnight and dried for 1 hour at room temperature. Samples from the fourth treatment were also fumigated overnight but they were dried for 1 hour in an oven set at 50°C. Finally, samples from the fifth treatment were only dried for 1 hour at 50°C but they were not fumigated. Once the fumigation and/or drying treatments were completed, each silver capsule was wrapped within a tin capsule. The certified standard material Sucrose (IAEA-CH-6; $\delta^{13}C$ = -10.45 ± 0.03 ‰) and Polyethylene (IAEA-CH-7; $\delta^{13}C$ = -32.15 ± 0.04 ‰), as well as β-alanine and sucrose, two in-house standards calibrated with these certified reference materials (Sigma-Aldrich; $\delta^{13}C$ = -26.18 ± 0.10 ‰ and -11.77 ± 0.09 ‰, respectively), were used for calibration purposes. The analyses were carried out using an EuroEA3000 CHNS-O analyzer (EuroVector S.p.A.) coupled to an Isoprime 100 IRMS (Elementar Americas Inc.). The stable carbon isotope ratios are expressed relative to the international reference standard VPDB. Precision and accuracy of the measured stable isotope signatures were assessed using a range of masses of the pure in-house calibrated compound (β-alanine) and IAEA certified sucrose covering the entire dynamic range of the instrument. The slope of the calibration curve built by plotting the known *vs.* measured values had a slope of 1.0355 with an R^2 of 0.9996. The precision obtained for the standards inserted between each series of six samples was 0.08‰ and 0.11‰ for β-alanine and sucrose, respectively.

Table 1. List of the different treatments applied to the spiked samples

Treatment	Fumigation	Drying conditions
1	None	None
2	Overnight	None
3	Overnight	1 hr, Room Temp.
4	Overnight	1 hr at 50°C
5	No	1 hr at 50°C

2.3.3. Statistical Analysis

Single-factor ANOVA was performed on the $\partial^{13}C$ average signatures obtained for the four enrichment levels to assess whether there were statistically significant differences between the five treatments. The confidence interval was set at 95%, with 12-15 degrees of freedom depending on the set of samples compared. When a statistically significant difference was found, a pair-wise post-hoc Tukey-Kramer for unequal sample size test was carried out to identify group pairs with different mean $\partial^{13}C$ signatures.

2.4. Assessment

The experiment was designed in a way that allowed assessing the influence of each factor separately (fumigation, drying time and drying temperature). Treatment #1 (no fumigation and no drying) served as the negative control, providing reference values at each enrichment level, while the 0‰ enrichment level allowed to assess whether any treatment, or combination of treatments, affected the signature of the native organic carbon present in the sediment sample.

The average OC content of the sample did not vary significantly between the sample treatments and enrichment level (average of 1.39 ± 0.10 %, data not shown) simply because the mass of carbon added to the sediment sample as ^{13}C-enriched acetate never exceeded 0.5% of the total OC mass in the non-treated sample. The stable carbon isotope signatures measured for the bulk lake sediment without enrichment for the diverse treatment varied between and -27.40 ± 0.39 ‰ to -28.08 ± 0.20 ‰, respectively (Table 2 and Figure 6). The differences between treatment were small and not significant at $p<0.05$ [$F(4,13)=2.74$, $p=0.74$].

Analyzing the enriched samples without any treatment (Treatment #1; Table 1) provides an accurate estimation of the exact enrichment levels obtained by spiking the sediment with 1-^{13}C sodium acetate (Table 2). The enrichment levels were close to but slightly lower than targeted values, with signatures of -22.89 ± 0.61 ‰ for the +5 ‰ enrichment level (an enrichment of +4.63 ‰ compared to the bulk, non-enriched sediment), -18.93 ± 0.41 ‰ for the +10 ‰ enrichment level (+8.59 ‰), and -9.81 ± 0.85 ‰ for the +20 ‰ enrichment level (+17.71 ‰).

No enrichment was found at any enrichment level for any of the other treatments (#2-5), except for an increase of about 1 ‰ for the fumigation only treatment (Treatment #2) at the highest enrichment level (+20 ‰). For all the other signatures, the measured values at any enrichment level were not significantly different from the non-treated samples.

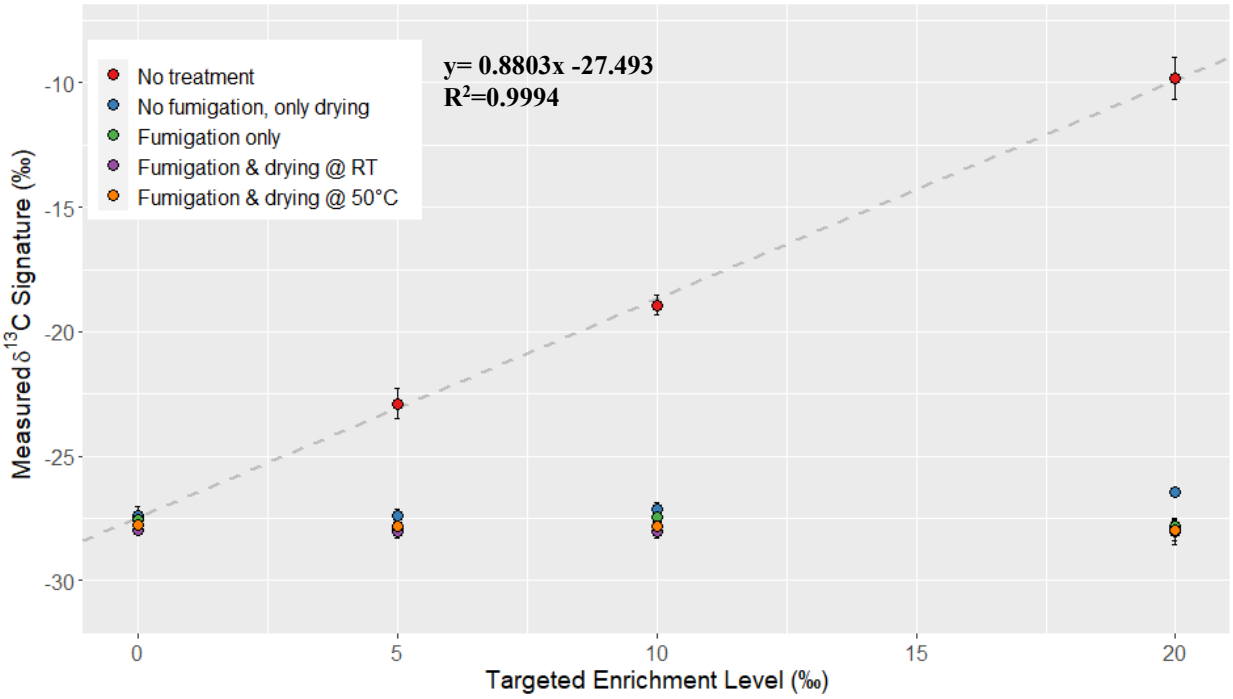

Figure 6. δ^{13}C signature of the carbonate-free lake sediment at diverse treatment and different enrichment levels. The equation and R^2 correspond to the regression obtained for the Treatment #1 (no fumigation and no drying).

Table 2. Average ∂^{13}C signature of the sediment sample for each treatment at different enrichment levels (n=4 in all cases)

Treatment	Enrichment level (‰)			
	0	+5	+10	+20
1	-27.52 ± 0.26	-22.89 ± 0.61	-18.93 ± 0.41	-9.81 ± 0.85
2	-27.40 ± 0.39	-27.38 ± 0.11	-27.14 ± 0.03	-26.47 ± 0.10
3	-27.55 ± 0.31	-27.87 ± 0.11	-27.44 ± 0.60	-27.82 ± 0.25
4	-28.08 ± 0.20	-28.05 ± 0.13	-27.83 ± 0.11	-28.06 ± 0.14
5	-27.77 ± 0.04	-27.85 ± 0.12	-28.04 ± 0.03	-27.98 ± 0.43

2.5. Discussion

The different treatments (Table 1) did not lead to significant differences in OC content and $\partial^{13}C$ signatures for the natural sample (no enrichment), confirming the absence of carbonates in the sample (e.g., no difference between fumigated and non-fumigated samples; Table 2), as well as the absence of an impact of the different treatments on the OC content and stable isotope signature of the natural organic matter in the sample. This result ensured that the potential changes in $\partial^{13}C$ signatures measured for the enriched samples reflected the impact of the treatment on the added 1-^{13}C sodium acetate only.

The exact enrichment levels obtained by spiking the sediment with 1-^{13}C sodium acetate followed by the measurement of their $\partial^{13}C$ signatures without treatment (Treatment #1; Table 1) were slightly less enriched than expected from the mass balance calculations (Table 2). These samples were weighed and wrapped immediately in tin boats, and then stored in a desiccator until analysis. Plotting the targeted $\partial^{13}C$ signatures (calculated assuming a purity of 99% for 1-^{13}C sodium acetate, as indicated on the certificate of the company) against the measured ones yielded a strong correlation (R^2=0.9994), but with a slope different from unity (0.88), suggesting that the actual purity of the product was lower than 99% (Figure 6). Alternatively, the slope of 0.88 could reflect a partial loss of the labeled spike by volatilization during storage in the desiccator, which would imply the protonation of 1-^{13}C acetate to 1-^{13}C acetic acid. The very strong correlation coefficient however argues against this explanation as the proportion of the spike lost through volatilization would have to be exactly the same for each enrichment level to obtain a regression with an R^2 of 0.9994. These results show however that without any treatment, the enrichment of the sediment worked and that most of the added 1-^{13}C acetate was not lost during sample homogenization, weighing or storage.

With the exception of a small enrichment of about 1 ‰ for the fumigation only treatment (#2) at the highest enrichment level (+20 ‰), the measured values at any enrichment level were not significantly different from the non-treated samples for treatments #2-5 (Tukey-Kramer post hoc test), indicating a complete loss of the 1-^{13}C sodium acetate spike in these samples. This result was particularly surprising for treatment #5 for which only heating the sample at 50ºC for 1 hr lead to its quantitative loss by evaporation.

Vapour-phase fumigation with concentrated HCl followed by drying is used routinely to remove inorganic carbon when seeking to measure the stable isotope signature of OC only, and it is preferred over the liquid HCl approach because the latter leads to leaching, and thus loss, of a fraction of total OC. The resulting vapour-phase acidification of the sample leads to the protonation of low molecular weight conjugate bases that can be present in a sample, such as acetate, and their subsequent loss as acids through volatilization. Such organic compounds of low molecular weight, with a high vapour pressure and a low capacity for forming hydrogen bonds can thus be lost upon fumigation even when the samples are dried at room temperature to remove excess acid (Treatment #3).

Surprisingly, the ^{13}C-labeled acetate spike was also lost when the only treatment applied to the non-fumigated sample was drying for one hour at 50ºC (Treatment #5, Table 1). It is highly possible that sodium acetate interacted with air moisture, acidic clays or humic matter during the weighing step and by drying it at a higher than room temperature (50ºC), it favoured the reaction to proceed from a conversion of sodium acetate to acetic acid mediated by the hydrolysis of water molecules. The high volatility of acetic acid (vapour pressure of 11.6 and 54 mm Hg at 20ºC and 50ºC, respectively) could explain the loss of the labelled substrate when dried in the oven only. The protonation of acetate did not happen during the homogenization of the sediment and the spike (homogenization as a sediment plus 1-^{13}C sodium acetate spike slurry followed by freeze-drying) since the spike was entirely recovered in Treatment #1 (no fumigation and no drying). Furthermore, the water used in the homogenization step and the resulting sediment slurry both had a pH above 6, which is at least 1.25 pH unit above the pKa for acetic acid (4.75). The exact mechanism causing this protonation and loss is still not fully understood.

2.6. Comments and Recommendations

The results presented above bring new insights on the precautions to take when using ^{13}C-labeled low molecular weight salts or organic acids in incubation experiments. Ideally, when the initial ∂^{13}C signature is needed, the cultivation medium or the sample matrix should not contain any inorganic carbon thus eliminating the need for its removal by fumigation. When this is not possible, the only alternative is to use a higher molecular weight labelled compound as the source of ^{13}C. Alternatively, if inorganic carbon is present in the matrix but is not essential to the process under study, fumigation followed by drying could be carried out prior to spiking the sample with the labeled compound. If ^{13}C-labeled acetate must be used in an experiment, the pH of the cultivation medium or the matrix should remain largely above its pKa of 4.75, and sublimation in a freeze-drier should be used when drying the samples.

Further investigation is needed in order to assess the extent of the loss of low molecular weight compounds (e.g., formate, C-acetate, propionate, ^{13}C-labelled or not, although the acid form of the latter is more than 20 times less volatile than that of acetic acid) during fumigation and/or drying. This is true not only in experiments such as described above, but also whenever natural samples that can be enriched in these compounds, such as anoxic soils or sediments, are fumigated and dried in preparation for analysis by standalone EA or EA-IRMS.

Chapter III: Stable Carbon Isotope Signature of Bacteria-Specific Fatty Acid to Assess the Reactivity of Terrestrial and Marine Organic Matter in Sediment of the St. Lawrence Estuary and Gulf

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3.1. Abstract

Hypoxia in the St. Lawrence Estuary and Gulf has been progressively worsening since the beginning of the century and has severely affected the health of this ecosystem. An increase in the flux of terrestrial OM and inorganic nutrients (NO_3^-, HPO_4^{2-}) discharged in this aquatic system by the St. Lawrence River has led to an increase in nutrient abundance leading to higher primary productivity in the surface waters of the estuary and thus to higher concentrations of dissolved and particulate OM in the water column, which results in an increased consumption rate of oxygen during the degradation of OM by microorganisms. Hypoxic conditions are worsened by global warming of the ocean, by which warmer oxygen-poor water from the North Atlantic is directed to the deep water of the estuary. To improve our understanding of the relative importance of terrestrial OM and nutrients in the onset of hypoxia in the St. Lawrence system, the lability of terrestrially-derived and marine OM was indirectly determined through the analysis of the natural abundance $^{13}C/^{12}C$ ratio of bacteria-specific fatty acid (*iso* C15:0 and *anteiso* C15:0) using gas chromatography coupled to an isotope ratio mass-spectrometer (GC-IRMS). First, to calibrate the relationship and isotopic fractionation between the ^{13}C signature of the bacteria-specific fatty acids and that of their food source, a ubiquitous bacterial strain (*Alteromonas macleodii)* responsible for the degradation of natural OM was cultivated in marine broth enriched in ^{13}C with ^{13}C-sodium acetate. Fatty acids from neutral and polar lipids were then extracted from surface sediment sampled at ten stations from a terrestrial-marine transect along the St. Lawrence Estuary and Gulf. The stable isotope ratios of the bacterial fatty acids were mapped along the transect and compared and those for bulk OM at the same stations. The fractionation patterns of the bacterial branched fatty acids and ubiquitous fatty acid (C12:0, C14:0, C16:0) was also explored. Our results will lead to a better understanding of the factors that have led to the current low oxygen levels in the deep waters of the estuary, which will help guiding remediation efforts to improve the health of this important ecosystem.

3.2. Introduction

As one of the largest enclosed seas in the world, the St. Lawrence Estuary and Gulf (SLEG) system connects the Great Lakes and the St. Lawrence River to the Atlantic Ocean. From the microscopic to the macroscopic world, this ecosystem is home to a vast diversity of species and its watershed is densely populated by humans as the majority of the population of the province of Québec (Canada) resides along the shores of the river and estuary. These waterways are thus affected by human activities that can cause changes in the biogeochemical cycles within this ecosystem and have an impact on its fauna (Rabalais et al., 2010). One such change that has occurred in the St. Lawrence Estuary (SLE) in the past century is the development of hypoxic conditions in the bottom waters of the lower estuary (Gilbert et al., 2005). Hypoxic conditions correspond to a level of dissolved oxygen in the water column below the threshold concentration of 62.5 μmol/L (Breitburg et al., 2018; Lefort et al., 2012). Hypoxia can be episodic or seasonal with a recurring pattern of low dissolved oxygen concentrations during the summer and higher concentrations the rest of the year. For example, the bottom waters of the northern Gulf of Mexico along the coast of the United States, or Saanich Inlet, Canada, become hypoxic mainly during the warmer summer months (Diaz & Rosenberg, 2008; Gray et al., 2002). Hypoxia can be permanent in other locations, such as in the bottom waters of the lower SLE, where consistently decreasing dissolved oxygen levels have been reported over the past 72 years (Gilbert et al., 2005b).

In the 1960s, the decrease in dissolved oxygen levels in the bottom waters was accelerated due to industrialisation, an increasing population density and intensification of agricultural activities along the shores of the St. Lawrence River. Such increase in human activities in the St. Lawrence River watershed lead to higher levels of nitrogen, phosphorus and organic matter being discharged in the estuary, which promoted primary production in the surface waters and lead to eutrophic conditions owing to the high injection rates of terrestrial and marine organic matter in the bottom waters of the lower SLE (Cordell et al., 2009; Goyette et al., 2016). This in turn lead to an increase in dissolved oxygen consumption through aerobic degradation of OM by bacteria, ultimately resulting in dissolved oxygen concentrations decreasing below the hypoxic threshold (Jutras et al., 2020b).

Eutrophication is not the only cause of the hypoxic bottom water of the lower SLE. Global warming and the changes in oceanic currents are also important contributors to hypoxia in this region (Jutras et al., 2020a). The lower SLE receives its water from three sources. The surface layer, flowing seaward, is fed by the St. Lawrence River discharge while the bottom layer flows inland and is sustained by two deep Atlantic Ocean currents, the cold and O_2-rich Labrador current and the warmer and more O_2-depleted North Atlantic Central current (Jutras et al., 2020a). The proportion of both currents feeding the bottom waters of the SLE determine the amount of dissolved oxygen available. Recent work suggests that the increase in the proportion of North Atlantic Central bottom waters entering the SLEG was responsible for 75% of the depletion in dissolved O_2 in the SLE between 2008 and 2018 (Jutras et al., 2020a). This decrease in dissolved

oxygen availability causes this coastal ecosystem to become even more sensitive to the stress caused by eutrophication.

Eutrophication caused by human activities may participate in the onset of permanent hypoxia in coastal ecosystems. It could have profound effects on the ecosystem, such as migration of species, decline in population health, and even their disappearance owing to low oxygen availability (Díaz & Rosenberg, 2011; Rabalais et al., 2010). Hypoxic conditions also severely affect biogeochemical cycles of several elements. An example of such impact is the modification of the chemistry in surface sediments owing to the decrease in redox potential of the sediment porewaters (Banks et al., 2012; Lenstra et al., 2020). Under oxic bottom water conditions, iron (III) and manganese (IV) hydroxides accumulate at the sediment-water interface where they efficiently sorbs OM (Lalonde et al., 2012) and toxic metals and metalloids such as zinc, arsenic and cadmium. Upon lowering of the redox potential, these iron and manganese complexes can eventually be reduced to water-soluble Fe(II) and Mn(II), thus releasing water soluble organic matter and metals/metalloids to the porewaters and the water column (Banks et al., 2012).

There are two main sources of OM reaching the sediments of the SLEG; terrestrial OM, originating from soils and vascular plants and comprising compounds such as lignin, tannin, cellulose and waxes, as well as marine organic matter mostly comprised of proteins and smaller proportions of polysaccharides and simple lipids (Hedges, 1992). Owing to their contrasting molecular composition, marine and terrestrial OM are characterized by differing bacterial lability, with the former considered more readily degradable to bacteria then the latter (Burdige, 2005). Terrestrial OM is for instance derived in large parts from highly altered polymeric degradation products such as lignin, a polymer composed of carbon-carbon or ether-linked phenylpropanoid building units with an irregular structure making it more difficult to degrade particularly under anoxic conditions (Ruiz-Dueñas & Martínez, 2009), while marine OM is composed primarily of major biomolecules such as proteins/peptides, carbohydrates and lipids that are more readily processed by bacteria under both oxic and anoxic conditions.

Bacteria are converting carbon atoms from organic matter they degrade as building blocks for their tissues, including their cell membrane fatty acids. Isotopically, “they are what they eat”, e.g., the stable carbon isotope signature of the source of organic matter that they degrade is reflected in that of their tissues and major biochemical components (Bouillon & Boschker, 2006; Bourgeois et al., 2011; Fang et al., 2014; Fry, 2006)). Theoretically, measuring the stable carbon isotope signature of bacteria-specific biochemicals, such as the branched *iso* C15:0 and *anteiso* C15:0 fatty acids, might thus reveal the origin of the organic matter that is preferentially degraded. Bulk terrestrial OM and marine OM stable carbon isotope signatures ($\delta^{13}C$) range from -30 to -26‰ and -22 to -18‰, respectively. This difference between terrestrial and marine OM is caused by the different source of carbon assimilated (dissolved HCO_3^- or atmospheric CO_2) and its fractionation by the enzyme RuBisCo (Berg et al., 2015). In the SLEG system, the terrestrial end-member for bulk sedimentary OM is about -27‰ while that of the more marine sediment end-member, where a minor terrestrial OM contribution is still detectable, is about -22‰. The stable carbon isotope signature of the bacteria-specific *iso* C15:0 and *anteiso* C15:0 fatty acids, which

increase the fluidity of bacterial membranes and help bacteria adapting to extreme environments (Kaneda, 1991), should thus reflect this pattern of progressive enrichment in compound-specific $\delta^{13}C$ values along the terrestrial-marine continuum.

The main objective of this work was to determine whether ubiquitous bacteria found in the sediment display a feeding preference for terrestrial or marine OM by comparing the stable carbon isotope signature of bacteria-specific fatty acids (*iso*-pentadecanoic acid and *anteiso*-pentadecanoic acid, *iso* C15:0 and *anteiso* C15:0, respectively), with that of bulk organic matter collected at each station. To demonstrate the concept that isotopically, "bacteria are what they eat", and to calibrate the relationship between the $\delta^{13}C$ signature of the two branched fatty acids and that of the food source at enrichment levels of 0 to 25‰ (Garcia-Martinez et al., 2002), a ubiquitous bacterium, *Alteromonas macleodii*, present in high abundance in the St. Lawrence ecosystem, was cultivated with ^{13}C-labeled compound (1-^{13}C-acetate) as its sole carbon food source. This calibration was then used to calculate the $\delta^{13}C$ of the OM being degraded by bacteria using the $\delta^{13}C$ signatures of their *anteiso*C15:0 extracted from a series of surface sediment collected along the terrestrial-marine continuum of the SLEG. Our initial working hypothesis was that bacteria preferentially degrade the more labile marine OM over terrestrial when both are available (Goto et al., 2017; Hedges et al., 2000). The SLEG is the ideal location for this study as the proportion of terrestrial OM and marine OM gradually changes along the continuum.

3.3. Methods

3.3.1. Study Site

The St. Lawrence is a large waterway connecting the Great Lakes to the Atlantic Ocean. It is divided in three distinct parts: River, west of Quebec City, the Estuary between Quebec City and Ste-Anne-des-Monts, and the Gulf. The shallow (20-100 m water depth) Upper Estuary, stretching between Québec City and Saguenay Fjord, is fed mostly by the St. Lawrence River, which is heavily impacted by human activities (high population density and intensive agriculture). In addition to the Upper Estuary, the Lower Estuary receives water inputs from the 90-km long Saguenay Fjord and is stratified with deep Atlantic bottom waters flowing inland from the Laurentian Channel in the Gulf and upwelling near the Saguenay Fjord outlet (Figure 7). The sample locations were chosen to cover the entire terrestrial to marine continuum and the transition between freshwaters and salty waters (Table 3).

Table 3. Station location and characteristics of the St. Lawrence Estuary and Gulf

Station	Latitude	Longitude	Distance from Quebec City (km)	Water column depth (m)	Surface water salinity (psu)	Bottom water dissolved oxygen (µM)
Sag05	48°24.798'N	70°49.307'W	-	89	0.87	275.26
DE	47°11.223'N	70°37.419'W	60	17	2.89	312.50
G	47°30.689'N	70°09.775'W	114	62	15.82	296.88
25	48°14.531'N	69°22.808'W	215	325	25.23	63.26
23	48°40.104'N	68°44.086'W	300	345	25.59	60.48
21	49°04.750'N	67°18.147'W	400	325	27.32	67.67
19	49°29.547'N	65°13.092'W	530	362	30.94	78.34
18.5	49°19.519'N	64°23.601'W	539	389	28.26	79.20
18	49°08.428'N	63°39.712'W	600	364	29.01	98.97
16	48°29.664'N	61°24.744'W	780	418	30.89	122.80

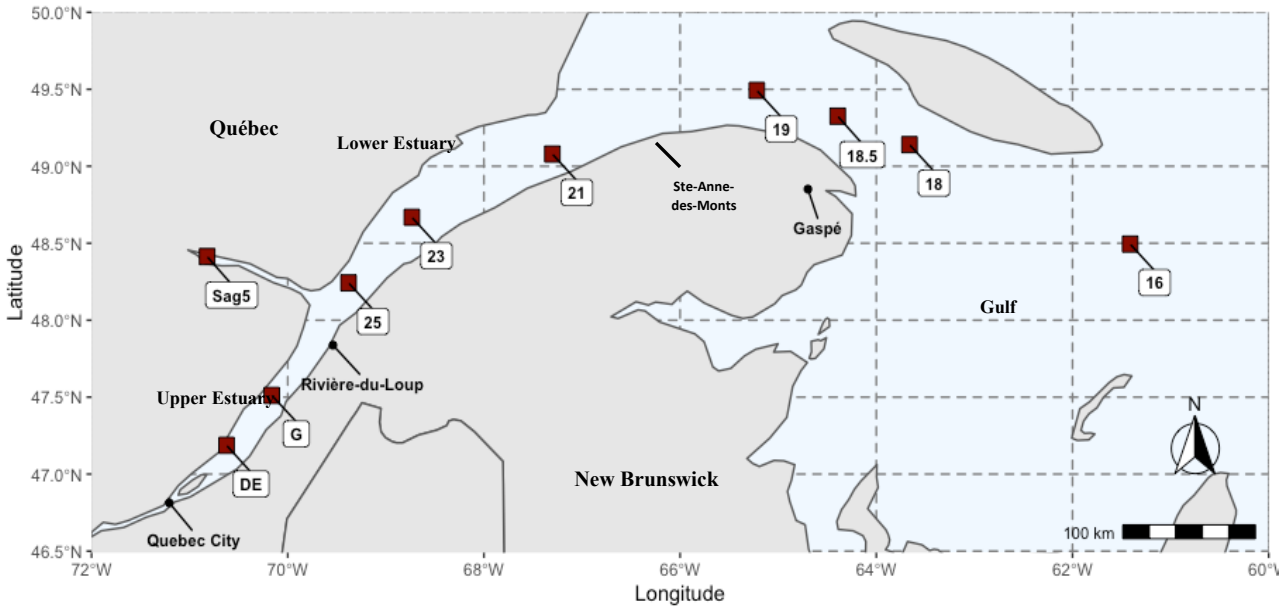


Figure 7. Map of the study area with the location of the 10 sampling stations

3.3.2. Sediment Collection

The sediments were collected during a sampling mission aboard the R*V Coriolis II* in June 2018 along the St. Lawrence Estuary, between Québec City and the Gulf of St. Lawrence, as well as at one location in the Saguenay Fjord (Figure 7). Box cores were used to collect sediments at stations SAG05, 25, 23, 21, 19, 18.5, 18 and 16. The well-mixed top 10 cm of sediment from the

box cores were used for this study. Surface sediments at stations DE and G were collected using a small Van Veen grab. The sediments were homogenized, centrifuged to remove excess pore water, freeze-dried and stored until further treatment.

3.3.3. Bacterial Broth Preparation and ^{13}C Enrichment

The bacterial broth was prepared specifically for this work in an autoclaved Fernbach 2-L flask using all but one of the ingredients present in a premade mix of DIFCO™ Marine Broth 2216 (VWR, BD Canada Microbiology). The premade mix could not be used because it contains peptone as the main source of carbon. The nitrogen source came from the yeast extract. In this study, in order to be able to precisely control the $\partial^{13}C$ stable isotope signature of the carbon source sustaining bacterial growth, peptone was replaced with sodium acetate and sodium acetate-1-^{13}C (Sigma-Aldrich, CAS 23424-28-4) whose proportions was adjusted in such a way to keep the same amount of carbon as in the premade marine broth recipe but with a known enrichment in ^{13}C. A mass balance approach was used to calculate the exact mass of sodium acetate-1-^{13}C needed to enrich each one of the five batches of broth with theoretical signatures of -35, -30, -25, -15 and -10 ‰, thus covering the range of $\partial^{13}C$ signatures routinely encountered for natural organic compounds in the environment.

3.3.4. Bacterial Inoculation and Clean-Up

The bacterial species cultivated in this experiment was *Alteromonas macleodii* (purchased from the University of Alberta, Canada). The bacteria were first cultivated on marine broth agar plates to assess their purity and inoculated separately in the five broths of different ^{13}C enrichment levels. The bacteria were incubated for two continuous days at constant temperature (18ºC) and constant shaking (2000 rpm). To extract the bacteria from the broth, the samples were spun down using a LYNXIII centrifuge (10ºC at 10000 rpm) to isolate the pellet containing the bacteria. The pellets were then rinsed with ultrapure Milli-Q water, spun down again and separated from the supernatant. Finally, the bacteria were freeze-dried and divided in triplicate samples for each enriched broth (five enrichment levels, n=3 for each enrichment level, total number of samples n=15). The total bacterial fatty acid fraction was then extracted following the same procedure as detailed below for the lipid extraction of the sediments.

3.3.5. Sediment Lipid Extractions

All glassware used was thoroughly washed and combusted at 450ºC for a minimum of 4 hours to ensure the removal of any trace carbon that may interfere with the stable carbon isotope analysis. Approximately 300 grams of dried sediments (100 g per replicate) for each station were homogenized with a mortar and pestle. Once homogenized, the sediments of each of the ten stations were separated in three aliquots (n=3) in PTFE tubes for lipid extraction. A 1:1

acetone:hexane mixture was used for the lipid extractions by sonication in an ultrasound bath (Fisher Scientific FS60H), followed by centrifugation at 9000 rpm (IEC-HN-S). Following sonication, ultrapure water was added and the organic layer containing the total lipid fraction was transferred to conical 50-mL glass tubes.

3.3.6. Separation of Lipids by Silica Gel Chromatography

The organic phase was evaporated under a gentle N_2 stream to about 1 mL and separated by silica gel chromatography, using about 1.5 cm^3 of sodium sulphate added on top of the silica to remove any trace of water remaining in the samples. The lipids were separated in three fractions of increasing polarity using hexane (straight, cyclic and branched hydrocarbons), 25% toluene in hexane (aromatic hydrocarbons) and methanol (neutral and polar lipids). Only the last lipid fraction was used in this work.

3.3.7. Saponification and Liquid-Liquid Extraction

The neutral and polar lipid fraction was evaporated to dryness under a gentle N_2 stream and then saponified using a solution of methanol:potassium hydroxide at pH=14 at 100ºC in a sand bath for 3 hours. The neutral lipids (alkanols and sterols/stanols) were extracted from the mixture by liquid-liquid extraction (dichloromethane and 5% sodium chloride (W/V), 3 times and preserved for future work). The samples were then acidified to pH < 1 with 12 M hydrochloric acid and the protonated fatty acids were then liquid-liquid extracted as above. To ensure the complete removal of water, the free fatty acid fractions were dried on a short column of solid sodium sulfate. The samples were then evaporated to almost dryness before derivatization.

3.3.8. Derivatization to Fatty Acid Methyl Esters (FAME)

The free fatty acids were methylated using 0.5 mL of a boron trifluoride-methanol solution (BF_3-MeOH) (Supelco, CAS 373-57-9). Derivatization was done in a completely anhydrous environment to avoid incomplete reaction. The solutions were heated to 100ºC in a sand bath for one hour to ensure complete conversion to methyl esters. The fatty acids methyl esters (FAMES) were then recovered by liquid-liquid extraction using hexane and a 5% sodium chloride solution and dried on another Na_2SO_4 drying column. The samples were concentrated to a volume between 75 and 500 µL (depending on the expected concentrations) under a gentle N_2 stream and stored at -20ºC until analysis.

3.3.9. Fatty Acids Identification

The fatty acids were identified using the Supelco-37 FAMES standard (Sigma-Aldrich, CRM47885) and *anteiso* 15:0 Fatty Acid (Avanti, Sigma-Aldrich, CAS 5746-58-7), as well as by

gas chromatography (Agilent 7890B) coupled with a mass spectrometer (Agilent 5977B), equipped with an Agilent DB5-MS 50 m x 0.20 mm x 0.33 µm column). The analyses were carried out under constant flow (1.5 mL min^{-1}) and used an optimized temperature gradient for the separation of the compounds. The carrier gas used was helium. The samples from each station were analyzed by GC-FID and GC-MS to identify the peaks of interest and to ensure there was no co-elution.

3.3.10. Analysis of Stable Carbon Isotopes in Bulk Samples

An EuroVector Elemental Analyzer (EuroEA3028-HT) coupled to an Isoprime 100 isotope ratio mass spectrometer (IRMS; Elementar Americas Inc.) was used to determine the bulk stable carbon isotope signatures of the sediments and the various broths. Each sample (bacterial broth or sediment) was freeze-dried and weighed in quadruple. The sediments were decarbonated by fumigation (Stern et al., 1984) prior to analysis. Each sample in silver capsules was wrapped within a tin capsule. The certified standard material Sucrose (IAEA-CH-6; $\delta^{13}C$ = -10.45 ± 0.03 ‰) and Polyethylene (IAEA-CH-7; $\delta^{13}C$ = -32.15 ± 0.04 ‰), as well as β-alanine and sucrose, two in-house standards calibrated with these certified reference materials (Sigma-Aldrich; $\delta^{13}C$ = -26.18 ± 0.10 ‰ and -11.77 ± 0.09 ‰, respectively), were used for calibration purposes. The stable carbon isotope ratios are expressed relative to the international reference standard VPDB. Precision and accuracy of the measured stable isotope signatures were assessed using a range of masses of the pure in-house calibrated compound (β-alanine) and IAEA certified sucrose covering the entire dynamic range of the instrument.

3.3.11. Compound-Specific Stable Carbon Isotope Analysis

Compound-specific stable carbon isotope analyses were carried out on an Agilent Gas Chromatograph (Agilent 6890N) coupled to an Isoprime 100 IRMS (Elementar Americas Inc.). The column used for peak separation was a DB-5MS (50 m, 0.20 mm, 0.33 µm; Agilent 128-5552). The purity of each sample peak was verified by GC-MS to check for co-eluting compounds that could affect the measured stable isotope signature. The GC method was optimized by adjusting the injection mode (splitless), column flow (constant at 1.5 mL min^{-1}) and temperature gradient. All peaks considered in this work were baseline separated. The calibration curve and quality control standards were prepared with the Fatty Acid Mixture F8-2 and n-Alkane Mixture A7 (both supplied by Arndt Schimmelmann, University of Indiana, USA) as their stable carbon isotope signatures are known. A correction factor was obtained from the 5-point calibration (5, 10, 20, 50, 100 mg L^{-1}) of measured *vs.* known FAMES and alkanes values. The samples were bracketed by a calibration and corrected accordingly to avoid instrumental drift. All values reported for stable isotopes are in per mil (‰) and relative to the international reference standard for stable carbon isotopes VPDB.

3.4. Results

3.4.1. Calibration Assessment of the Enriched Broth: Are Bacteria What They Eat?

Bacteria were cultivated in a ^{13}C-enriched medium to assess the relationship between the $\delta^{13}C$ signature their source of carbon and that of their specific fatty acids. To verify the accuracy of the calculation when preparing the cultivation medium using ^{13}C-enriched acetate, the calculated $\delta^{13}C$ values of each broth enriched at different levels were plotted against the measured bulk $\delta^{13}C$ values obtained via EA-IRMS. The relationship between the measured and calculated $\delta^{13}C$ values is linear with a slope of 0.92 and intercept of -3.25 (R^2= 0.9986). Interestingly, the measured $\delta^{13}C$ values of the bacterial broth were more depleted than the calculated theoretical values (Table 4), which suggests that the purity of the enriched acetate product used in this work was not as high as stated by the provider. The difference was small however and had no impact on the experiments carried out in this work.

Table 4. Bulk stable carbon isotope signatures for the bacterial broth enriched with different proportion of sodium acetate-1-^{13}C, broth #1 not enriched to broth #5 as being the most enriched

Broth Number	Calculated $\delta^{13}C$[a] (‰)	Measured broth $\delta^{13}C$[b] (‰)	isoC15:0 $\delta^{13}C$ (‰)	anteisoC15:0 $\delta^{13}C$ (‰)	C12:0 $\delta^{13}C$ (‰)	C14:0 $\delta^{13}C$ (‰)	C16:0 $\delta^{13}C$ (‰)
1	-36.50	-35.99 ± 0.12	-35.78 ± 0.72	-34.27 ± 0.14	-36.37 ± 0.20	-35.01 ± 0.25	-34.58 ± 0.45
2	-31.00	-31.81 ± 0.04	-31.38 ± 0.42	-30.74 ± 0.41	-31.38 ± 0.15	-31.21 ± 0.09	-30.90
3	-26.00	-27.35 ± 0.22	-29.04 ± 0.21	-28.02 ± 0.20	-27.54 ± 0.18	-26.90 ± 0.22	-
4	-16.54	-18.69 ± 0.14	-21.39 ± 0.79	-21.87 ± 0.60	-19.16 ± 0.96	-17.75 ± 0.23	-17.38 ± 0.12
5	-11.65	-13.90 ± 0.07	-16.46 ± 0.64	-17.26 ± 0.21	-13.93 ± 0.54	-	-

[a] Theoretical signature based on the mass and purity of acetate-1-^{13}C used in the preparation, [b] n=4, the standard deviations show the precision between the measurements

A calibration curve was then built to find the relationship between the $\delta^{13}C$ signature of the food source (bulk) and that of specific individual fatty acids. In this experiment, the bacteria's sole carbon source for fatty acid synthesis was coming from sodium acetate (labelled ^{13}C and unlabelled) that was added to the cultivation broth. There was a positive linear relationship between the $\delta^{13}C$ signature of both bacteria-specific fatty acids (*iso* and *anteiso* C15:0) of *A.*

macleodii and that of the broth (Figure 8), with slopes of 0.85 and 0.75, respectively, different from unity. The standard deviations for measurements of compound-specific $\delta^{13}C$ of *iso* C15:0 and *anteiso* C15:0 varied between 0.21-0.79 ‰ and 0.14-0.60 ‰, respectively.

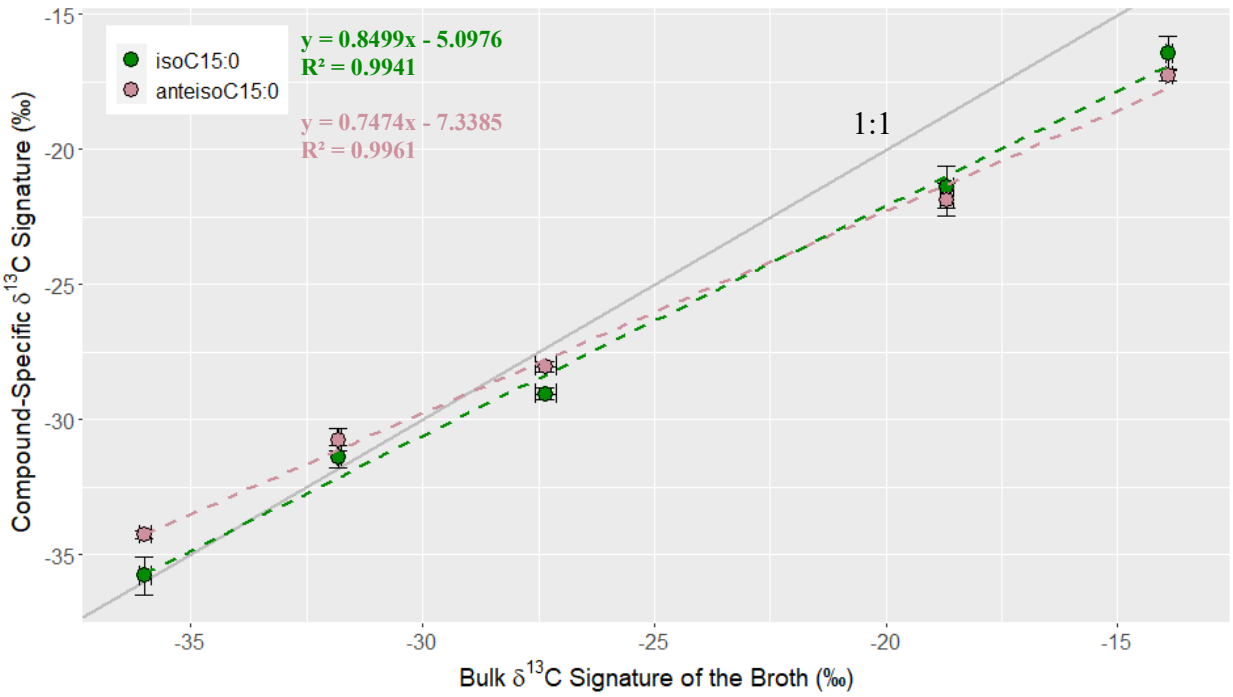


Figure 8. Relationship between the compound-specific $\delta^{13}C$ signature for *anteiso* C15:0 and *iso* C15:0 at different enrichment levels of the cultivation broth. The standard deviations show the precision between the measurements

The $\delta^{13}C$ of other fatty acids were also analyzed to investigate whether the same relationship as for *iso* and *anteiso* C15:0 existed between the bulk and the compound-specific $\delta^{13}C$ signatures. The $\delta^{13}C$ signatures of the straight-chain C12:0 (lauric acid), C14:0 (myristic acid) and C16:0 (palmitic acid) fatty acids varied linearly with bulk $\delta^{13}C$ signatures of the broth (Figure 8), with slopes very close to unity, intercepts near zero and R^2>0.99 (Table 4). There is a significant difference between the slopes of the straight-chain fatty acids and the *iso*/*anteiso* forms.

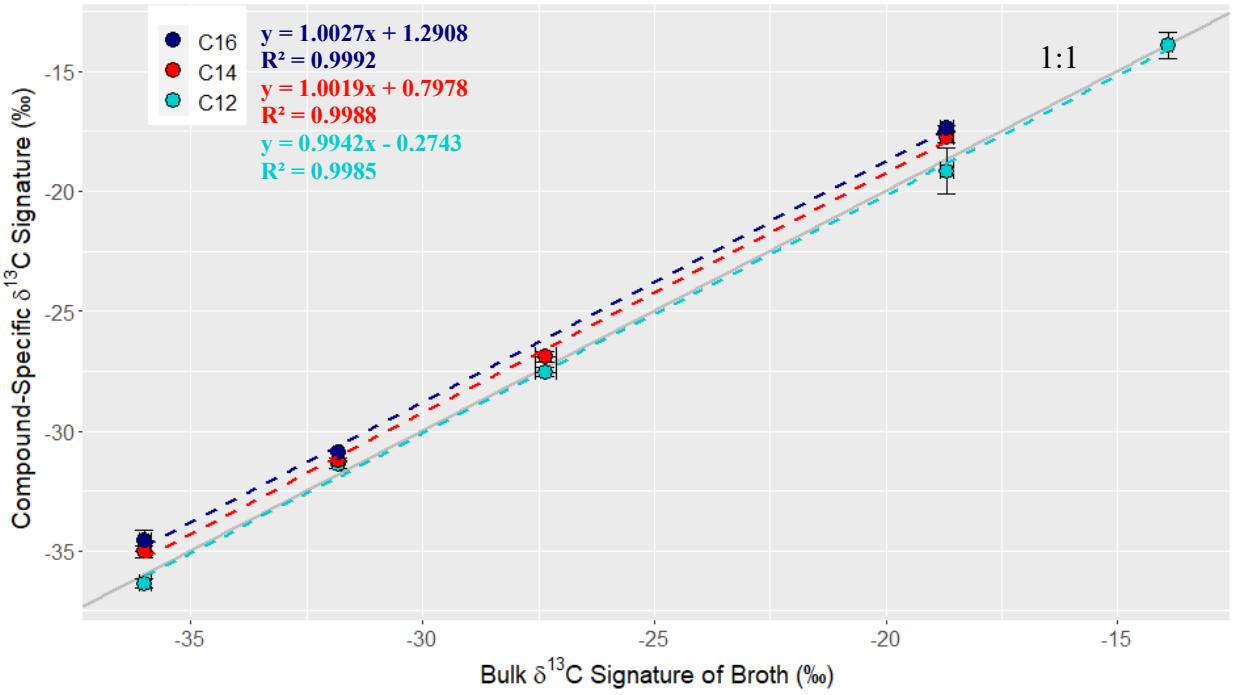


Figure 9. Relationship between the δ^{13}C signature of the straight-chain fatty acids C12:0, C14:0 and C16:0 with that of bulk ^{13}C-enriched broth for *A. macleodii*. The standard deviations show the precision between the measurements

3.4.2. Analysis of the Fatty Acids Extracted from Sediments of the St. Lawrence Estuary and Gulf

The δ^{13}C signature of bulk sedimentary OM was measured at each of the ten stations to assess its origin (terrestrial vs. marine). The signatures varied from -22.39 ± 0.09 to -27.33 ± 0.02 ‰ (n=4). As shown on Figure 10 the δ^{13}C signatures are getting progressively more enriched in ^{13}C along the terrestrial-marine continuum. Stations DE, G and Sag5 are located near the discharges of the St. Lawrence and the Saguenay Rivers and receive predominantly terrestrial OM leached from plants and soils. At the opposite end of the continuum, the marine stations 19 to 16 are located in the St. Lawrence Gulf and thus a higher proportion of organic matter originating from plankton. The δ^{13}C signatures of bulk organic matter at these more marine stations plateau at between -22.39 ± 0.09 to -22.59 ± 0.04 ‰, and are not significantly different from one another (p=0.37). All δ^{13}C are summarized in Table 5.

The δ^{13}C signatures of the bacteria specific fatty acids also get more enriched along the continuum (Figure 10), but with an offset of between about 1 to 2.5 ‰ towards more depleted values. Similar to the signature of bulk organic matter, a gradual enrichment was found between stations DE and 19, but then the signatures decreased between stations 19 and 16 instead of reaching a plateau. This depletion is more pronounced for the *anteiso* C15:0 fatty acid.

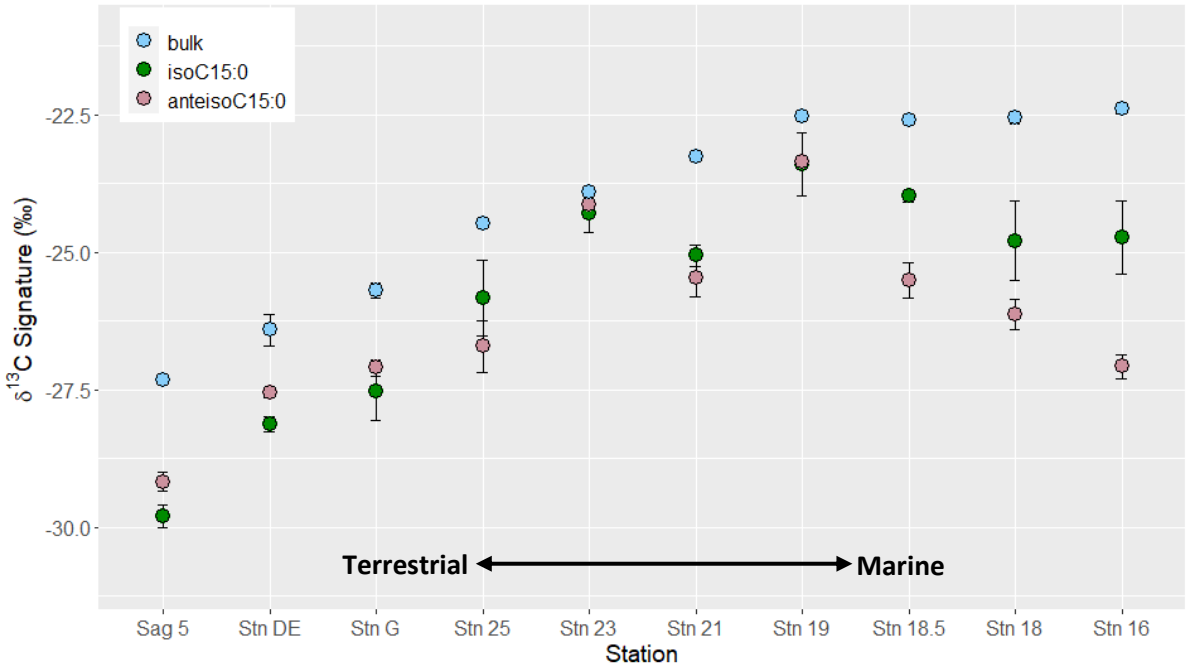


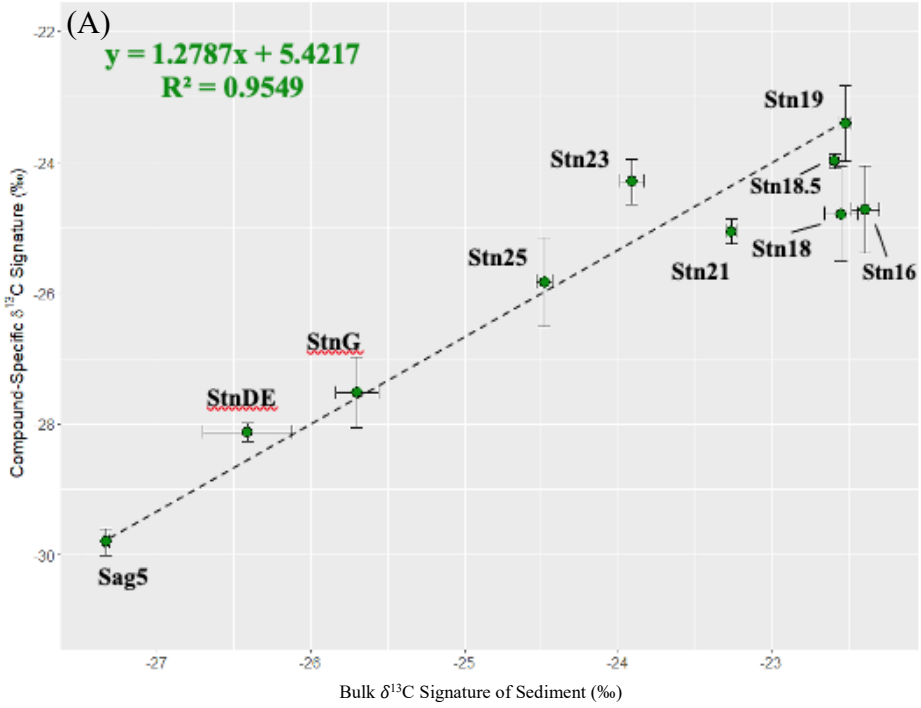
Figure 10. Compound-specific δ^{13}C of *anteiso* C15:0 and *iso* C15:0 and δ^{13}C signature of bulk sedimentary organic matter collected at the different sampling stations. The stations closer to land are on the left-hand side while those located in the gulf are toward the right side. The standard deviations show the precision between the measurements.

Table 5. Summary of the bulk δ^{13}C and the two branched fatty acids (*iso* and *anteiso* C15:0) along the 10 sampling stations

Stations	Bulk δ^{13}C (‰)	*iso* C15:0 (‰)	*anteiso* C15:0 (‰)
Sag5	-27.33 ± 0.02	-29.80 ± 0.20	-29.18 ± 0.17
DE	-26.41 ± 0.29	-28.13 ± 0.14	-27.56 ± 0.09
G	-25.7 ± 0.14	-27.52 ± 0.54	-27.10 ± 0.15
25	-24.48 ± 0.05	-25.83 ±0.68	-26.71 ± 0.48
23	-23.91 ± 0.08	-24.30 ± 0.34	-24.12 ± 0.07
21	-23.26 ± 0.03	-25.06 ±0.19	-25.46 ± 0.35
19	-22.52 ± 0.04	-23.40 ± 0.58	-23.36 ± 0.09
18.5	-22.59 ± 0.04	-23.98 ± 0.11	-25.51 ± 0.32
18	-22.55 ± 0.11	-24.79 ± 0.73	-26.13 ± 0.27
16	-22.39 ± 0.09	-24.72 ± 0.67	-27.08 ± 0.23

3.4.3. Bacteria-specific Fatty Acids

To decipher the relationship between bulk sedimentary OM and endogenous bacteria, the $\delta^{13}C$ signatures of the bulk sedimentary OM and that of the *iso* and *anteiso* C15:0 fatty acids were compared (Figures 11A and 11B). For both fatty acids, a positive relationship with slopes of 1.28 (*iso*, $R^2 = 0.955$) and 1.09 (*anteiso*, $R^2 = 0.871$) was found along the terrestrial-to-marine transition portion of the continuum (stations Sag5 to 19). This relationship breaks down in the Gulf of St. Lawrence for the more marine stations (18.5 to 16) where a near constant value (*iso* C15:0) or a decrease (*anteiso* C15:0) in $\delta^{13}C$ signatures of the fatty acids to more depleted values is evident. The data for the anteiso fatty acid is also more scattered than for the iso C15:0 acid.



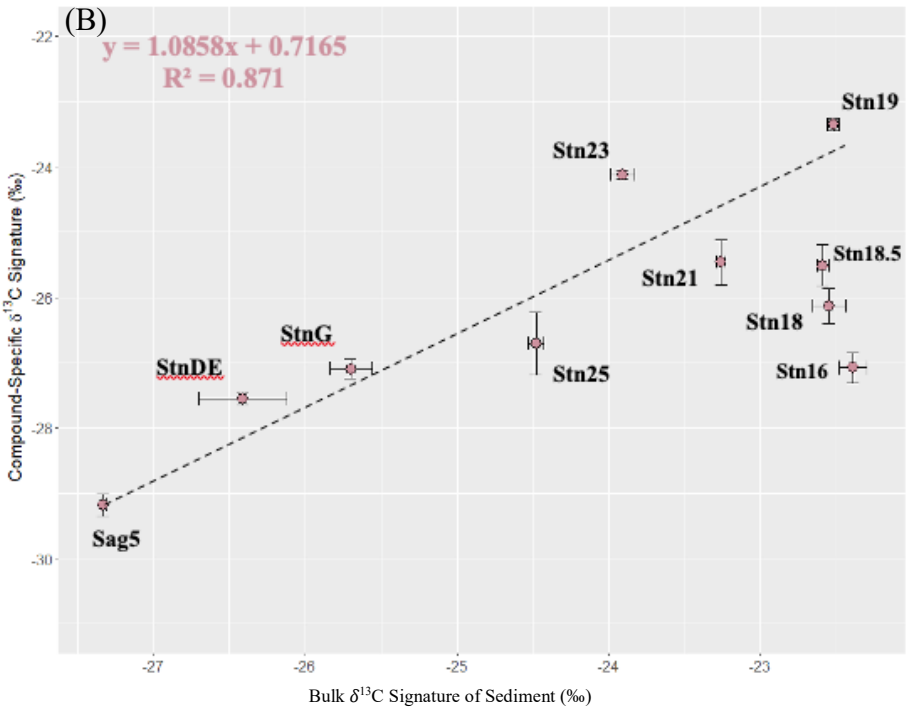


Figure 11. Relationship between the bulk δ^{13}C signature of sedimentary organic matter at different stations and those of (A) the *iso* C15:0 fatty acid, and (B) the *anteiso* C15:0 fatty acid. The standard deviations show the precision between the measurements

3.5. Discussion

The approach used in this research was to cultivate the ubiquitous marine bacteria *Alteromonas macleodii,* mainly found in the Mediterranean and North Atlantic Ocean, in a controlled environment containing only 1-^{13}C-sodium acetate as carbon source to comprehend if the δ^{13}C signature of the "food" source is correlated to their fatty acids δ^{13}C signature (Garcia-Martinez et al. 2002). Then, this concept was applied to analyse the δ^{13}C signature of bacterial fatty acids extracted from sediments collected along the continuum (10 stations) of the SLEG.

3.5.1. Cultivation Study

The data obtained from the enrichment cultivation study of the marine *A. macleodii* shows that bacteria generally "are what they eat" with respect to their fatty acids δ^{13}C, although with an offset. As shown in Table 4, the calculated δ^{13}C signature of the bacterial broth is more enriched than the measured δ^{13}C values, which we assume is due to a slightly lower purity of the sodium acetate-1-^{13}C compared to the information provided by the supplier. When calculating the mass of sodium acetate-1-^{13}C needed to get a given enrichment level, it was assumed that the labelled sodium acetate was made up of 99% purity sodium acetate-1-^{13}C with only one of the two carbons of the molecule being labelled with ^{13}C. Because the measured δ^{13}C signature of the broth is

depleted relative to the targeted values, it is likely that the labelled acetate was not as pure as the 99% announced on the product label. These deviations did not affect the results of the study but show that using stable isotope analysis is delicate and it is critical to pay attention to these small details.

To assess the hypothesis that the stable carbon isotopic signatures of the carbon food source are reflected in the fatty acids of bacteria, the $\delta^{13}C$ signatures of the bulk bacterial broth were compared to the $\delta^{13}C$ signatures of the individual bacterial branched fatty acids *iso* and *anteiso* C15:0 and some ubiquitous straight-chain fatty acids such as lauric, myristic, and palmitic acids (C12:0, C14:0, and C16:0, respectively). The relationship between the bulk and bacterial derived fatty acids should be close to unity if our hypothesis is correct. However, it was found that the slope between the $\delta^{13}C$ of branched fatty acids (*iso* and *anteiso* C15:0) and the bulk $\delta^{13}C$ of the bacterial broth (Figure 8) is lower than unity. The confidence intervals for the regressions overlap at α=0.05, signifying no statistically significant difference between the slopes. This difference suggests that the $\delta^{13}C$ of the food source is not exactly reflected in the $\delta^{13}C$ of bacterial branched fatty acids. For the straight-chain even carbon numbered fatty acids, the slope is closer to unity indicating the $\delta^{13}C$ of the organic carbon food source is conserved during the fatty acid synthesis. The branched fatty acids are more depleted than the straight-chain fatty acids relative to the total organic carbon $\delta^{13}C$. This can, in part, be explained by the way that each fatty acid is synthesized. The *iso* and *anteiso* C15:0 are synthesized from the amino acid precursor leucine and isoleucine, respectively. Once the leucine or isoleucine is biosynthesized or uptaken from the environment via transporters, it has to go through an enzymatic reaction (Hosie & Poole, 2001; Sun et al., 2012). The leucine and isoleucine undergo branched-chain amino acid aminotransferase to remove the amine group of the amino acid, making an alpha-keto acid. The enzyme branched-chain alpha-keto acid dehydrogenase then modifies these alpha-keto acids to become isobutyryl-CoA and isovaleryl-CoA for the *iso* and *anteiso,* respectively. Finally, once in their coenzyme form, they can undergo chain elongation. However, for the straight-chain fatty acids, the precursor molecule is acetyl-CoA and undergoes the regular fatty acid synthesis chain reaction (Berg et al., 2015). The biosynthesis of the branched fatty acids requires additional steps compared to the straight fatty acid synthesis cycle. Therefore, more enzymes are needed to obtain the final product making the branched fatty acids more prone to enzymatic fractionation, which is then reflected in their $\delta^{13}C$ signatures. These enzymes discriminate between the lighter (^{12}C) and the heavier (^{13}C) isotopes and favour the lighter isotopes during synthesis as their utilization requires less energy (Fry, 2006). This kinetic enzymatic fractionation results in more depleted branched fatty acid $\delta^{13}C$ than their original carbon source, thus resulting in a slope lower than unity (Figure 8)

Some fatty acids may be incorporated directly, with or without minor modifications, from the food source to the microbial biomass and tend to retain a similar $\delta^{13}C$ signature as the bulk food source $\delta^{13}C$. However, branched fatty acids are *de novo* synthesized, thus supporting the argument that a greater number of enzymes are required for their synthesis and thus that the potential for more enzymatic fractionation is higher (Ruess & Chamberlain, 2010). In other controlled feeding experiments with fungi and microorganisms, studies have shown that most *de*

novo fatty acids undergo enzymatic discrimination and the stable carbon isotopic signatures of the fatty acids is dependent on the level of fractionation by the enzyme (Ruess et al. 2005; De Niro and Epstein, 1977). These studies showed that the difference was statistically significant between the $\delta^{13}C$ of *de novo* synthesized fatty acids and the bulk $\delta^{13}C$ of the food source. The results in this study agree with this work reporting that *de novo* fatty acids are more depleted relative to their organic carbon source. For common fatty acids such as C12:0, C14:0, and C16:0, there was no noticeable enzymatic fractionation and the $\delta^{13}C$ signature of food sources is reflected in the bacteria's C12:0, C14:0, and C16:0 signatures. In addition to using fewer enzymatic steps for the *de novo* synthesis of these fatty acids, they can be used exogenously from food sources via diffusion to the cell's membrane (Yao et al., 2016). However, using C12:0, C14:0, and C16:0 as biomarkers to assess the lability of organic matter is not ideal as they are ubiquitous and are found in most living organisms.

Interestingly, the correlation between the bulk broth $\delta^{13}C$ signatures and the *iso* and *anteiso C15:0* is not identical (0.85 and 0.75, respectively; Fig. 8). While there is a difference in the regressions, they are not significantly different. Again, the confidence interval around the regression overlapped (α=0.05) indicating that the slopes are not significantly different. This may make it tempting to use the two interchangeably as is often done in studies such as observing the changes of allochthonous and autochthonous organic carbon in a lake, assessing the bacterial contribution to total OM in soils or sediments, or simply characterizing the sources of OM in a river (Arnold et al., 2019; Zhang et al., 2015). However, these stable carbon isotope results suggest that the two branched fatty acids may reflect different sources or reaction histories in more complex settings such as in the St. Lawrence water way.

3.5.2. Bulk Sedimentary Organic Matter $\delta^{13}C$ Signatures Along the Terrestrial-Marine Continuum

Many biogeochemical studies use bulk $\delta^{13}C$ to determine the origins of OM found in sediments (Hedges & Parker, 1976; Yu et al., 2010). The SLEG represents an interesting study site for such analyses owing to the freshwater to saltwater gradient along its course and the distinct sources of OM. The analysis of the bulk sediment $\delta^{13}C$ signatures along this continuum (Figure 10) revealed a linear trend with values being more enriched from station DE to station 19 and then plateauing from station 19 to 16. These results reflect the fact that stations closer to Quebec City receive higher inputs of terrestrial OM from the river and land runoff, and thus OM accumulating in sediments shows $\delta^{13}C$ signatures indicative of terrestrial OM. At stations more distanced from the river and closer to the Gulf, the sediment OM is more enriched in ^{13}C, indicating a change in proportion of the terrestrial OM and marine OM along the transect towards the Gulf. The sedimentary OM at the four stations located in the Gulf is characterized by similar bulk $\delta^{13}C$ signatures of about 22.5 ‰ (-22.39 ± 0.09 to -22.59 ± 0.09 ‰) implying that the organic matter at these stations originate from the same general source. The most depleted $\delta^{13}C$ signatures are found at Sag5 (-27.33 ± 0.04 ‰) located in the Saguenay Fjord, which, because it is surrounded by forested land, receives the majority of its inputs as OM derived from soils and terrestrial plants.

3.5.3. CSIA of the *Iso* and *Anteiso* C15:0 Fatty Acids at the Different Sampling Stations

The compound-specific stable carbon isotope analysis of *iso* and *anteiso* C15:0 along the 10 stations of the SLEG show a regular enrichment from stations DE to 23 (Figure 11). This result was expected as the OM source is changing along the freshwater-sea water continuum, as shown above. Thus, the stable carbon isotope signatures of the branched fatty acids follow the same trend as the $\delta^{13}C$ values of bulk sediment OM by becoming more enriched in ^{13}C seaward. However, unlike the sediment bulk $\delta^{13}C$ measurements at the marine stations (from stations19 to 16), no plateau is observed. Another interesting observation is that there is a break in the quasi-linear enrichment trend seen at station 21. A clearly more depleted $\delta^{13}C$ signature was found for *iso* and *anteiso* C15:0 compared to what is predicted from the regression. This could be partially explained by the geographical location of station 21 as it is located downstream of three major rivers on the north shore of the estuary (Manicouagan River, des Outardes River, and Betsiamites River). These rivers drain the boreal forest and have a high flow rate particularly in the spring, likely leading to higher inputs of terrestrial OM at this station compared to neighboring stations. These fresh terrestrial inputs likely are readily degraded and as a result, the $\delta^{13}C$ signature of bulk OM accumulating in the first 10 cm of the core is in line with the regression found for stations DE to 19.

Interestingly, the $\delta^{13}C$ signatures of *iso* and *anteiso* do not behave similarly along the transect. At the more terrestrial stations, the *iso* C15:O is depleted in ^{13}C relative to the *anteiso* C15:0, whereas at the more marine stations, the reverse is true; the *anteiso* C15:0 is more depleted relative to the *iso* C15:0. The $\delta^{13}C$ signatures of *iso* and *anteiso* C15:0 differ in the Gulf with a difference as high as 2‰, which supports the argument that both forms should not be used interchangeably. The *anteiso* form of fatty acids is synthesized from isoleucine while the *iso* form is derived from leucine, thus leading to different enzymatic fractionation because of the different pathways and enzymes involved in the process (Tang et al., 2009). However, this explanation goes against the results found in the cultivation experiment of *A. macleodii*, where the difference between the *iso* and *anteiso* is minimal. This suggests that the synthesis of these fatty acids in the sediment is more complex and could be affected by different factors (e.g., slightly different substrates, different bacterial communities, etc.). In the gulf (stations 19-16), the $\delta^{13}C$ signatures of both *iso* and *anteiso* C15:0 fatty acids become increasingly more depleted. This was unexpected as it suggests that bacteria degrade increasingly more terrestrial-like OM going seaward in the gulf. Even though the bulk sediment $\delta^{13}C$ signatures suggest that marine-like organic matter is predominant at these stations, this is a clear indication that bacteria preferentially degrade a sub-component of bulk sedimentary OM at these stations.

The main purpose of this study was to determine whether bacteria preferentially degrade a specific type of OM (terrestrial *vs.* marine) in the SLEG, thus providing clues on the relative biological lability of the two organic matter pools. To assess the relative biological lability of the two OM pools, we compared to the compound-specific $\delta^{13}C$ signatures of the branched fatty acids with those of bulk sediment organic matter at each station (Figure 11). We found a linear relationship between the $\delta^{13}C$ signatures of both *iso* or *anteiso* C15:0 and those of bulk sediment

OM along the terrestrially-influenced stations (Sag5 and DE to station 23, inclusively), with a slope slightly above unity. This covariation of the stable carbon isotope signatures of the branched fatty acids with those of the bulk sediment organic matter agrees with the results of Bourgeois et al. (2011) in the Rhône prodelta and adjacent shelf sediments in France. In their study, the compound-specific $\delta^{13}C$ signatures of the branched fatty acids were more depleted, and thus more terrestrial-like, near the river mouth than in areas where phytoplankton and algal primary production was higher and thus more marine-like (Bourgeois et al., 2011). In the St. Lawrence system, stations 18.5, 18, and 19 are located directly in the gulf where most of the OM inputs are attributed to marine primary production. However, the $\delta^{13}C$ signatures of the *iso* and *anteiso* C15:0 are significantly depleted relative to those of the bulk sediment OM. This is surprising result as we expected that the compound-specific $\delta^{13}C$ signatures of the branched fatty acid would follow the same trend as for the bulk sediments OM. One explanation for this apparent depletion of the branched fatty acids $\delta^{13}C$ signatures relative to those of bulk sedimentary OM at these marine stations could be due to the fact that the sedimentation rates are getting increasingly lower with distance from the river mouth owing to a lower discharge of terrestrial organic matter and a lower primary productivity in the water column. The Gulf of St. Lawrence is surrounded by forested land and thus the inputs of terrestrial organic matter through rivers and brooks as well as from the lower estuary are not negligible. The majority of the OM inputs are however derived from primary productivity in the surface waters, as suggested by the $\delta^{13}C$ signatures bulk OM at these stations. However, because of the low sediment accumulation rates, this marine OM has probably been extensively reworked, leaving behind the most refractory components of marine OM. The $\delta^{13}C$ signatures of the top 10 cm of bulk sedimentary OM represents a mixture of highly degraded organic matter that has accumulated in the past several decades, as confirmed by the high amino acid degradation index values found at these stations (Alkhatib et al., 2012). With most of the metabolizable marine OM degraded, bacteria might degrade an increasingly more important fraction of terrestrial OM, possibly explaining the disconnect between the compound-specific and the bulk $\delta^{13}C$ signatures.

Contrary to our initial working hypothesis, bacteria therefore do not appear to show a preference for marine organic matter, as best illustrated by the fact that the compound-specific $\delta^{13}C$ signatures for both bacterial fatty acids at station 16, the furthest station away from Quebec City in the gulf, were close to those of station G, where the OM is mainly derived from terrestrial inputs (Figure 11). Between stations DE and 23 (including Sag5), the stable carbon isotope signature of the bacterial fatty also closely reflected that of OM in surface sediment, which are composed of a mixture of terrestrial and marine organic matter.

3.5.4. Conclusion, Limitations/Future Works

The onset of hypoxia in the SLEG is a growing environmental and economic concern in Eastern Canada. Eutrophication and global warming of the ocean remain the main cause of hypoxia in this system (Jutras, et al., 2020a). The increase in the discharge of anthropogenic nutrients and organic matter from the watershed ultimately enhances bacterial OM degradation,

which eventually results in an increased consumption of dissolved oxygen. Knowing whether terrestrial or marine OM has proportionally a greater impact on oxygen consumption would help understanding the factors that drive and sustain hypoxia in this system, and most importantly, it could guide our efforts targeting remedial action that could be taken to decrease the pressure on dissolved oxygen levels in the deep waters of the SLEG. To our knowledge, this is the first time that the biological lability of sedimentary OM is assessed in this system using bacterial branched fatty acids and their stable carbon isotope signatures.

To better understand the relationship between bacteria and their food source in terms of stable carbon isotope signatures, other ubiquitous strains of marine bacteria should be cultivated in a cultivation broth enriched in ^{13}C as in this study. This would allow deciphering whether different strains of bacteria fractionate the stable carbon isotopes in the same way as *A. macleodii* when synthesizing their *iso* and *anteiso* C15:0 fatty acids. Incubation studies using controlled sources of OM could also better highlight the links between the $\delta^{13}C$ signatures of bulk sedimentary OM and the bacterial fatty acids, as would the analysis of surface sediment samples (0-1 cm) at different times of the year as the proportion of marine and terrestrial OM at each station varies owing to seasonal differences in primary productivity and in terrestrial OM discharge. Other (e.g., lignin oxidation products, stigmasterol, ß-sitosterol, campesterol, etc.) to better constrain the contribution of terrestrial and marine OM at each station.

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Chapter IV: Conclusions and Future Work

4.1. Conclusions

To the best of our knowledge, this project was the first-time stable carbon isotope analysis of bacterial specific fatty acids (*iso* and *anteiso* C15:0) were mapped along the terrestrial-marine continuum of the SLEG. The overall objective of this master's project was to investigate the biological lability of terrestrial and marine OM when used for bacterial growth, leading to their degradation. Our initial hypothesis was that marine OM would be more readily degraded by marine bacteria. A smaller project emerged when some issues arose during the sample preparation of the main project. This additional investigation emphasizes precautions needed during acid fumigation for the removal of carbonates in samples.

In the main project (Chapter III), the $\delta^{13}C$ signature of branched bacterial fatty acids such as *iso* and *anteiso* C15:0 were used as biomarkers to determine the source of OM degraded by bacteria. Through a cultivation study, it was found that the fractionation factor for straight-chain fatty acids and branched fatty acids were not the same. The $\delta^{13}C$ signature for straight-chain fatty acids did not show major fractionation and is related directly to the $\delta^{13}C$ signature of the organic carbon source. Fractionation is seen between the $\delta^{13}C$ signatures of the branched fatty acids and those of their organic carbon sources, but they are strongly correlated albeit with a slope lower than unity. This was explained by the different biosynthesis pathways of each type of fatty acid. Additionally, as shown before, *iso* and *anteiso* C15:0 are often used interchangeably in the literature. However, our research shows that this is not completely accurate when using complex samples such as sediments as the $\delta^{13}C$ signatures of the *iso* and *anteiso* C15:0 fatty acids were often significantly different in our sediment samples. Most importantly, the results showed that bacteria do not appear to have a feeding preference when it comes to the degradation of marine or terrestrial OM. The $\delta^{13}C$ of the *iso* and *anteiso* C15:0 followed the trend of the bulk $\delta^{13}C$ of sediment of the terrestrial-marine transect of the SLEG. However, at the marine stations, $\delta^{13}C$ of the branched fatty acids became increasingly more depleted, indicating that terrestrial OM was being preferentially degraded. The main hypothesis was not confirmed but resulted in new insights in the OM cycling in the St. Lawrence waterway.

In the second project (Chapter II), carbonate-free lake sediment samples were enriched with 1-^{13}C- sodium acetate and underwent 5 different treatments before the acid fumigation to investigate the reasons for the loss of sodium acetate during decarbonisation. This chapter reports work designed to highlight a potential caveat in studies making use of small organic compounds enriched in ^{13}C when decarbonisation by acid fumigation is needed before EA-IRMS. Our results suggest that acetate is lost during fumigation regardless of the treatment. We suggest using higher molecular weight labeled compounds where possible when doing enrichment studies. If carbonates are not important in the matrix of the process under study, it is suggested that the labeled compound be added after the acid fumigation step.

By understanding what bacteria preferentially degrade, this work provides better insight on the marine organic carbon cycle with respect to bacterial degradation, which leads to oxygen depletion in the SLEG. It also leads to a better understanding of the onset of hypoxia. Even though the contribution of global warming of oceans is now the major contributor to hypoxia in the bottom

waters of the estuary, our research could lead to targeted remedial solutions for the hypoxia in the estuary. Global warming is a bigger problem that needs to be addressed internationally, however, eutrophication is still an issue and can be dealt with nationally and even locally.

Future Works

Since the hypothesis of the main project was not confirmed, additional avenues of investigation arose. Future work would include $\delta^{13}C$ analysis of long-chain fatty acids (C20:0 and up), which are typical for vascular terrestrial plants. This would provide a more complete mapping of the 10 stations transect for most fatty acids found in sediments as it will help in the characterization of OM at these stations. Additionally, different strains of marine bacteria should be cultivated to assess the fractionation of the *iso* and *anteiso* C15:0 relative to the organic carbon source degraded by the bacteria. It would be interesting to cultivate other strains of marine bacteria to verify if the relationship between the stable carbon isotope signature of the fatty acids and the bulk organic carbon source is similar to the *Alteromonas macleodii.* The analysis of bacterial community structure at each station could be done to further understand what type of bacteria are degrading OM at particular locations. Incubation studies in controlled laboratory experiments could also be carried out to assess the thermodynamics and kinetics of terrestrial and marine OM degradation by these bacteria.

Lastly, the analysis of other biomarkers for terrestrial organic matter such as lignin oxidation products, β-sitosterol, and stigmasterol could be carried out to further assess the contribution of terrestrial organic matter at each station.

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