UNIVERSITY OF SOUTHAMPTON

FACULTY OF NATURAL AND ENVIRONMENTAL SCIENCES

School of Ocean and Earth Sciences

# Stable carbon isotope analysis of seawater samples: a new approach to assess $CO_2$ effects on the marine carbon cycle

by

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Thesis for the degree of Doctor of Philosophy

January 2017

# ABSTRACT

Stable isotope ratio analyses offer a unique opportunity to obtain information about ecosystem dynamics, patterns and processes. The anthropogenic contribution to the global atmospheric $CO_2$ rise through fossil fuel combustion, deforestation and other related human activities has changed the stable carbon isotope composition ($\delta^{13}C$) of the atmosphere over the past 200 years. Changes in the carbon isotopic patterns of terrestrial biosphere, lithosphere and oceans are also expected. The global ocean has been acting as a net sink for $CO_2$ emissions and although it moderates the climate, it is currently in a critical state of health. While the physico-chemical consequences (ocean acidification) of the increasing $CO_2$ uptake by the ocean are fairly well known, the perturbation to marine ecosystems and the related effects on biota still entail large uncertainties. This thesis investigates the feasibility of using measurements of $\delta^{13}C$ of seawater samples to increase our understanding of the biogeochemical responses of marine ecosystems to human $CO_2$ perturbation.

The isotopic composition of all the individual inorganic and organic carbon species from three long term mesocosm experiments (Sweden 2013, Gran Canaria 2014, Norway 2015) was determined. To have accurate and precise isotopic measurements, mass spectrometry instrument calibrations and method validation procedures were performed. Universal and inter-laboratory accuracy of the analysis was assessed by running standard materials provided by the International Atomic Energy Agency (IAEA, Vienna) and by the Scottish Universities Environmental Research Centre (SUERC) stable isotope laboratory, respectively. Precision and internal consistency was assessed from isotopic measurements of seawater reference materials from A.G. Dickson and D. Hansell for dissolved inorganic and organic carbon, respectively. A novel accurate, precise and rapid method, coupling a Shimadzu 5000A total organic carbon (TOC) analyser to an isotope ratio mass spectrometer (Thermo Scientific Delta V Advantage IRMS), was successfully developed in order to determine the $\delta^{13}C$ of dissolved organic carbon in seawater samples which, due to analytical challenges, is currently not a widespread technique.

The combination of isotopic and non-isotopic measurements from mesocosm experiments provided a holistic view of the biogeochemical mechanisms that affect carbon dynamics under different $CO_2$ conditions (up to 2000 ppm). A clear $CO_2$ response was detected in the isotopic datasets, but increased $CO_2$ levels had only a subtle effect on the concentrations of the dissolved and particulate organic carbon pools. Distinctive $\delta^{13}C$ signatures of the particulate carbon pool both in the water column and the sediments were detectable for the different $CO_2$ treatments and they were strongly correlated with the $\delta^{13}C$ signatures of the inorganic carbon but not with the $\delta^{13}C$ of the dissolved organic pools. Phytoplankton fractionation was positively affected by high $CO_2$ either because of the higher $CO_2$ availability or because of a shift in phytoplankton community composition, however, phytoplankton bloom intensity and evolution was independent of $CO_2$ concentrations and higher $CO_2$ levels had no significant effect on inorganic nutrient uptake or carbon production/consumption.

Overall this study proved the stable carbon isotope approach to be an effective tool for the assessment of the major biogeochemical interactions among individual compartments within the marine system opening the door to new interpretations for past, present and future changes of the global carbon cycle.

# Table of Contents

# List of Tables

# List of Figures

# DECLARATION OF AUTHORSHIP

I, Mario Esposito, declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

**Stable carbon isotope analysis of seawater samples: a new approach to assess $CO_2$ effects on the marine carbon cycle**

I confirm that:

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6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. None of this work has been published before submission

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# Acknowledgements

First of all, I would like to thank my supervisors, Prof. Eric Achterberg and Dr. Douglas Connelly for giving me the opportunity to undertake this PhD, for their guidance and for all the support and prompt help they provided during these past years.

The realisation of this project could not have been possible without the immense trust that both my supervisors have given to me when I had to operate and modify the very expensive laboratory equipment for testing analytical methods and undertake measurements of samples. I extensively thank them for letting me have a full and exclusive management of their state of the art laboratory here at NOCS.

I also would like to thank them for giving me the opportunity to actively take part in two of the KOSMOS experiment projects. Accordingly, I also thank the KOSMOS core team (Ulf Riebesell, Lennart Bach, Tim Boxhammer, Jan Czerny, Andrea Ludwig and Michael Sswat). I would like to thank the members of the KOSMOS consortiums (María Algueró-Muñiz, Dana Hellemann, Thomas Hornick, Jana Meyer, Alice Nauendorf, Paul Stange and Maren Zark) at the Sven Lovén Centre for Marine Sciences (Kristineberg, Sweden) and at Plataforma Oceánica de Canarias (PLOCAN) for sharing their data, knowledge and ideas during and following the mesocosm experiments. Special thanks go to JC (Jaw Chuen Yong) who collected all my samples during the Bergen mesocosm experiment.

At NOCS, I would like to thank Kate Peel for helping me to purchase the laboratory standards and consumables used for the analysis of my samples. Special mention goes to the lunch break Mediterranean crowd (Giuseppe, Katerina, Julian, Amani, Hachem, Maria, Ismael, Hector, Eleni) who managed to turn the strenuous and long period of write-up to a more relaxed and enjoyable time.

Many thanks to my friends who made Southampton a beautiful place to stay during the past years. Last but not least, I would like to thank my family for having, up to now, positively supported my decisions and choices on all occasions.

# Contribution of Authors

This section lists the contributions of each of the authors included on peer-reviewed and soon to be peer-reviewed manuscripts.

Chapter 2 is a review of the main techniques for the determination of stable carbon isotopes in aquatic samples. The chapter has been converted into a manuscript format and following revisions by both my supervisors (Eric Achterberg and Douglas Connelly), it has just been submitted to the journal *Analytica Chimica Acta*.

Chapter 3 describes the development of a new combined system for concentration and isotopic measurements of dissolved organic carbon in seawater samples. Based on Panetta et al. (2008) and Lalonde et al. (2014) systems I optimised the method and carried out all the required tests and analysis. My supervisors have extensively commented and reviewed both the practical and written work. We believe the chapter is publishable and it will be soon submitted as a technical note article to a still to be defined journal.

Chapter 4 and 5 present data from mesocosm experiments. Chapter 4 will be converted into a manuscript and published within the framework of the BIOACID II long-term mesocosm study as part of the PLOS collection although further work is required. Chapter 5 will be part of another collection within the "Impacts of $CO_2$ Perturbation on the Ecology and Biogeochemistry of Plankton Communities during a simulated upwelling event: A mesocosm experiment in oligotrophic subtropical waters" research topic of *Frontiers in Marine Biogeochemistry.* Both papers would benefit from the integration of additional biological data (phytoplankton community structure) which have been already requested.

# List of Acronyms and Abbreviations

CA: Calcium carbonate, laboratory standard for $\delta^{13}C$ analysis

CM: Carrara marble, laboratory standard for $\delta^{13}C$ analysis

CRM: Certified reference material

CS: Calcium carbonate, laboratory standard for $\delta^{13}C$ analysis

DIC: Dissolved inorganic carbon

DOC: Dissolved organic carbon

EA: Elemental analyser

GC: Gas chromatography

GF/F: Glass fiber filter

HTC: High temperature combustion

IAEA: International Atomic Environmental Agency

IAEA-600: Caffeine, international certified standard for $\delta^{13}C$ analysis

IAEA-CH-6: Sucrose, international certified standard for $\delta^{13}C$ analysis

ICP-OES: Inductively coupled plasma – optical emission spectrometer

IRMS: Isotope ratio mass spectrometry

KOSMOS: Kiel Off-shore Mesocosms for future Ocean Simulation

M (1-9): Mesocosm unit (numbered from 1 to 9)

MAB: Marble, laboratory standard for $\delta^{13}C$ analysis

NA: Sodium bicarbonate, laboratory standard for $\delta^{13}C$ analysis

NBS-18: Calcite, international certified standard for $\delta^{13}C$ analysis

NDIR: Non-dispersive infrared detector

NF: Sodium carbonate, laboratory standard for $\delta^{13}C$ analysis

NFL: Sodium carbonate in solution, laboratory standard for $\delta^{13}C_{DIC}$ analysis

NS: Sodium carbonate, laboratory standard for $\delta^{13}C$ analysis

NSL: Sodium carbonate in solution, laboratory standard for $\delta^{13}C_{DIC}$ analysis

PDB: Pee De Belemnite

PIC: Particulate inorganic carbon

POC: Particulate organic carbon

TOC: Total organic carbon

TPC: Total particulate carbon

V-PDB: Vienna Pee De Belemnite

WCO: Wet chemical oxidation

$\delta^{13}C_{DIC}$: Stable isotope signature of dissolved inorganic carbon

$\delta^{13}C_{DOC}$: Stable isotope signature of dissolved organic carbon

$\delta^{13}C_{PIC}$: Stable isotope signature of particulate inorganic carbon

$\delta^{13}C_{POC}$: Stable isotope signature of particulate organic carbon

$\delta^{13}C_{Sed}$: Stable carbon isotope signature of sediment trap material

$\varepsilon_{phyto}$: isotope fractionation between the inorganic carbon and phytoplankton

# Chapter 1: General Introduction

## 1.1 Anthropogenic perturbation to the global carbon cycle

The carbon cycle involves a complex series of processes that interconnect carbon compounds in different reservoirs of the environment. Carbon is the 4$^{th}$ most abundant element in the universe (Cameron, 1973) and its unique chemical properties make it a key component of all known life on Earth. Carbon is exchanged among the four major Earth system reservoirs (atmosphere, terrestrial biosphere, lithosphere and oceans) by physical and biological processes including incorporation of carbon dioxide ($CO_2$) into living matter through photosynthesis, return to the atmosphere through passive exchange and respiration, and storage through decay and deposition of dead organisms. Fluxes of carbon between each reservoir vary substantially, with carbon residence times within each compartment differing by several orders of magnitude (Archer & Brovkin, 2008). Long time-scales (millions of years) are generally needed to store carbon in geological reservoirs while carbon transfer between atmosphere, terrestrial biosphere and ocean can occur over much shorter periods (days to centuries). Human activities are currently altering the natural global carbon cycle. Fossil fuel burning, deforestation and overexploitation of natural resources are causing an exponential increase of the $CO_2$ concentrations in the atmosphere. Air records from ice cores and time series measurements of atmospheric $CO_2$ have shown an increase from about 280 part per million (ppm) before the industrial revolution in the 19$^{th}$ century to an average of 404.48 ppm recorded in December 2016 at the Mauna Loa observatory (http://www.esrl.noaa.gov/gmd/ccgg/trends/index.html). As $CO_2$ is considered to be one of the most abundant greenhouse gases, rises in its atmospheric levels are contributing to global warming. The combination of $CO_2$ and other greenhouse gases has caused an increase in the surface Earth temperature of 0.85 °C, over the period 1880-2012 (Hartmann et al., 2013) with associated harmful consequences for the environment including desertification, sea level rise, stronger storms and extreme events.

## 1.2 Anthropogenic $CO_2$ perturbation to the Ocean system

The Ocean performs numerous vital functions for planet Earth. It controls weather systems, provides food supplies and is a source of many commercially valuable products. It is well known that the Ocean is currently in a critical state of health. The oceans act as a climate modulator and today they have already absorbed more than 80 % of the heat added to the climate system and about 25 % of the $CO_2$ emitted by humans (Canadell et al., 2007). Each year approximately 90 Pg of carbon are transferred between the atmosphere and oceans (Prentice et al., 2001; Ghosh &

Brand, 2003; Houghton, 2007) through physico-chemical as well as biological processes (Figure 1-1).

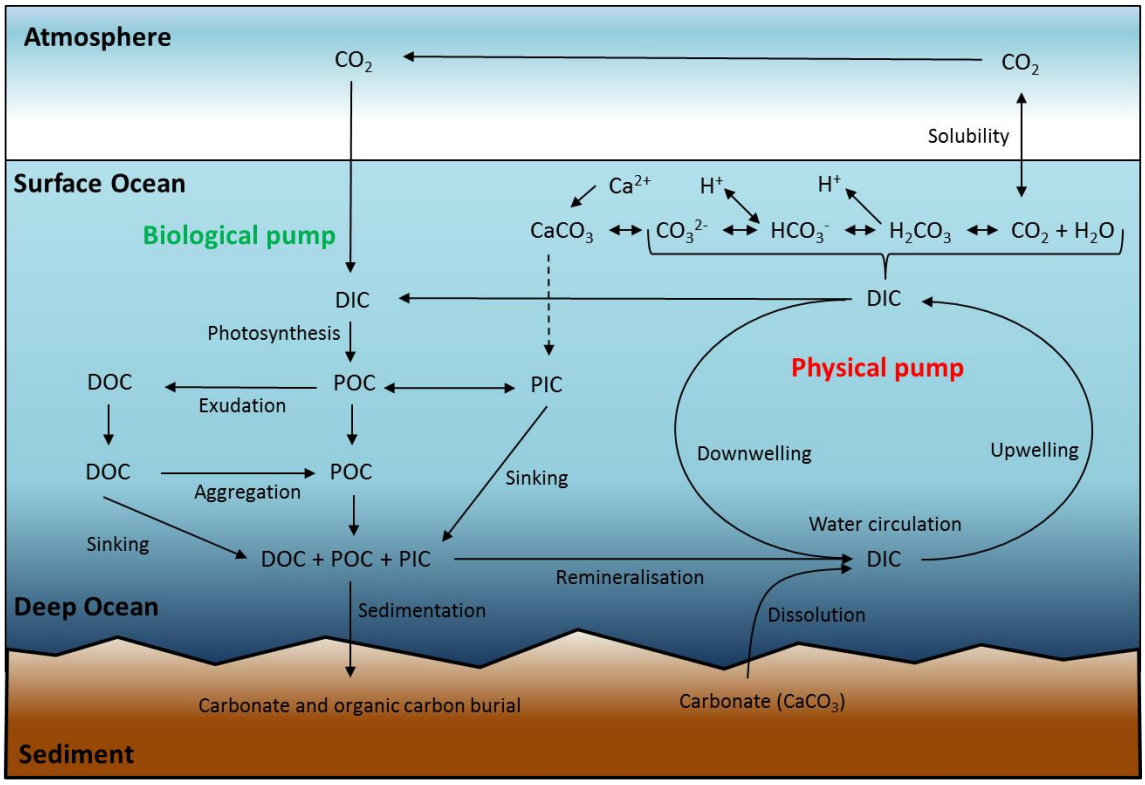


**Figure 1-1**. Schematic of carbon transfer mechanisms between the atmosphere and the ocean.

The physical dissolution of atmospheric $CO_2$ into the oceans through the solubility pump (Volk & Hoffert, 1985) is controlled by the partial pressure gradient of $CO_2$ ($pCO_2$) between the atmosphere and surface waters. Most of the $CO_2$ that moves into the ocean reacts with water molecules to form carbonic acid ($H_2CO_3$) which consequently dissociates into the forms of bicarbonate ($HCO_3^-$) and carbonate ($CO_3^{2-}$) ions. During the dissociation process, protons ($H^+$) are released and most of them react with $CO_3^{2-}$ to produce additional $HCO_3^-$ ions. As atmospheric $CO_2$ concentrations are increasing, ocean $CO_3^{2-}$ concentrations are reduced along with seawater pH (increase of $H^+$) due to a process known as ocean acidification (Caldeira & Wickett, 2003). The influx of anthropogenic $CO_2$ to the oceans has already caused a drop in surface seawater pH of 0.1 units (from about 8.2 to 8.1, total scale) since the onset of the industrial revolution and a further decrease of 0.3 to 0.4 units is predicted to occur by the end of this century under a business as usual scenario (Orr et al., 2005; Feely et al., 2009). The decrease in pH and available $CO_3^{2-}$ in the water column is believed to reduce the ability of some calcifying marine organisms to form solid calcium carbonate ($CaCO_3$) structures both as a consequence of increased $CaCO_3$ dissolution rates and because of potential physiological reduction of marine species calcification rates (Guinotte & Fabry, 2008). Changes in species distribution and abundance are therefore expected, with effects on marine ecosystems being still under investigation.

Ocean biology is acting as a second modulator for the climate system. In the sunlit surface ocean (euphotic layer), phytoplankton convert inorganic carbon into organic carbon assimilating it within their cells through the complex process of photosynthesis. The products resulting from the biological fixation of dissolved $CO_2$ include particulate organic carbon (POC) which consists mainly of phytoplankton aggregates and zooplankton faecal pellets, particulate inorganic carbon (PIC) which consists of $CaCO_3$ structures produced by planktonic calcifying organisms and dissolved organic carbon (DOC) which is mainly a by-product of POC decomposition. While about 80 % of the produced biomass is estimated to be converted back into $CO_2$ via community respiration in the euphotic zone (Andersson, 2004), between 0.3 and 30 % of it escapes remineralisation and is transported to depth as sinking biogenic particles (Lutz et al., 2002). During the descent the majority of the particles are re-mineralised by bacteria and other heterotrophic organisms releasing $CO_2$ into the surrounding water. On average about only 1 % of carbon derived from the primary production reaches the seafloor (depths greater than 1.5 km) and becomes incorporated into marine sediments (Lutz et al., 2002). This vertical transport of organic carbon is known as the biological carbon pump (Volk & Hoffert, 1985). It has been estimated that, globally, the biological carbon pump exports below the photic zone between 5 and 13 Gt of carbon per year (Henson et al., 2011; Laws et al., 2011) and without it, atmospheric $pCO_2$ concentrations would be more than the double what they are today (Maier-Reimer et al., 1996).

Three main mechanisms control the efficiency of the biological pump: primary production in the euphotic zone, export to depth and remineralisation rates during the descent. The balance between these mechanisms regulates the vertical transport of carbon in the ocean and its sequestration at depths with direct consequences on the concentrations of $CO_2$ in the atmosphere. Primary production and export contribute to an increase in ocean $CO_2$ sequestration from the atmosphere while community respiration tends to decrease it. Moreover, it has been postulated that anthropogenic perturbation has a direct effect on photosynthesis with an observed enhanced inorganic carbon consumption relative to nutrient uptake under high $CO_2$ levels (Riebesell et al., 2007). The enhanced carbon uptake was reflected in an increased export although no increased organic matter production was observed (Schulz et al., 2008). Interestingly, de Kluijver et al., (2010) found an opposite response to $CO_2$ increase: no indication of enhanced sinking during the bloom phase although an increase in phytoplankton biomass was observed in the surface layer. Several other studies have been carried out in order to understand the effect of rising $CO_2$ on primary production and export, however, interpretation of the data has led in some cases to conflicting results.

The impacts of increasing $CO_2$ on the marine carbon cycle and the quantification of the anthropogenic perturbation of the ocean system therefore still entail large uncertainties. The challenge with ecosystem response prediction is that it requires sufficient understanding of small

and large scale processes which can only be revealed through the combination of a vast array of accurate *in situ* measurements. Because anthropogenic activities are directly and indirectly leading to a number of amplification and feedback mechanisms involving changes in $CO_2$ concentrations both in the atmosphere and oceans, carbon is the key element to study. Estimations of the infiltration of anthropogenic $CO_2$ into the ocean started more than two decades ago through assessment of the changes in dissolved inorganic carbon (DIC) concentrations (Gruber et al., 1996; Sabine et al., 1999) and stable carbon isotope ratios (Gruber et al., 1999; Sonnerup et al., 1999; Quay et al., 2007). However, none of the previous studies appeared to have linked the variations in the concentrations or isotopic signatures of DIC to potential changes in the other carbon species present in the oceanic system. The global difference in pool size of the various carbon species (DIC pool is about 50 bigger than DOC pool) could be the reason, however, detection and quantification of local variations might allow us to gain further insights of specific biogeochemical interactions in the ocean system. Accurate measurements of carbon concentrations and stable isotopes in all its forms (inorganic and organic, dissolved and particulate) are fundamental to obtain a firm understanding of the whole picture. In particular, isotope measurements allow detailed nuanced views of elemental cycling and provide a different perspective to assess biogeochemical connections that often leads to new discoveries (Fry, 2006). In a coastal environment study, Currin et al. (2003) showed through stable isotope analysis that in tidal marshes, seagrass, although highly abundant, is not always the dominant food web supporting source and different contribution for saltmarsh and phytoplankton to the DOC and POC pools can be found.

## 1.3 Stable Isotopes: mixing and fractionation

Isotopes are forms of the same element that differ in the number of neutrons in their nucleus. The nucleus of each atom contains protons and neutrons. While the number of protons defines the element and the sum of the protons and neutrons gives the atomic mass, the number of neutrons defines the isotope of that element. Today we know that all elements have one or more isotopes. While radioisotopes decay or change into new elements with time, others stay in the same form remaining stable over geological time scales. Persisting as such, stable isotopes provide a way to directly trace the origin and follow the circulation of elements in our Earth's system (Fry, 2006). The stable isotope composition of the various elements is expressed as a difference measurement. The commonly used term is the δ value which denotes a part per thousand (‰) difference from a standard. The values are expressed as:

$$\delta^H X = \left[\left(\frac{R_{sample}}{R_{standard}}\right) - 1\right] * 10^3 \quad (1.1)$$

where the superscript H represents the heavy isotope mass of the element X, and R represents the ratio of the heavy isotope to the light isotope for the element of interest. The multiplication by 1000 is a simple amplification of the value, as isotope differences found in nature are in general very small.

In the natural world, stable isotopes are in continuous movement separating and recombining within the atmosphere, terrestrial biosphere, lithosphere and oceans. The two processes of mixing and fractionation control isotope cycling and circulation. In any thermodynamic reaction there is an isotopic distribution of elements according to the rates of reaction of the different molecular species. The same is valid for biochemical reactions in which similar molecules of slightly different mass react at different rates (Peterson et al., 1987). The formation and breaking down of chemical bonds leads therefore to an uneven distribution of one isotope over the other according to their weights. Overall, for either bond formation or rupture, less energy input is needed for the light isotopes, accounting for the important fractionation rule that lighter isotopes react faster (Peterson et al., 1987; Fry, 2006). Although the continuous mixing and fractionation of isotopes within the environment could lead to think of a chaotic distribution, Earth's reservoirs naturally exhibit characteristic isotopic patterns. Peterson & Fry (1987) schematised the carbon isotopic signature ($\delta^{13}C$) of the main natural reservoirs through a general overview of the carbon cycle (Figure 1-2).

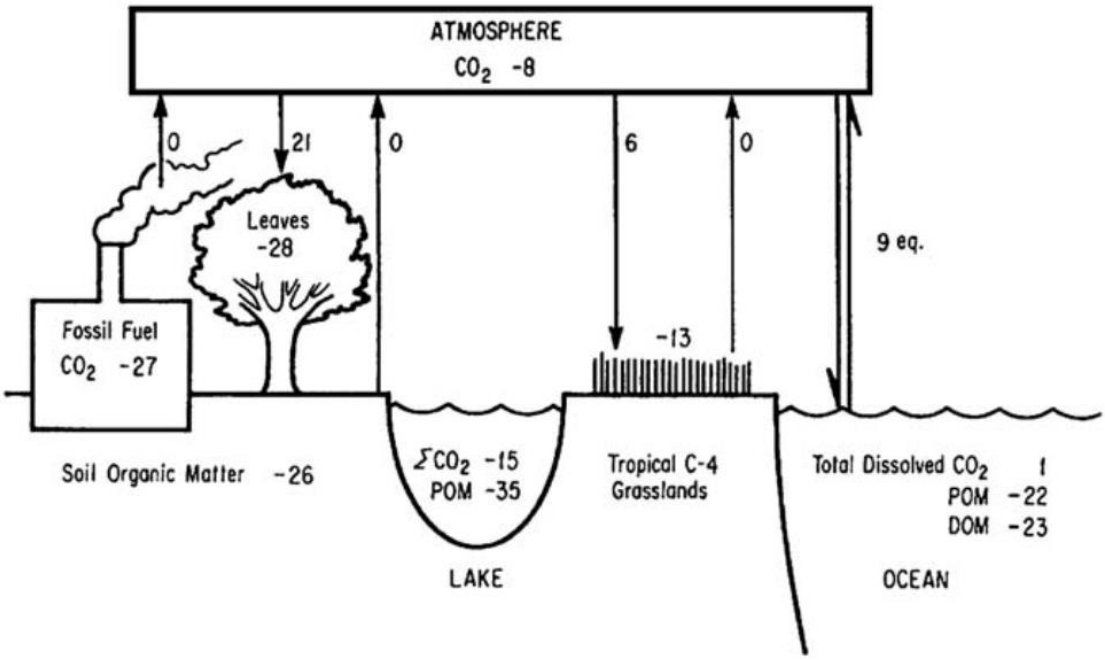


**Figure 1-2**. $\delta^{13}C$ distribution in ecosystems. Single arrows indicate $CO_2$ fluxes. The double arrow signifies an equilibrium isotope fractionation. Numbers for pools indicate $\delta^{13}C$ values (‰) and numbers for arrows indicate the fractionation (Δ, ‰) occurring during transfers. Figure taken from Fry (2006).

## 1.4 Stable carbon isotope distribution in the ocean

Similarly to carbon concentrations, the $\delta^{13}C$ distribution in the ocean is determined by the combination of thermodynamic and biological processes. However, typical $\delta^{13}C$ profiles in the ocean strongly deviate from the common inorganic and organic carbon distribution patterns. During the air-sea gas exchange, kinetic isotopic fractionation occurs. The difference between the fractionation during the transfer of $CO_2$ from the atmosphere to the ocean (-2 ‰) and from the ocean to the atmosphere (-8 to -10 ‰) leads to a more enriched $\delta^{13}C$ signature in the ocean relative to the atmosphere when equilibrium is reached (Gruber et al., 1999). This air-sea isotopic fractionation is strongly dependent on temperature (Zhang et al., 1995) however $\delta^{13}C$ distribution does not follow surface ocean temperature variations because equilibrium conditions are almost never reached as $^{13}C$ takes approximately 10 times longer to equilibrate with the atmosphere compared to total carbon in the ocean (Lynch-Stieglitz et al., 1995).

Biological processes contribute to further alter the $\delta^{13}C$ distribution in the surface ocean. During photosynthesis the uptake of $^{12}C$ is preferentially selected compared to the heavier isotope $^{13}C$ resulting in higher $\delta^{13}C_{DIC}$ values of the surrounding surface waters and consequently $^{13}C$ depleted organic matter (commonly expressed as $\delta^{13}C_{POC}$). The inorganic carbon source utilised by phytoplankton either diffuses or is actively transported across the membrane into the cell. Once in the cell, carbon is fixed by an enzymatic reaction mainly driven by the enzyme ribulose-1,5-bisphosphate Carboxylase/Oxygenase (RubisCO) to produce phytoplankton biomass or diffuses back into the environment (Francois et al., 1993). The combination of the different isotopic fractionations associated with each step, determines the overall photosynthetic fractionation. Therefore, the carbon isotopic composition of phytoplankton is determined by the isotopic composition of the source of inorganic carbon ($CO_2$ or $HCO_3^-$), isotope fractionation during transport into the cell, leakage of $CO_2$ out of the cell and isotopic discrimination during enzymatic carboxylation.

After leaving the surface layer of the ocean, the $\delta^{13}C$ of organic matter is subjected to remineralisation and physical mixing. Isotopic fractionation during these processes contributes to further alter the carbon isotope composition of the water column leading to $^{13}C$ depleted subsurface waters that move according to ocean circulation pattern. However a small fraction of biologically derived carbon (mostly carbonates) reaches the bottom and remains preserved into the sediments. As such the carbon isotopic signature of biogenic sediment reflects the distribution, abundance and isotopic ratio of the organisms in the upper layer. Stable carbon isotopes measurements of sediment material have been widely used to infer past climate changes. Foraminifera and coccolithophorids have been major producers of pelagic sediments for the past 200 million years and isotopic analyses of their fossils have provided important

information about the major global changes in past photosynthetic activity (Berner et al., 1983; Derry et al., 1992). However, the interpretation of sedimentary $^{13}C$ records is not straightforward and it must account for changes in the $\delta^{13}C$ of seawater, potential phytoplankton community shifts and variations of the biological pump strength (Spero et al., 1999). The combination of isotopic measurements of all the carbonate system parameters with measurements of planktonic species composition and physiological processes such as photosynthesis and respiration in today's ocean can provide essential information to link variations in sedimentary records to past environmental conditions.

Although the isotopic composition of carbon species in the marine system is both regionally and temporally variable, it generally lies within specific isotopic ranges (Figure 1-3).

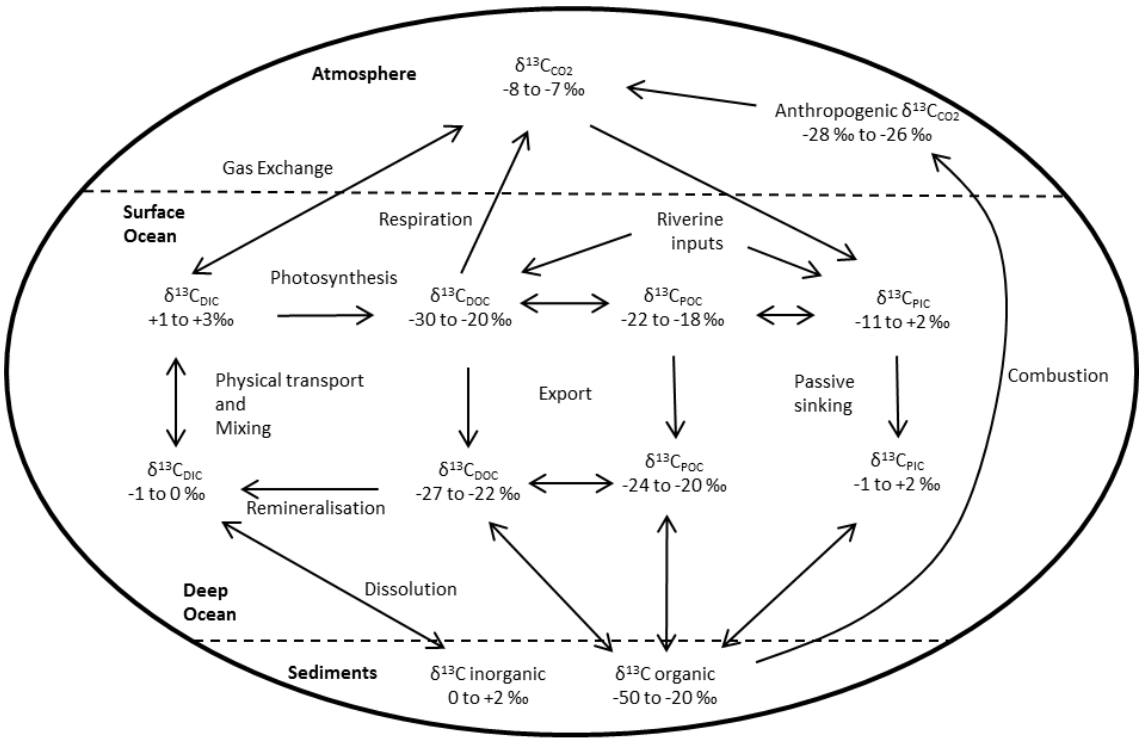


**Figure 1-3.** Stable carbon isotope cycle in the marine environment. Isotopic values are approximations obtained from various sources.

Each compartment is characterised by a typical carbon isotopic signature that defines background patterns. These characteristic isotopic patterns result from several factors, including large reservoirs, fixed enzymatic reactions and common ecological stoichiometries that have linked together the carbon species over geological time scales (Fry, 2006). However, the identification of local deviations from background patterns and the assessment of the factors associated to these variations can be crucial for reducing the number of assumptions related to estimations of past climate conditions.

## 1.5 Anthropogenic $CO_2$ perturbation to the stable carbon isotope distribution in the marine system

The anthropogenic contribution to the global atmospheric $CO_2$ rise through fossil fuel combustion, deforestation and other related human activities is thought to be changing the isotopic patterns of the individual components in the various compartments. The $\delta^{13}C$ value of atmospheric $CO_2$ is decreasing as a consequence of increasing inputs of $^{13}C$ depleted $CO_2$ derived from fossil fuel combustion and from the decomposition of newly formed organic matter. Carbon isotope measurements from air trapped in ice cores and atmospheric $CO_2$ samples, showed a decrease of $\delta^{13}C$ in our atmosphere from -6.3 ‰ (Francey et al., 1999) before the industrial revolution to values of about -8 ‰ (http://www.esrl.noaa.gov/). As the global ocean has been acting as a net sink for the current $CO_2$ emissions, the direct consequence of the net uptake is a decrease in the $\delta^{13}C$ of surface water DIC. Keeling (1979) named this isotopic shift "Suess effect". Modelling studies have estimated the magnitude of the oceanic Suess effect between 1860 and 2000 to be -0.07 ‰ per decade (Tagliabue & Bopp, 2008). Various authors have already used this $^{13}C$ oceanic Suess effect signal as an additional constrain for assessing the spatial distribution and inventory of anthropogenic $CO_2$ in the ocean (Gruber et al., 1999; Sonnerup et al., 1999; Quay et al., 2007). Although the oceanic $^{13}C$ Suess effects may reflect the accumulation of anthropogenic DIC, regional and seasonal variability strongly affect surface water $\delta^{13}C_{DIC}$ and potentially mask the $^{13}C$ Suess effect in the surface water (McNeil et al., 2001). Besides thermodynamics and ocean circulation, the natural variability is related to biological processes which are considered one of the main factors driving $\delta^{13}C_{DIC}$ distribution. On the global scale, the relatively small range of variability of $\delta^{13}C$ in surface waters, compared to the large potential range, indicates that biological effects on $\delta^{13}C$ nearly offset thermodynamic effects (Broecker et al., 1992; Gruber et al., 1999). Therefore, when estimation of anthropogenic induced changes on the oceanic $\delta^{13}C$ distribution are performed, in order to minimise the error associated with natural variability, biological processes must be considered. This leads us to turn our attention to the dynamics of organic matter cycling. As mentioned above, during photosynthesis the preferential uptake of light carbon ($^{12}C$) leaves the surrounding surface waters and subsequent carbonates structures formed from it, enriched in $\delta^{13}C$. Most of the formed $\delta^{13}C$ depleted organic matter is however remineralised, cancelling out the enrichment due to photosynthesis. However, increases in export rates and subsequent remineralisation at depth, would cause $\delta^{13}C$ depletion in deep waters and consequently in sedimentary calcareous structures (Figure 1-4).

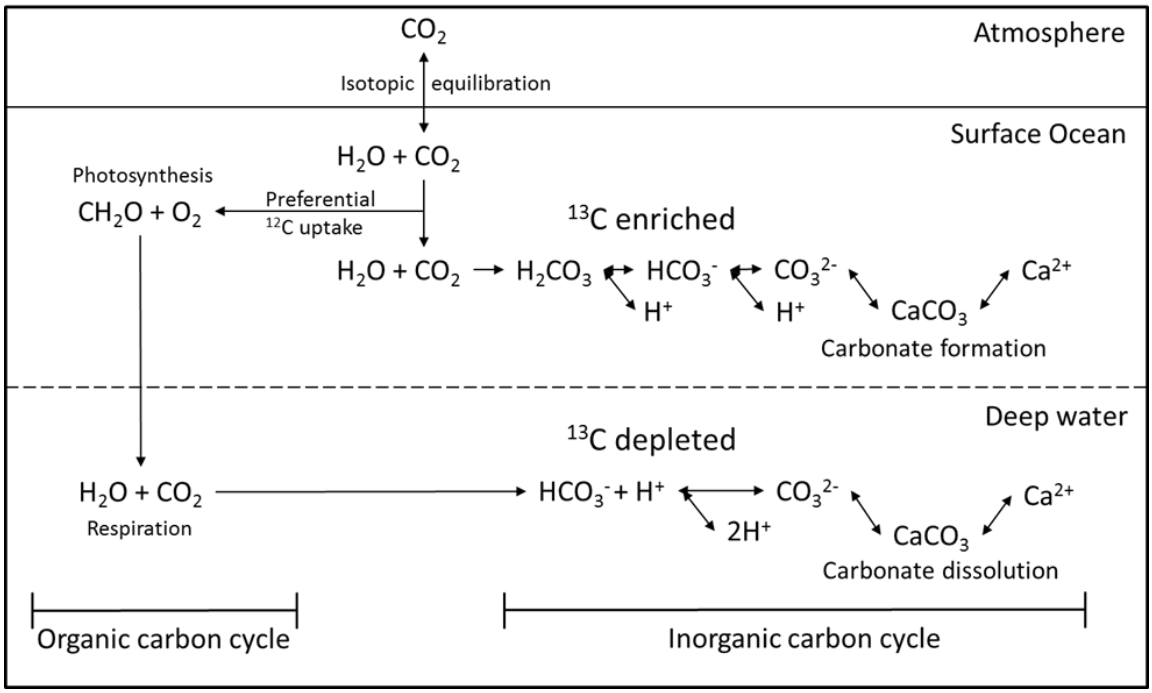


**Figure 1-4.** Stable carbon isotope flow from surface to depth due to export production with basic schematic of the interaction between the marine organic and inorganic carbon cycle (Adapted from Cooke & Rohling, (2001)).

If photosynthesis and export are enhanced under elevated $CO_2$ concentrations (Riebesell et al., 2007), the increasing inputs of anthropogenic light carbon ($\delta^{13}C$ = -28 ‰) into the ocean should theoretically decrease the $\delta^{13}C$ signature of the ocean and consequently deep waters through a direct relationship. However, the net effects are not yet fully understood as many other factors, including changes in calcification rates and depths, phytoplankton growth rates, shifts in planktonic communities contribute to alter the stable carbon isotope equilibria. Over the past decades, many studies have shown that carbon isotope fractionation in marine phytoplankton varies according to the $CO_2$ concentrations of surface water (Hinga et al., 1994; Rau et al., 1996; Burkhardt et al., 1999) with a general increase in $^{13}C$ fractionation under higher $CO_2$ levels. However no universal relationship exists as most of the responses appeared to be clearly species specific (Hoins et al., 2015). Recently, Boller et al. (2011) have re-examined the isotopic discrimination from the coccolithophore *Emiliania huxleyi* and found a surprisingly low fractionation value ($\varepsilon$ = 11.1 ‰ against the usual fractionation range of 18-29 ‰) which might lead to a re-evaluation of $\delta^{13}C$ values from environmental and geological studies. To accurately assess the anthropogenic $CO_2$ perturbation to the stable carbon isotope distribution in the marine system it is therefore important to include isotopic values of phytoplankton which are often cast in terms of isotopic composition in POC (or derived from it). As POC and DOC are potentially interconnected, stable isotope measurements of DOC could provide information about the contribution of specific sources of carbon to the DOC pool. Combined measurements of the

isotopic composition of POC and DOC have been successfully used in rivers and estuaries (Raymond & Bauer, 2001; Bianchi et al., 2007; Vargas et al., 2013) to assess source contribution and cycling patterns, however, very few studies have examined the variability of particulate and dissolved organic $\delta^{13}C$ distribution in the marine system (Bauer et al., 2002; Hedges, 2002). The similarity in total sizes of the DOC and atmospheric $CO_2$ pools (~700 Pg and ~750 Pg, respectively) highlights the importance to link DOC dynamics and fluxes to the global carbon cycle. It has been postulated that the net oxidation of only 1 % of the DOC in the global ocean would be sufficient to produce a $CO_2$ flux larger than the one annually produced by the combustion of fossil fuels (Hedges, 2002). Therefore, $\delta^{13}C_{DOC}$ measurements of seawater might shed light on the relationship between the inorganic and organic carbon pools in the ocean by monitoring potential isotopic shifts due to anthropogenic changes during the processes of transport, remineralisation and sedimentation. However, although DOC is often the dominant organic carbon source in the marine environment, analytical problems related to the very low concentrations of DOC in the ocean water and to the presence of salt in seawater samples made and continue to make $\delta^{13}C_{DOC}$ measurements highly challenging.

## 1.6 The mesocosm study approach

In light of the recent focus on the increase in anthropogenic $CO_2$ concentrations in the atmosphere and the related uncertainties of its effects on the marine system, a wide range of research and development activities have been conducted, and among those mesocosm experiments. The **K**iel **O**ff-Shore **M**esocosms for future **O**cean **S**imulations - **KOSMOS** - is a newly established ocean observatory and experimentation system for deployment in open waters. A mesocosm unit consists of a fiber glass and stainless steel flotation frame, a flexible 2 m in diameter and up to 20 m long thermoplastic polyurethane bag and a 2 m long funnel-shaped sediment trap (Riebesell et al., 2013). The KOSMOS facilities were successfully employed in long-term experiments in different climate zones, ranging from the high Arctic to temperate and subtropical waters. In this thesis, three mesocosms studies are considered, two from the high latitude North Atlantic subarctic zone and one from the oligotrophic environment of subtropical Canary Island waters (Figure 1-5).

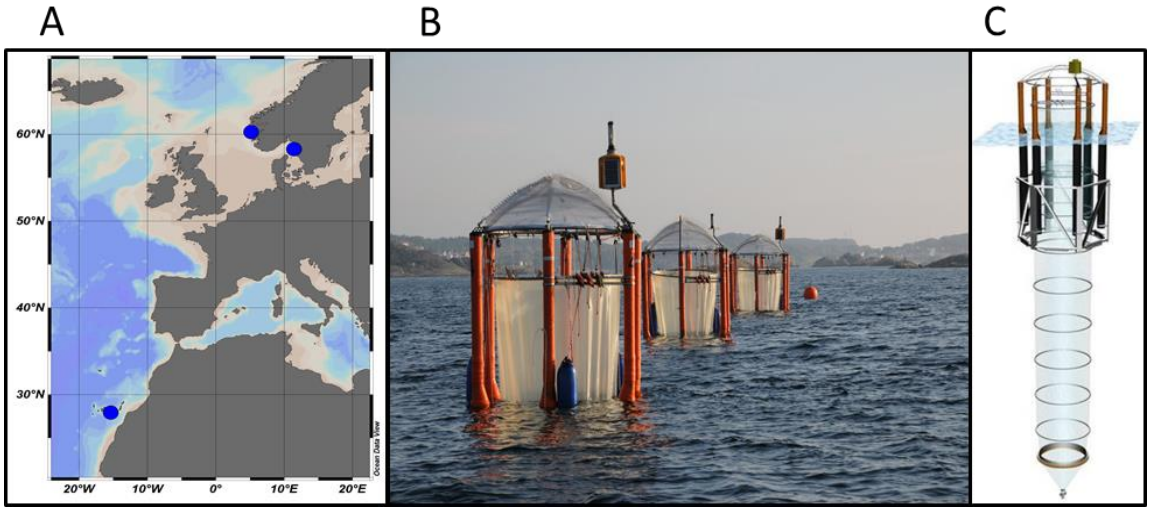


**Figure 1-5.** Study locations (A) of KOSMOS experiments with a picture at Gullmar fjord, Sweden (B) and a sketch of a mesocosm unit (C).

The three experiments were coordinated by GEOMAR Helmholtz Centre for Ocean Research Kiel and conducted in the framework of the German project on ocean acidification BIOACID (Biological Impacts of Ocean ACIDification).

Enclosing a large amount of water (up to 75 $m^3$), mesocosms have the advantage of representing near natural ecosystems in which experimental science can be conducted and researchers can test scientific hypothesis using parallel multiple controls and/or variable setups. As such, mesocosms represent ideal platforms for investigating the effects of $CO_2$ on the marine ecosystem. The main aim of the recent mesocosm experiments was to investigate the effects of elevated $CO_2$ on natural plankton communities and previous studies have already proved the validity of these platforms as tool to identify and quantify biogeochemical and physiological responses not only at organism level but also at community and ecosystem level (Engel et al., 2005; Delille et al., 2005; Riebesell et al., 2007; Schulz et al., 2008; de Kluijver et al., 2010, 2013; Endres et al., 2014; Bach et al., 2016). However no mesocosm study has been carried out so far to directly assess the effect of increasing $CO_2$ concentrations on the distribution of stable carbon isotopes in the marine system. Due to their semi-enclosed nature, mesocosms provide ideal settings to study the dynamics and cycling of stable carbon isotope within the various marine system compartments. Measurements of the variations of the $\delta^{13}C$ fractionation in both organic and inorganic carbon from the water column and trapped in the sediments, in natural versus simulated future oceanic conditions will potentially yield essential information about the mechanisms driving the fate of carbon. Being closed systems (except $CO_2$ air-sea exchange), mesocosms have the advantage of representing an environment in which physico-chemical and biological parameters can be strictly connected. This enables us to investigate directly the processes driving isotopic changes of the various compartments identifying sources and sinks for

carbon with the potential of providing a baseline for reconstructing past and predicting future climatic conditions.

## 1.7 Thesis aims and objectives

The principal motivation for this thesis is to increase our understanding on the role that processes such as photosynthesis, bacterial degradation and sedimentation in the ocean play in the global carbon cycle. Measurements of the isotopic composition of all the carbon system species in the marine system were used to assess that. In this project the biogeochemical responses of different marine ecosystems to human $CO_2$ perturbation were investigated by looking at the marine carbon cycle in terms of isotopic composition of all the carbon system species. The aim is to assess how the anthropogenically induced change in the stable isotope signature of the atmospheric $CO_2$ affects the stable isotopic distribution of the inorganic and organic carbon species in the ocean. In theory, linking the $\delta^{13}C$ signatures of the organic material to the inorganic pool, a better understanding of the rates and dynamics that affect carbon concentrations within the marine system can be obtained and the key processes that control the fate of anthropogenic carbon can be better identified and quantified.

The first objective was to establish valid protocols for the analysis of stable carbon isotopes in seawater samples. Although instrument calibration procedures are reported in the appendixes as supplementary information, the validation method procedures for the carbon isotope standards constitute an important part of the present work and should be considered of prime importance as they represent the main stage for the generation of accurate and precise isotopic datasets. In line with the implications for the establishment of high quality data, a crucial objective of this thesis was to have precise and accurate measurements of the isotopic composition of DOC in seawater samples, which currently are not widespread. Development and optimisation of the most appropriate analytical technique was therefore performed.

Having the possibility of compiling a complete dataset for the stable isotope composition of all the individual carbon parameters in a marine system, mesocosm experiments were used as platforms to study the isotopic variations under different $CO_2$ conditions with the aim of identifying specific pathways for the anthropogenic $CO_2$. Through isotopic and non-isotopic carbonate system parameter data and in conjunction with ancillary and plankton community datasets additional aims of this thesis were:

- to track the transfer of carbon from $CO_2$ to seawater and marine biota
- to identify potential $CO_2$ threshold levels for the development and growth of primary producers

- to quantify the contribution of degradation and sinking to the transfer of carbon within the marine system.

## 1.8 Thesis structure

This thesis is based on four main chapters which include stable isotope analysis of carbon in seawater samples. All chapters are formatted in the same manner with Figures and Tables numbered according to the chapter they belong (e.g., Figure 3-1 defines Figure 1 of Chapter 3).

Prior the actual analysis of samples, **Chapter 2** reviews the main techniques for the determination of stable carbon isotopes in aquatic samples, providing a state of the art on the current methodologies. The analytical principles employed by some of the commercially available instruments are outlined and an overview of the procedures, from sampling to analysis, is presented. Analytical challenges associated with each type of measurement are discussed and recommendations for high quality results are provided.

From Chapter 2, it is clear that analytical challenges have precluded routine measurements of the stable isotope composition of dissolved organic carbon ($\delta^{13}C_{DOC}$) in seawater samples. This was the pushing factor for the development of a novel analytical procedure. **Chapter 3** describes the development of a new combined system for natural abundance concentration and isotopic measurements of DOC in seawater samples. The chapter covers instrument modifications, analytical procedures and method validation for accurate and precise $\delta^{13}C_{DOC}$ measurements of marine samples.

**Chapter 4** presents and describes sample collection and analytical procedures for the determination of $\delta^{13}C$ signatures of inorganic and organic dissolved and particulate carbon both in the water column and deposited in sediment traps of samples collected during long term mesocosm experiments. Following a detailed description of raw data processing and calibration procedures for ensuring accurate and inter-comparable measurements, the effects of high $CO_2$ levels on the isotopic distribution of carbon species in the mesocosm systems are determined. Mesocosm experiments allow for different $CO_2$ conditions in semi-enclosed controlled environments and as such, will provide ideal settings for the assessment of the dynamics of stable carbon isotopes within the marine system. Although the current unavailability of supplementary biological and oceanographic data precludes an in-depth assessment of underlying biogeochemical processes, **Chapter 4** provides a holistic view of the variation of the stable isotope distribution of carbon in response to future $CO_2$ concentration changes within the marine system.

In **Chapter 5**, stable carbon isotope measurements from the Gran Canaria mesocosm experiment are examined in combination with a wide range of oceanographic and biological measurements in order to assess fluxes among the carbon system compartments and determine

the effects and fate of the natural carbon component in a phytoplankton succession. The stable isotopic signature of the added $CO_2$ will be used as a tracer to follow the carbon through the various compartments of the marine system within mesocosm platforms. Variations in the isotopic composition of specific carbon species will be assessed and the contribution of physico-chemical processes such as air-sea gas exchange and biological processes such as photosynthetic carbon fractionation will be quantified.

Finally, **Chapter 6** concludes on the major considerations of the present work, and outlines some directions for future work.

# Chapter 2: Determination of stable carbon isotopes in seawater samples: analytical procedures and applications

Mario Esposito, Eric P. Achterberg, Douglas Connelly

Submitted to Analytica Chemica Acta as "Stable carbon isotope composition of seawater: A review on isotope ratio mass spectrometry analytical methods"

## 2.1 Introduction

The atmosphere, oceans and land exchange large amounts of carbon through physical and biogeochemical processes and this transfer determines the global carbon cycle. Each year the atmosphere and land exchange approximately 120 Pg of carbon ($10^{15}$ g C), and another ~90 Pg C is transferred between the atmosphere and oceans (Prentice et al., 2001; Ghosh & Brand, 2003; Houghton, 2007).The physical and biogeochemical processes responsible for the carbon fluxes between the atmosphere and coastal and open oceans have been studied for many years (Bindoff et al., 2007), but the perturbation generated by the anthropogenic $CO_2$ emissions are only now emerging. The quantification of anthropogenic perturbations associated with the inputs of carbon to the oceans still involves large uncertainties mostly due to the diversity of data types (Regnier et al., 2013), and a lack of a direct global approach to the determination of the fluxes. Assessments of environmental, ecological and socio-economic impacts resulting from changes in $CO_2$ levels are often evaluated by use of general circulation models which simulate system responses under different anthropogenic emission scenarios (Eby et al., 2009; Barnett et al., 2006). However the precision of these predictions is affected by large uncertainties, particularly at regional scale, associated with model parameterization, natural variability and exact future $CO_2$ emissions (Barnett et al., 2006). An effective direct and independent way to examine the $CO_2$ exchange processes between compartments and quantify the contributions of different components to individual compartments is through parallel measurements of the isotopic compositions of all organic and inorganic carbon species. These include dissolved inorganic carbon (DIC), dissolved organic carbon (DOC) and particulate organic and inorganic carbon (POC and PIC, respectively).

The isotopic composition of these carbon species is both regionally and temporally variable but generally lies within specific isotopic ranges. The surface water DIC isotopic signature is determined by the isotopic composition of atmospheric $CO_2$, preferential biological uptake towards lighter carbon ($^{12}C$) and vertical and horizontal physical transport of waters with

individual isotope signatures (Lynch-Stieglitz et al., 1995). Isotopic signatures of the dissolved and particulate organic carbon vary according to the origin of the material (autochthonous or allochthonous) (Bauer & Bianchi, 2012) and the isotopic fractionation during photosynthetic fixation and bacterial mediated dissolution of calcium carbonate (Schmittner et al., 2013). Accurate knowledge of the isotopic signature of each of the carbon species allows us to differentiate between sources of carbon and determine the exchange rates between the various compartments of the marine system. Carbon production and consumption can be determined *in situ* and correlations with both environmental and biological variables can be established. Measurements of stable carbon isotopes in seawater samples will therefore improve our understanding of the processes controlling $\delta^{13}C$ distribution in the present ocean and provide a powerful tool to investigate the anthropogenic carbon component within the marine carbon cycle.

Different methodologies for the determination of stable carbon isotopes in aquatic samples exist and most of them rely on the use of isotope ratio mass spectrometry (IRMS), although they are specific for every sample type. The measurement principle of mass spectrometry is based on the separation of accelerated positively charged ions that, passing through a magnetic field under controlled vacuum (pressure in the $10^{-6}$ mbar range), deviate according to their mass and charge. The resulting ion beams are focused on an array of collectors (Faraday cups), amplified and processed by computer software. In the last ten years, the introduction of commercially available instrumentation has made mass spectrometry a popular and relatively easy to use tool. However, the rapid growth in applications has created a large number of different analytical protocols that researchers have to carefully assess according to their required measurement purposes. In the following sections an overview of the procedures for the determination of stable carbon isotopes in natural water samples is presented. For each type of sample the distinct stages of sampling, preservation, conversion into $CO_2$ and analysis are outlined. Sources of contamination and various available analytical systems are discussed, and recommendations on how to obtain high quality results are provided. Particular focus is put on the methods for the determination of stable isotopes in marine carbon samples.

## 2.2 Analytical strategies for stable carbon isotope measurements in water samples

### 2.2.1 Analytical principle

Isotope ratio mass spectrometers allow the determination of the relative abundance of a specific isotope in a given sample. In order to eliminate bias, the stable isotopic composition of a sample is always measured relative to working standards which are calibrated against internationally recognised materials which are often provided by the International Atomic Energy Agency (IAEA, Vienna). It is now common practice to express the carbon isotopic composition of a sample as a difference measurement using the δ value which denotes a difference in parts per thousand (‰) from a Vienna Pee Dee Belemnite (V-PDB) standard. The isotopic values are expressed as:

$$\delta^{13}C = \left[\left(\frac{R_{sample}}{R_{standard}}\right) - 1\right] * 10^3 \quad (2.1)$$

where R represents the ratio of the heavy isotope to the light isotope ($^{13}C/^{12}C$ in the case of carbon isotopes). For the determination of carbon isotopes ratios, the sample materials must be converted into gaseous $CO_2$ and introduced into an IRMS instrument. Off-line dual inlet and on-line continuous flow configurations are the two common ways of gas introduction. In dual inlet systems, the gases are introduced through capillaries connected to a computer controlled bellows that allow delivery of constant gas volumes. Although dual inlet is generally considered the most precise method (precision of 0.01‰ for $\delta^{13}C$) for measurement of the isotope ratios of light elements (Carter & Barwick, 2011), it is often not the method of choice for the analysis of a large number of water samples as pre-analysis operations can be time consuming (1-3 hours per sample depending on analysis type and concentration). The use of this setup could however be employed in comparative studies or as additional data validation tool. Continuous flow measurements are generally preferred as sample handling and other time consuming pre-analysis operations are reduced. In continuous flow systems a stream of helium gas is used to carry the analyte and reference gas into the mass spectrometer. However continuous flow configurations require the IRMS instrument to be interfaced with peripheral devices which convert carbon in the sample into a gaseous form and deliver the resulting $CO_2$ gas to the IRMS for analysis. Commonly employed peripheral instruments for stable carbon isotope analysis are elemental analysers (EA), total organic carbon analysers (TOC) and GasBench devices (Figure 2-1).

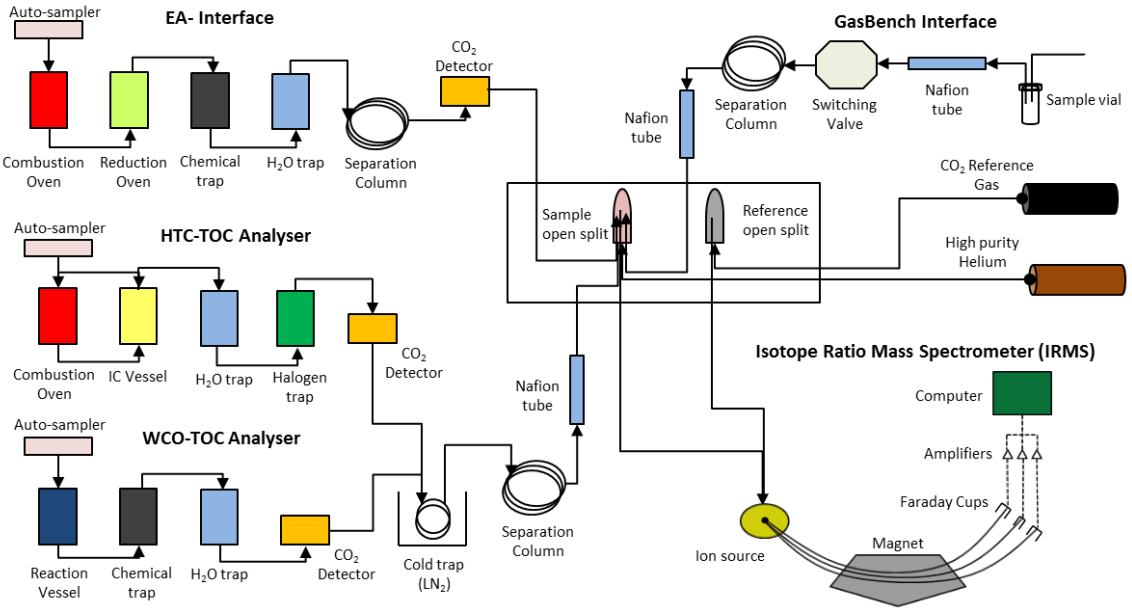


**Figure 2-1** Schematics of the most common interfaces for stable carbon isotope measurements of seawater samples in continuous flow mode. EA=Elemental Analyser, HTC-TOC= High Temperature Combustion Total Organic Carbon Analyser, WCO-TOC= Wet Chemical Oxidation Total Organic Carbon Analyser.

A range of analytical protocols are available and with careful attention to pre- and post-analytical steps, each instrumental configuration can potentially be used to perform stable carbon isotope measurements in seawater samples. The purpose of this review is to discuss available instruments and analytical procedures for the determination of stable carbon isotope ratios in seawater samples, with the intention of providing the most suitable directives and recommendations for each sample type to facilitate the generation of accurate and internationally comparable results.

### 2.2.2 Dissolved inorganic carbon sampling

Atmospheric $CO_2$ is the most common source of contamination for DIC analysis. Sample collection protocols are designed to minimise the exchange of $CO_2$ with the atmosphere during seawater sampling, preservation and storage (Dickson et al., 2007). The most common preservation technique consists of addition of a small aliquot (0.05-0.02% of the total sample volume) of saturated mercuric chloride ($HgCl_2$) solution (Dickson et al., 2007). Addition of 2 to 5µl $ml^{-1}$ of $HgCl_2$ has no detrimental influence on the $\delta^{13}C$ analysis, however at additions higher than 5µl $ml^{-1}$ DIC isotopic fractionation is shifted towards heavier $^{13}C$ values (up to 0.4‰) as a result of reduced $CO_2$ solubility (Torres et al., 2005). As mercury compounds are toxic and hazardous to the environment, an alternative solution is to inject the seawater sample directly into Exetainers vials (12 ml butyl rubber septum capped) and acidify to pH <2 using $H_3PO_4$ in the field for subsequent

headspace sampling. With this method both the $\delta^{13}C$ values and carbon concentrations were shown to be stable for up to 6 months (Taipale & Sonninen, 2009).

### 2.2.3 $\delta^{13}C_{DIC}$ analysis

Several methods for $\delta^{13}C_{DIC}$ measurements have been developed (Table 2-1). Commonly used approaches involve acidification of the samples with orthophosphoric acid ($H_3PO_4$) to extract $CO_2$, collection of the produced gas and analysis through IRMS. The conventional method consists of extracting the $CO_2$ gas offline under vacuum followed by cryo-collection (liquid nitrogen) and sealing in glass tubes for subsequent manual injection into a mass spectrometer (Tan et al., 1973). This method provides excellent precision (0.04 ‰), but involves preparation times for a single sample of about 1 hour (Tan et al., 1973) and DIC concentrations are not determined when this technique is employed. As samples have to be prepared individually, the procedure is clearly not applicable for routine analysis of the generally large number of water samples collected during oceanographic studies.

With the increasing attention on DIC isotope research, new devices have been developed. The most recent (GasBench II, Thermo Fisher Scientific) employs a continuous flow analysis approach and minimises manual operations and analysis time (usually about 10 min per sample). The method is based on acidification ($H_3PO_4$) of water samples contained in septum sealed vials with automated headspace gas sampling after a set equilibration time (24 h). The analyte stream is passed through a semipermeable membrane (Nafion) where a dry helium counter-flow removes the water vapour. The dry gas is then carried into a fixed volume sampling loop (100 µl for typical marine DIC concentrations of 2.0-2.4 mmol $kg^{-1}$) and sequentially injected into a gas chromatography column (Agilent PoraPlot Q). The eluted sample stream ($CO_2$ + He) passes through a second Nafion water trap and is directed to the IRMS via a capillary (Figure 2-2). Excellent precision (better than 0.1 ‰) has been reported for this method (Torres et al., 2005; Assayag et al., 2006). Nevertheless, in order to obtain high precision isotopic data, knowledge of the sample DIC concentration range is required prior to isotopic analysis for calibration of the IRMS signal intensity. Variations in the size of the sampling loop (50 µl, 100 µl, 250 µl) and/or sample dilution with helium allow the determination of isotopic ratios in samples with a wider range of DIC concentrations.

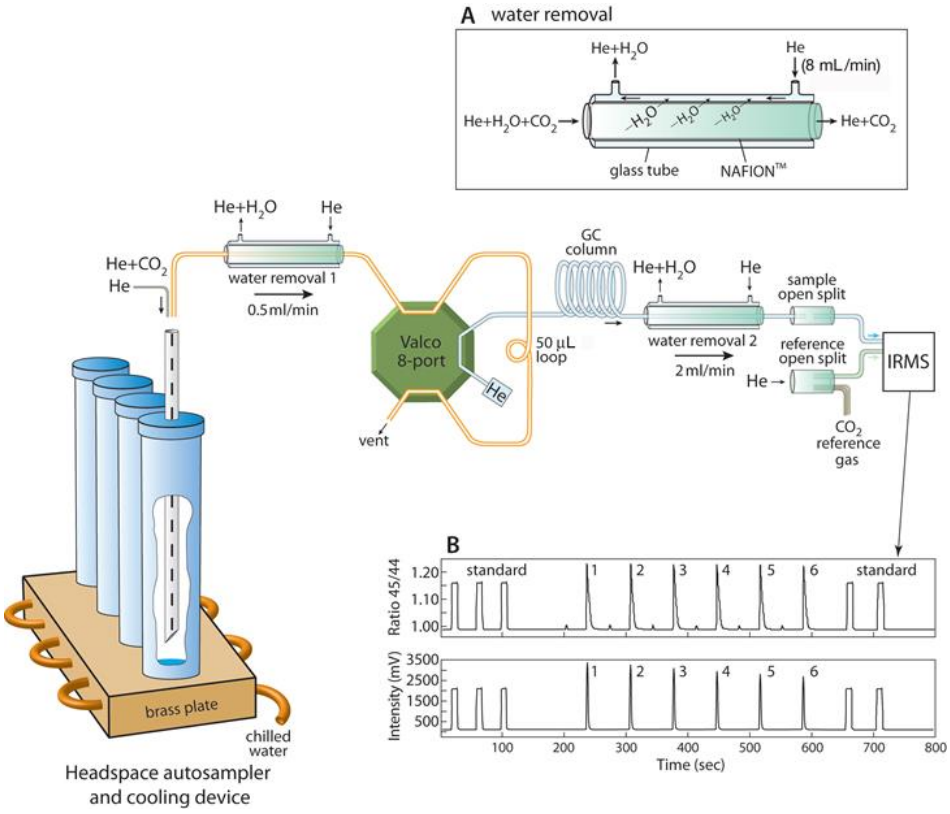


**Figure 2-2.** Schematic drawing of the GasBench-II system, interfaced to a gas source stable IRMS, showing the various modular components (modified from Finnigan-MAT 1999). Inset A shows the water removal NAFION trap. Inset B shows the 44/45 ratios and the signal intensity for mass 44. Figure taken from Torres et al. (2005).

Recently, Yang & Jiang (2012) suggested an alternative method to $H_3PO_4$ acidification to extract $CO_2$ from a seawater sample. The method is based on the $CO_2$-water equilibrium principle where DIC and gaseous $CO_2$ equilibrate in the vials according to carbon speciation of the solution (DIC concentration), pH and temperature. The sample is injected into pre-filled (0.3% $CO_2$ in He mixture) vials and after overnight equilibration, the headspace is sampled. This technique may better represent the actual sample values as it reduces signal intensity variations due to different sample DIC concentrations and it does not alter sample composition due to acid addition. However isotopic data may be subjected to a higher degree of error as values can only be retrieved after complex post run calculations. The $\delta^{13}C_{DIC}$ values are expressed as a function of the isotopic signature of the flushing gas (0.3% $CO_2$ in He mixture), the sample DIC content and the fractionation factor between gaseous $CO_2$ and DIC which can be obtained when the temperature (± 0.02°C accuracy) and pH (± 0.005 accuracy) of the sample are known.

These methods provide high precision isotopic DIC data but not a direct quantification of DIC concentrations. The coupling of non-dispersive infrared detectors (NDIR) in continuous flow with IRMS instruments (St-Jean, 2003; Osburn & St-jean, 2007) has allowed direct analysis of the generated $CO_2$ gas, first quantitatively and then isotopically. The hyphenated systems employ the

$H_3PO_4$ acidification procedure for gas extraction with $CO_2$ equilibration undertaken in reaction vessels where sample aliquots are injected. The advantages are that analyses are performed directly from the sampling bottles without the need of manual sample transfer to specialised vials, analysis times are relatively short (<10 min per sample) and precision on quantitative analysis is 2 ppb of C. However, isotopic measurements are potentially affected by instrumental background carbon contamination, but with appropriate corrections, a precision similar to that of EA-IRMS (≤ 0.2‰) has been reported (St-Jean, 2003).

| **Method** | **Instrument** | **Precision** | **Comments** | **Ref.** |
|---|---|---|---|---|
| Off-line $CO_2$ extraction with $H_3PO_4$ under vacuum. Cryo-collection and manual injection into IRMS | Home-made extraction system. Dual-inlet IRMS | ± 0.04‰ | Long sample preparation time (about 1 h) | (Tan et al., 1973) |
| $CO_2$ extraction with $H_3PO_4$ and head space gas sampling. Continuous flow | Varian 3400 GC, combustion reactor, Finnigan MAT 252 | ± 0.2‰ | Partial extraction of DIC as $CO_2$ Correction for isotopic fractionation between $CO_{2(g)}$ and $CO_{2(aq)}$. | (Salata et al., 2000) |
| $CO_2$ extraction with $H_3PO_4$ and head space gas sampling. Continuous flow | Thermo-Finnigan GasBench II, Delta plus XL IRMS | ± 0.15‰ | Knowledge of sample DIC concentration range required. Semi-quantitative | (Torres et al., 2005) |
| $CO_2$ extraction with $H_3PO_4$ and head space gas sampling. Continuous flow | Gilson 22X autosampler, GV Instruments 2003, IRMS | ± 0.1‰ | Isotopic fractionation and salinity effect corrections are included in the analytical protocol | (Assayag et al., 2006) |
| $CO_2$-water equilibration and head space gas sampling. Continuous flow | Thermo-Finnigan GasBench II, Delta plus XP IRMS | ± 0.2‰ | Knowledge of sample temperature, pH and $\delta^{13}C$ of flushing gas required | (Yang & Jiang, 2012) |
| Direct sample injection in $H_3PO_4$ reaction vessel for $CO_2$ extraction. Continuous flow | OI Analytical 1010 TOC analyser, Delta Plus IRMS | ± 0.2‰ | System blank interference Background correction procedures required | (St-Jean, 2003; Osburn & St-jean, 2007) |

**Table 2-1.** List of commonly used methods for the determination of the stable isotope signature of dissolved inorganic carbon in seawater samples

### 2.2.4 Dissolved organic carbon sampling

Dissolved organic carbon in seawater is defined as the carbon remaining in a seawater sample after removal of all particulate carbon by filtration (typically ashed GF/F filters, 0.7 µm nominal pore size) and all inorganic carbon by acidification and sparging (Farmer et al., 2007). After collection, samples for DOC are preserved by poisoning, acidification or freezing. Poisoning with $HgCl_2$ has been employed in the past, however, its use appeared to affect the analysis output by deactivating Pt-containing catalysts of high temperature combustion analysers (Bauer et al., 1993). Freezing, although suitable for long term sample stability, if not done rapidly may lead to formation of micro-particulate material and 'salting out' of DOM may occur (Sharp et al., 1993; Fellman et al., 2008). Acidification of the samples to a pH<2 with either concentrated HCl or $H_3PO_4$ is in general the most common method used both for biological preservation and inorganic carbon removal (Sharp et al., 1993; Farmer et al., 2007). None of the above preservation methods have yet been extensively tested for $\delta^{13}C_{DOC}$ alteration.

### 2.2.5 $\delta^{13}C_{DOC}$ analysis

Measurements of the stable isotope composition of DOC ($\delta^{13}C_{DOC}$) in marine samples tend to be long and problematic. The general principle is based on the oxidation of the DOC into gaseous $CO_2$ and subsequent analysis by IRMS (Table 2-2). While several methods and instruments for the determination of the total DOC concentrations have been developed and are currently well established, the determination of $\delta^{13}C_{DOC}$ still entails analytical uncertainties and is far from being a routine analysis.

The first carbon isotopic measurement of a seawater DOC sample was undertaken in the 1960s and consisted of acidification (pH=2), photo-oxidation (UV lamp), cryogenic collection (LN) and vacuum $CO_2$ extraction of a volume of 3-5 l of filtered seawater over a time span of more than 6 h (Williams & Gordon, 1970). Although a precision of ±0.05 mg C $l^{-1}$ and ±0.2 ‰ (DOC and $\delta^{13}C_{DOC}$, respectively) was achieved, the method is not suitable for the high sample numbers of typical oceanographic studies.

In order to reduce analysis time and sample handling, in-house and commercial total organic carbon (TOC) analysers have been developed and various authors have interfaced these devices to IRMS (St-Jean, 2003; Bouillon et al., 2006; Osburn & St-jean, 2007). Their setups, based on wet chemical oxidation (WCO) of water sample with potassium persulfate at 130 °C (Menzel & Vaccaro, 1964), are able to provide isotopic precision of ≤0.3 ‰, using small sample volumes (2 ml) and short analysis time (20 min per sample) (Osburn & St-jean, 2007). With careful attention to blank contribution, the isotopic composition of marine samples with DOC concentrations down to 45 µM can be determined. However, formation of chlorine gas and salt deposition in the flow

lines limit their use for long term routine analysis and system maintenance is required after only about 15-20 measurements. Moreover results from a recent inter-comparison study showed differences in $\delta^{13}C_{DOC}$ values of up to 21 ‰ for natural water samples and a general enrichment of 10 ‰ for seawater samples analysed by WCO techniques (Van Geldern et al., 2013).

An alternative method for DOC analysis is lyophilisation of aliquots (25 ml) of seawater and subsequent sealed tube oxidation at high temperature (580 °C) with copper oxide (Fry et al., 1993). The main advantage of this technique is the production of enough $CO_2$ for highly accurate (±0.04 ‰) measurements but the laborious and time consuming (24-48 h) operations preclude its use for routine determination of $\delta^{13}C_{DOC}$. The extensive time required for sample lyophilisation is eliminated by Gandhi and colleagues (Gandhi et al., 2004) who employed the rotatory evaporation technique to concentrate the samples and measured $\delta^{13}C_{DOC}$ using an EA coupled to an IRMS in a continuous flow setup. However, their measurements were mainly done in stream water samples as the high inorganic salt/organic carbon ratio in a seawater sample represents a problem for DOC recovery and introduces a higher degree of uncertainty, negatively affecting the reproducibility of the analysis.

The most common technique for DOC measurements in marine environments involves high temperature combustion (HTC) of samples (typically 100 µl) with a catalytic oxidation of organic matter. In general, HTC methods are preferred over WCO techniques as sample matrix does not affect analysis output and they require low sample volumes allowing for replicate measurements. The recent coupling of HTC-TOC analysers to IRMS instruments greatly improved sample processing and $\delta^{13}C_{DOC}$ measurements of deep seawater had a precision of ± 0.3‰ (Panetta et al., 2008). Carbon dioxide produced by high temperature combustion of multiple sample aliquots (100 - 300 µl) is cryogenically (Lang et al., 2007; Panetta et al., 2008) or chemically (Lalonde et al., 2014) trapped and, following drying and chromatographic separation, is transferred to the IRMS device. Although cryogenic collection is not $CO_2$ selective and potential condensation and clogging of the flow lines can occur, liquid nitrogen must be preferentially selected compared to chemical trapping techniques as variations in the molecular sieves (zeolite, ascarite) trapping capacity could lead to substantial isotopic fractionation of the trapped $CO_2$ gas. One of the major limiting factors in the HTC-TOC-IRMS setup is the blank contribution from the system and reagents. The problem is overcome and maximal performance is obtained when blank value is constant and measurable so that an appropriate background correction procedure can be applied.

Other methods used for carbon isotope measurements in marine DOC include supercritical oxidation (le Clercq et al., 1998) and moving-wire (Sessions et al., 2005) techniques. The former method involves oxidation of the organic carbon sample inside an alumina tube at 650 °C and 350 bars in the presence of pure oxygen and a copper catalyst, with subsequent cryo-trapping of the evolved $CO_2$ and analysis using dual-inlet IRMS. This method is believed to prevent the formation

of chlorine gas and achieve complete oxidation even for solutions with low carbon concentration, high salt content and refractory compounds. However, large sample volumes (500 ml) and long analysis times (1 h) are required. The moving-wire technique requires small sample volumes (1 µl) and involve rapid analysis times (30-40 s per sample) with an excellent precision of <0.2 ‰ for samples containing as little as 10 nmol of organic carbon. The method consists of injection of a drop of sample on a continuously spooling nickel wire which passes through a combustion oven where the carbon is oxidised to $CO_2$ and successively transferred to the IRMS ion source via a silica capillary. Although this is probably the fastest method for non-volatile carbon isotopic analysis, with excellent precision, the analytical system is complex with high running costs (nickel wire is single use) thereby preventing wide scale routine analysis. Moreover interferences from inorganic salts can potentially reduce the combustion efficiency affecting both the accuracy of carbon yields and isotopic measurements (Sessions et al., 2005).

| **Method** | **Instrument** | **Precision** | **Comments** | **Ref.** |
|---|---|---|---|---|
| Photo-oxidation, cryogenic collection and vacuum extraction of $CO_2$<br>Manual sample injection | Home-made<br>Dual-inlet IRMS | ± 0.2‰ | High sample volumes (3-5 l)<br>Long analysis time (6 h) | (Williams & Gordon, 1970) |
| Wet chemical oxidation<br>Continuous flow | Modified OI Analytical 1010 TOC analyser/ Thermo HiPerTOC<br>Delta Plus IRMS | ± 0.3‰ | High system maintenance<br>Salt interferences<br>Poor inter-comparability | (St-Jean, 2003; Osburn & St-jean, 2007; Bouillon et al., 2006) |
| Lyophilisation and high temperature oxidation<br>Manual sample injection | Home-made lyophiliser, sealed combustion tube<br>Finnigan 251 IRMS | ± 0.04‰ | Time consuming sample preparation procedures (24-48 h) | (Fry et al., 1993) |
| Rotatory evaporation<br>High temperature combustion<br>Continuous flow | EA3000 Eurovector GV Instruments IRMS | ± 0.3‰ | Data reproducibility affected by sample salt content<br>DOC recovery problems | (Gandhi et al., 2004) |
| High temperature combustion<br>Continuous flow | MQ-1001-Delta Plus<br>Shimadzu-Isoprime<br>Aurora 1030-Isoprime | ± 0.3‰ | High system blanks<br>Background correction procedures required | (Lang et al., 2007; Panetta et al., 2008; Lalonde et al., 2014) |
| Supercritical oxidation (650 °C at 350 bar), cryogenic collection. Dual-inlet | Home-made design<br>VG SIRA 9 IRMS | ± 1‰ | Large sample volumes (500ml)<br>Long analysis time (1 h) | (le Clercq et al., 1998) |
| Moving nickel wire<br>Continuous flow | Home-made design<br>Finnigan 252 IRMS | ± 0.2‰ | High running costs<br>Possible salt interferences | (Sessions et al., 2005) |

**Table 2-2**. List of commonly used methods for the determination of the stable isotope signature of dissolved organic carbon in seawater samples

### 2.2.6 Particulate carbon sampling

Total particulate carbon (TPC) in oceanic waters is typically collected by the filtration of known volumes of seawater through 0.7 µm pore size pre-combusted GF/F filters. Filtration can take place either *in situ* (in the water column) using specialised pumps deployed at a fixed depth (Moran et al., 1999), or in laboratories with discretely collected water samples (typically 1-2 l) and reduced pressure (< 200 mbar) conditions. Various sample storage treatments have been reported in the literature, however, when immediate analysis is not feasible, drying (overnight at 60°C) and subsequent freezing (-20 °C) of the filters is the most adequate sample preservation method, with no significant changes in the stable carbon isotope composition of the samples (Lorrain et al., 2003).

### 2.2.7 $\delta^{13}C_{PIC}$ and $\delta^{13}C_{POC}$ analysis

The total particulate carbon collected on filters consists of particulate organic carbon (POC) and particulate inorganic carbon (PIC). High temperature combustion methods have been widely employed both for quantitative and isotopic measurements by interfacing EA to IRMS instruments (Fry & Brand, 1992). The analytical operation of a commercial elemental analyser is based on flash combustion where the sample is placed in tin capsules and automatically transferred into the combustion furnace held at 950-1030 °C. The eluted combustion gases are carried by a He gas stream into a reduction reactor, water trap and chromatography column in order to purify, dry and separate the combustion products. The generated carbon is quantitatively detected by a thermal conductivity detector (TCD) and isotopic ratios are determined by IRMS. The gas from the EA is sent to the IRMS either via cryogenic trap (Fry & Brand, 1992) or directly via open split interface (Figure 2-1, Thermo Finnigan set-up). In case of separate analysis of the organic and inorganic components, duplicate filters are required. One set of the replicate filters is acid fumed (HCl) in a desiccator for at least 4 h in order to remove the inorganic component (Lorrain et al., 2003) and analysed for the remaining particulate organic carbon component, while the other set is analysed for the total particulate carbon. It has been shown that even low carbonated samples (< 5 % of the total) can lead to a difference of 1‰ in the isotopic signature (Lorrain et al., 2003). An isotope mass balance calculation (Hayes, 2004) is therefore necessary to obtain the isotopic value of the inorganic and organic components.

## 2.3 Analytical challenges and limitations

Current techniques for stable carbon isotopes analysis in seawater samples can provide high precision data, however analytical methods encompass some challenges associated with the

sample matrix and treatment, and analysis itself. As measurements of stable carbon isotopes in seawater samples can be performed only after conversion of the sample into $CO_2$ gas, preparatory analytical steps between sample collection and final analysis are required and each of these steps could potentially impact on the accuracy and precision of the results. Contamination is one of the main concerns in isotopic studies. The variation in natural abundance of carbon isotopes is in the order of 0.11 atom % and to magnify (one order of magnitude) the relatively minute changes, the δ notation was adopted (Fry, 2006). Modern IRMS instruments are able to reliably detect these small variations but to assess at an early stage whether these changes are associated with natural variability or contamination is difficult.

Day to day variations in the IRMS performance is checked by regular analysis of a pure reference gas which is characterised by a reasonably constant isotopic ratio. This gas should not be used as reference material as shifts in the carbon isotopic composition can occur when the liquid phase $CO_2$ in the tank starts to decrease. In order to obtain reliable results, sample values are calibrated against material of known isotopic composition. The matrix of the chosen material should be as close as possible to the sample to be analysed and it should be subject to the same reaction conditions as the samples to avoid any isotopic alteration during the gas extraction process. Potential sample isotopic fractionation is normalised when reference materials and samples are run alongside and under the same analytical conditions (Werner & Brand, 2001). No prepared reference material for stable carbon isotope analysis of seawater samples exist at present.

The composition of a seawater sample provides limitations on estimation of the isotopic signature of the sample. Stable isotope measurements performed at bulk levels describe the signature of samples that contain a mixture of compounds. The various forms of carbon both inorganic ($CO_2$ and carbonate minerals) and organic (carbohydrates, proteins, amino acids and lipids) contained in marine samples are subject to different biochemical processes and different turn-over times. The partitioning processes between the various forms are determined by isotopic fractionation equations but the preferential assimilation or degradation of one component over the other cannot be determined as the isotopic signature of the sample reflects the mean isotopic value of the source or product and not a specific compound. Isotopic patterns must therefore be carefully interpreted as both environmental stressors and organisms metabolic conditions would affect the kinetics of the reactions.

Other issues relate to specific analytical techniques and instrument operations. The online method for analyses of inorganic carbon isotopes is believed to provide only partial extraction of $CO_2$ from the samples and accuracy of the measurements could be affected by a fractionation effect between the aqueous and gaseous phases (Yang & Jiang, 2012). Although this might be the case for samples with low DIC concentrations, typical marine samples with ca. 2.2 mmol $kg^{-1}$ DIC

could potentially suffer from the opposite effect, with precision negatively affected by a saturation of the mass spectrometer signal. Conversely, too low and sometimes inaccurate isotopic signals are produced when DOC analysis of deep water samples (concentrations in the 40-80 µM C range) is performed as analytical methods suffer from important instrument blanks (4-8 µM C, (Sharp et al., 2002)) and potentially incomplete sample oxidation. Sources of background $CO_2$ may be derived from reagents (in WCO methods), catalysts (in HTC methods), external air that may have permeated through mechanical components, or impurities contained in the analytical gases. Oxidation efficiency is affected by the presence of refractory organic compounds and high inorganic salt concentrations.

## 2.4 Quality assurance

### 2.4.1 Performance of analysers and mass spectrometers

In order to maintain high precision analysis output, good working knowledge of isotope ratio mass spectrometers and peripheral devices is required. Modern IRMS are designed to separate, detect and count ion currents from a sample gas but the precision of the measurements can be strongly reduced by additional ion fluxes entering the system. In the case of $\delta^{13}C$ measurements of water samples, contamination of the ion source with water promotes the formation of $H_3^+$ ions which produce interference at mass 45 through formation of $CO_2H^+$ (Brand, 2004). In order to correct for the isobaric interference, water background must be carefully monitored especially when sample preparation steps cannot further reduce the water signal. Isobaric interferences are however reduced by optimisation of the ionisation efficiency. The stability of the ion extraction conditions is met when a linear relationship between the intensity of the ion currents and the measured ratio occurs. This can be reached through accurate focusing of the ion beams by careful alignment of the lens. In general, for good instrument performances, system stability tests (zero enrichment and linearity) are performed on the $CO_2$ working gas and they require standard deviation to be better than 0.05 ‰ and linear regression variation better than 0.06 ‰/V (Thermo Electron Corporation, 2005).

Isotopic variations due to analytical procedures are minimised through constant control and monitoring. Maintaining records of the analytical system performances is important so that laboratories can easily assess repeatability and reproducibility of their measurements. Monitoring the correct execution of all the analytical procedures, system operations and results of control material measurements - internal quality control (Thompson & Wood, 1995) - is essential for the distribution of high standard quality data.

### 2.4.2 Certified reference material and inter-laboratory comparisons

The isotopic ratio of a substance is expressed as a variation (on δ-scales) from an international calibration material. Stable carbon isotope data are reported on the V-PDB scale. Various standards for carbon measurements were established after the original PDB material became unavailable (Coplen, 1996). Current certified reference materials (CRM) for the analysis of inorganic carbon are minerals (calcite, lithium carbonate, barium carbonate) or sediments (limestone, marble), while for analysis of organic carbon, L-glutamic acid, caffeine, and/or sucrose are generally used. These materials are distributed only in limited quantities as their availability is restricted. For routine analysis it is convention to use an in-house standard (secondary standards) which individual laboratories choose and calibrate against the international certified materials. In compliance with the principle of "Identical Treatment" (Werner & Brand, 2001), the selection of the right standard is fundamental for optimal results. As no liquid reference material for $\delta^{13}C_{DIC}$ exists at present, the majority of laboratories use common carbonates ($CaCO_3$, $Na_2CO_3$ and $K_2CO_3$) as secondary standards. These materials are used because they are available as pure single compounds, are easy to handle and replace if exhausted and they exhibit isotopic homogeneity and consistent isotopic composition over time if stored in appropriate conditions. However, standard preparation errors could alter their isotopic composition hence the validity of the results. An effective alternative could be the use of CRMs obtained from A.G. Dickson (Scripps Institution of Oceanography, USA). These are widely distributed natural seawater reference materials for oceanic DIC and total alkalinity measurements. Although no carbon isotopic composition is reported with their certificate of analysis, each batch is prepared under controlled conditions and should theoretically exhibit a specific and defined isotopic signature. Analysis of this material would highly represent the principle of identical sample treatment thus avoiding the potential errors related to synthetic sample solution preparation. In a recent study, Humphreys et al. (Humphreys et al., 2016), reported carbon isotopic signatures of seawater CRMs to be 1.15‰ and 1.27‰ for batches 141 and 144, respectively, with calculated precisions substantially better (0.08‰) for the CRMs compared to those of the calibration standards (precision between 0.13 and 0.46‰). Similarly, consensus reference material distributed from the laboratory of D. Hansell (University of Miami) and routinely used for validation and quality control of TOC instruments could be employed as $\delta^{13}C_{DOC}$ standards as they would be representative of natural DOC seawater samples. Previously reported isotopic values for Hansell DOC CRMs were -19.5 ± 0.4‰ (Bouillon et al., 2006) and -19.9 ± 0.4‰ (Lalonde et al., 2014), reflecting the predominant marine origin of these water samples. Periodic reporting of δ values of these new consensus reference materials could provide new guidelines for stable carbon isotope laboratories performing analysis on seawater.

Today, various laboratories around the world perform measurements of $\delta^{13}C$ on DIC, DOC and TPC as standard analytical procedures, inter-laboratory comparison exercises should be periodically performed in order to check the quality of the measurements. The only inter-laboratory comparison study for the determination of stable carbon isotope ratios of DIC and DOC in natural water samples to date was announced in April 2012 and involved five different laboratories around the world. Only two out of five were able to provide measurements in seawater samples using wet chemical oxidation. However, results from this test revealed an unexpected large enrichment in $\delta^{13}C_{DOC}$ values (-9.3 and -11.0 ‰) and differences of up to 1.1 ‰ for seawater $\delta^{13}C_{DIC}$ samples (Van Geldern et al., 2013). Although the discrepancies were explained mostly by variations in sample composition more than by analytical errors, uncertainties remain and should be further investigated through more frequent inter-laboratory exercises and regular collaboration among institutions.

### 2.4.3 Isotopic corrections and data reporting

Stable carbon isotope data have to be reported with documentation of correction procedures, precision of replicate measurements and uncertainties of $\delta$-values. Different mathematical corrections can be applied to isotopic data, but in order to have inter-comparable results common procedures should be employed.

The first correction applied to $\delta^{13}C$ measurements accounts for the contribution of $^{17}O$ species ($^{12}C^{17}O^{16}O$) to the total abundance of m/z 45 in the $CO_2$ mass spectrum. The $^{17}O$ correction is often automatically applied by IRMS software but different algorithms can be chosen. The Craig (1957) and SSH (Santrock et al., 1985) algorithms are the most commonly applied, but the choice of one over the other will produce different $\delta^{13}C$ results if applied on the same dataset as the values of the fractionation factor $\lambda$ ($^{17}O/^{16}O$ over $^{18}O/^{16}O$) are different. In 2010 the International Union of Pure and Applied Chemistry (IUPAC) published a technical report (Brand et al., 2010) with the purpose of setting a universal correction algorithm. A single $\lambda$ value ($\lambda$=0.528) was suggested and for data consistency and inter-comparability it was recommended that specific deviations from this value are documented.

Besides isotopic fractionation due to temperature and pressure changes, $\delta$ values of an analytical sequence can drift with time and may vary according to the size of the sample. The drift is corrected by the analysis of identical standards placed within the analysis sequence while the variation of the measured $\delta$ value versus the respective signal intensity is corrected through a mathematical linear regression. The slope is determined by plotting the $\delta^{13}C$ values of repeated measurements of a standard with increasing concentrations versus the respective peak amplitudes. A further normalisation correction is applied so that all carbonate $\delta$ values are

referenced to the international standard V-PDB. The recommended normalisation requires at least two reference materials (e.g. NBS19 and L-SVEC for inorganic carbon (Coplen, 1996)) that cover the range of the $\delta$ values assumed by the samples. The calculation consists of determining a "stretch factor" m and an "off-set" b linked by the following mathematical equation:

$$\delta_{true} = m * \delta_{raw} + b \quad (2.2)$$

where $\delta_{true}$ is the internationally accepted value and $\delta_{raw}$ is the measured value. The stretch factor m indicates the gradient of deviation of the measured values from the certified isotopic values of the two reference materials. To achieve better estimates more than two points can be used and a best fit regression line can be derived.

In order to accurately measure stable carbon isotopes, it is necessary to identify, quantify and correct for blank effects. In general two types of blanks can be identified: (1) a system blank which is associated to the carbon introduced by analytical gases, reagents and instrument itself and (2) a blank deriving from the carbon present in the water used to dissolve standard material. The quantification of the blank can be undertaken indirectly through iterative nonlinear optimisation procedures (Bouillon et al., 2006) if the isotopic signal is too low to be accurately measured, or directly through an isotopic mass balance equation (Panetta et al., 2008) if the signal is measureable and relatively constant. Once the isotopic ratio and signal intensity of the blank are determined, the following equation is applied to the measured samples in order to correct for the blank contribution:

$$\delta^{13}C_{sample} = \left[\frac{(\delta^{13}C_{meas} * A_{meas}) - (\delta^{13}C_{blank} * A_{blank})}{(A_{meas} - A_{blank})}\right] \quad (2.3)$$

where $\delta^{13}C_{meas}$ and $\delta^{13}C_{blank}$ are the isotopic ratios of sample and blank, respectively and $A_{meas}$ and $A_{blank}$ are the respective peak areas (signal intensities).

Together with isotopic results, it is important to assess and report the uncertainties of the measurements. The $\delta$ value uncertainties are derived from the precision of replicate measurements and from all the above correction and calculation procedures. The combined uncertainty ($u_c$) estimate can be mathematically expressed by the square root of the sum of the individual uncertainties squared (Eq. 2.4)

$$u_c = \sqrt{u(x_1)^2 + u(x_2)^2 + \cdots u(x_n)^2} \quad (2.4)$$

where $u(x_1)$… $u(x_n)$ define the contribution of the different uncertainties expressed as standard deviations. It is important to consider the different sources of error as independent variables and calculate the uncertainty directly from the raw values. Kragten (1994) introduced a quick and applicable spreadsheet method for the calculation of the uncertainty and his approach, if correctly performed, can be adapted to estimate the error in stable carbon isotope

measurements. As no universal protocols for stable carbon isotopes analysis in seawater samples yet exist, a description of the method used along with the correction procedures applied must be reported every time data are submitted.

## 2.5 Applications and data interpretation

Stable carbon isotope data are widely used as natural tracers in marine systems. They are applied to determine the accumulation rates of anthropogenic $CO_2$ (Quay et al., 2007), define carbon utilisation pathways in microbial communities (Druhan et al., 2014), estimate variations in the paleo-oceanic productivity and phytoplankton composition/preservation patterns (Veizer et al., 1999), and identify and quantify fluxes of carbon between the inorganic and organic pools in mesocosm experiments (de Kluijver et al., 2010). In light of the recent focus on global change and the related uncertainties on the effects of the increasing anthropogenic $CO_2$, many outstanding questions on carbon fluxes and dynamics in the marine environment remain. The $CO_2$ added to the atmosphere as a result of the combustion of fossil fuels contains on average approximately 2% less $^{13}C$ per mole than ambient atmospheric $CO_2$ (Keeling et al., 1979). As most of the $CO_2$ that is transferred into the ocean is dissolved in the form of bicarbonate and carbonate ions, it is generally assumed that there is an isotopic equilibrium between the atmospheric $CO_2$ and the DIC. The conversion of DIC into organic matter during primary production and the conversion into inorganic form during remineralisation contribute to generate large variations in the oceanic carbon isotope distribution. When stable isotopes are applied to investigate carbon dynamics and the effects generated by the anthropogenic carbon component on the marine system, good knowledge of the signature of all the potential carbon sources and pathways is required. Measurements of $\delta^{13}C$ in all the organic and inorganic seawater samples could help us to follow the anthropogenic carbon component within the marine carbon system and provide useful additional and independent information about the temporal and spatial distributions of this component in the oceans.

The advantage of using stable isotopes is based on the fact that each carbon source is characterised by a specific signature which can be measured and traced through natural processes. However isotopic studies may not always provide clear results and interpretation of the data can be challenging. When statistical tests are used for data interpretation, the methods must be carefully chosen according to the number, structure and distribution of the samples, and according to the study objective (Oulhote et al., 2011). Discrimination between sources can be obtained using simple statistical analyses (t-tests, ANOVA and scatter plots), however when isotopic signatures among compartments are similar and different sources are involved, more complex linear mixing models (Phillips et al., 2005) should be employed. Recently, Bayesian

approaches for the interpretation of isotopic datasets have been proposed (Semmens et al., 2009). This statistical analysis has the advantage of taking into account the distribution of actual values including their variability and analytical uncertainties.

## 2.6 Conclusion and recommendations

Stable carbon isotope measurements in seawater samples can improve our understanding of natural processes underlying carbon dynamics and provide useful information for predicting the effects of environmental changes in marine ecosystems. The development of modern isotope ratio mass spectrometers has offered the advantages of higher sample throughput and reduced manual operations, with also an increased number of analytical protocols and procedures which need to be inter-calibrated and/or certified in order to produce universally comparable isotopic datasets. A main concern in isotopic analysis is contamination during sampling and preparation of samples and standards. Sources of error in the measurements are derived from analytical systems and from inaccurate correction procedures. Instruments should be periodically checked and analytical performances should be monitored daily through stability and reproducibility tests. Data correction procedures must be carefully applied and recommended algorithms should be used. The accuracy of analytical methods must be assessed by running international CRMs and continuous inter-laboratory comparisons. As no isotopic certified reference materials that represent the complex structure of seawater samples exist, the use of A.G. Dickson and D. Hansell CRMs is encouraged. Reporting isotopic values of these consensus reference materials to an international database will improve the global accuracy of isotopic measurements of marine water samples. Data interpretation can be complex and statistical methods that allow comparisons among different studies should be established. Despite these challenges, stable carbon isotope measurements can offer an additional line of evidence for understanding carbon sources and cycling of the complex marine system.

# Chapter 3: Coupling of a total organic carbon analyser to an isotope ratio mass spectrometer

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In preparation, to be submitted to the journal ACA or Talanta.

## 3.1 Introduction

The mechanisms underlying the global and oceanic carbon cycles can be better understood when the sources and dynamics of organic carbon are revealed. Many questions on the marine sources and pathways of dissolved organic carbon (DOC) remain, as a full understanding of the mechanisms of formation and removal of the semi-liable and refractory fractions in the surface and deep ocean is lacking. Stable isotope analysis offers a powerful tool for identifying sources and defining transport pathways. Measurements of the stable isotopic composition of DOC ($\delta^{13}C_{DOC}$) have been used in the past for carbon source identification and to track carbon dynamics. However most of the studies have focused on rivers and estuaries (Bauer & Bianchi, 2012) and only a few articles describing open ocean $\delta^{13}C_{DOC}$ dynamics are found in literature (Bauer, 2002; Hansell & Carlson, 2014). The reason is mainly due to analytical challenges that have precluded the wide-spread determination of $\delta^{13}C_{DOC}$ measurements of saline samples. The isotopic signature of the DOC pool in the marine environment is difficult to determine because samples consist of a heterogeneous mixture of compounds (sometimes refractory), concentrations are generally low (< 80 µM) and inorganic salt content is typically relatively high (30-35 g/l); these issues make $\delta^{13}C_{DOC}$ measurements long and problematic.

To date there is no commercially available instrument or a universal analytical method able to fully overcome these challenges and although several analytical strategies have been employed, these have precluded routine measurement of $\delta^{13}C_{DOC}$ in marine samples. High temperature catalytic oxidation (HTC) is the most widespread method of choice for quantitative DOC measurements, and hence hyphenation of HTC total organic carbon (TOC) analysers to isotope ratio mass spectrometry (IRMS) instruments appears to be the most attractive option for $\delta^{13}C_{DOC}$ measurements in marine samples. Some of the advantages of using HTC-TOC analysers include high oxidation efficiency, minimal and no direct interference of inorganic salt on the measurements, high precision and reproducibility, small sample volumes allowing multiple injections and rapid sample throughput. However, to accurately measure the isotopic composition of a small amount of carbon in samples with a low DOC concentrations (as low as 0.5

mg C/l for deep seawater samples), commercial HTC-TOC analysers need to undergo some major modifications so that specific analytical conditions can be satisfied. Moreover, interface devices or additional analytical steps are required for IRMS analysis.

The first direct coupling of an HTC-TOC analyser to an IRMS for the simultaneous concentration and stable isotope measurements of DOC in seawater samples was described by Lang et al. (2007). Here the $CO_2$ gas resulting from the combustion was cryogenically trapped and focused before isotopic measurements, Panetta et al. (2008) and De Troyer et al. (2010) developed comparable systems and greatly improved the precision of the method (±0.3‰). The earlier approach had a high $CO_2$ background interference and potential carryover effect, and the latter set-up was assembled for analysis of high carbon content soil solution samples only. A more recent combination of a DOC analyser to an IRMS (Lalonde et al., 2014) greatly increased sample throughput and reduced user error through a full automation of the system. Sample injection volumes were 0.5-1 ml and a precision of ±0.5‰ was obtained on deep seawater samples. However appropriate and careful blank corrections were needed as system blank isotopic signature was quite variable and ranging between -20‰ and -12‰.

In this chapter a new TOC-IRMS combination for both quantitative and isotopic measurements of DOC samples is described. A widely used TOC analyser has been mechanically modified along with the chemical sample handling system, and subsequently connected to an IRMS instrument with the aim of performing highly accurate and precise analyses of the natural abundance stable carbon isotopes in seawater samples. Modifications addressed minimising background noise problems and enabling a simple maintenance of the system. Analysis time was reasonably short (about 20 min per sample) and running costs were low, thereby making the system suitable for routine $\delta^{13}C_{DOC}$ analysis of marine samples.

## 3.2 Experimental section

### 3.2.1 Total Organic carbon analyser

The TOC analyser used in this study is a Shimadzu TOC-5000A. The analyser consists of a quartz total carbon combustion tube filled with platinum-coated (0.5 %) alumina beads (Shimadzu) and heated to 680 °C, an inorganic carbon (IC) reaction vessel, a humidifier, a dehumidifier and a non-dispersive infrared detector (NDIR). Moistened oxygen carrier gas is supplied to the combustion tube at a flow rate of 150 ml/min. Small aliquots (typically 100 µl) of sample are injected directly into the combustion tube, where the organic carbon is oxidised and thereby generating $CO_2$ gas. The gas is then carried through the IC reaction vessel, cooled and dehumidified. A halogen scrubber and a dust-eliminating membrane (0.45 µm) filter the

generated gas before it enters the NDIR, where the $CO_2$ is detected. The TOC Control software processes the NDIR signal generating peaks that are proportional to the amount of infrared light absorbed by $CO_2$ molecules. Measurements of dissolved inorganic carbon (DIC) are carried out by bypassing the TC combustion step and direct injection of the sample into the IC reaction vessel which contains a few drops of a 25 % phosphoric acid (ACS grade, Fisher Scientific). Because of basic incompatibilities between the standard TOC analyser set-up and the IRMS, a simple and direct coupling of the two instruments would not satisfy the essential analytical conditions. For a successful coupling of TOC analysers to an IRMS device, specific modifications are needed. The changes have to fulfil the following criteria:

- The system must be closed to any inflow of atmospheric air as even trace amounts of atmospheric $CO_2$ can disturb the isotopic signal.
- The $CO_2$ generated from the combustion or from the acidification of the sample has to be moisture-free to prevent interferences by water.
- High purity helium (Grade 5.0 or above) instead of $O_2$ gas should be used to carry the generated $CO_2$ into the mass spectrometer, as the presence of $O_2$ in the ion source would negatively affect the accuracy and precision of the measurements and reduce the lifetime of the IRMS filament (Hettmann et al., 2007).
- Sample carrier flow rate has to match the IRMS requirements. An interface device able to deliver the sample gas at the appropriate pressure (≈1 bar) and flow rate (≈0.3 ml/min) is necessary.
- The sample analysis time must be controlled and constant. Different timing would result in different sample amounts being analysed and hence potentially different isotopic ratios, especially if the effluent gas is trapped cryogenically or on a molecular sieve (Bauer et al., 1992; Lalonde et al., 2014). Full automation of the system through software communication between instruments is a benefit.

In order to satisfy these criteria, the Shimadzu 5000A TOC analyser was modified in some of its chemical, mechanical and software components. Details of the modified TOC analyser are shown in Figure 3-1. A description of the changes applied follows below.

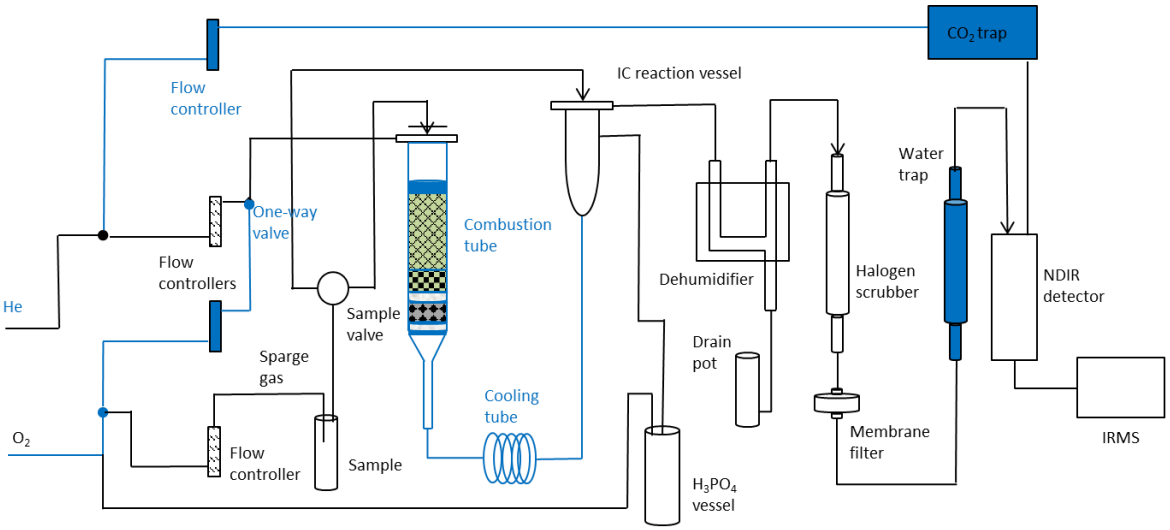


**Figure 3-1**. Schematic of the Shimadzu 5000A TOC analyser. The main modifications are marked in blue colour.

### 3.2.1.1 Chemical modification

The use of oxygen as carrier gas in the standard TOC analyser setups is to ensure complete oxidation of the organic material contained in a water sample injection. However, $O_2$ carrier gas is not suitable for $\delta^{13}C_{DOC}$ measurements as high levels of oxygen would alter the IRMS signal and hence change the carbon isotope signature. Furthermore, the IRMS instrument requires high purity helium as carrier gas. The first TOC analyser modification, therefore, had to account for a change in the gas. The use of He instead of $O_2$ as carrier gas in an unmodified TOC analyser would most likely reduce the oxidation efficiency in the combustion tube, in order to avoid this, a new column design was employed. The original Pt-coated alumina beads were replaced by new packing material placed in the column in the following order: an upper layer of fused silicon dioxide granules (4-20 mesh size, Sigma Aldrich) mixed with copper oxide (3-5 mm rods size, Elemental Microanalysis), a middle layer of solely CuO rods and a lower layer filled with silvered cobaltous/ic oxide (0.85-1.7 mm granules size, Elemental Microanalysis). Two platinum gauze disks (Elemental Microanalysis) were placed at the top of the column and two more at the bottom. A schematic of the modified combustion column layout is shown in Figure 3-2. The use of quartz beads mixed with CuO has already been previously reported by Qian & Mopper (1996) and Panetta et al. (2008) and it proved to produce lower blanks with oxidation efficiencies comparable to Pt-based catalysts. The silvered cobaltous/ic oxide acts as a first halogen trap while the platinum meshes catalyse the oxidation reaction.

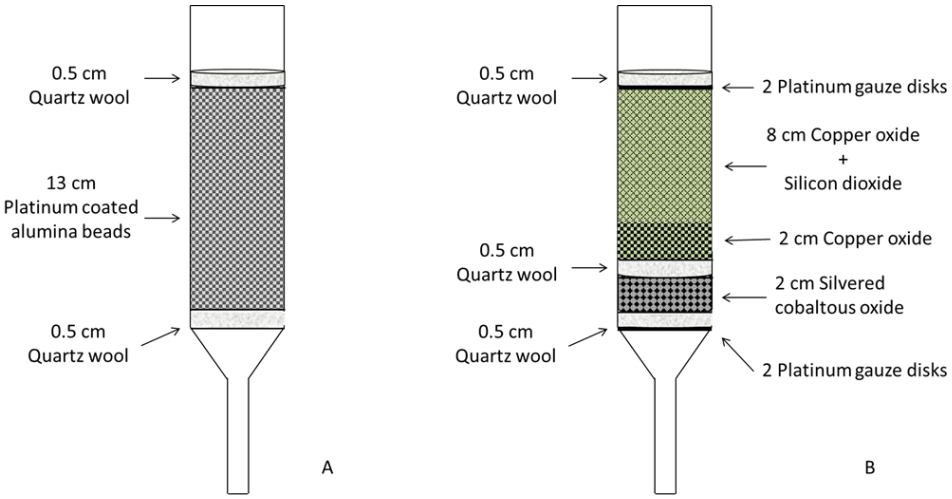


**Figure 3-2.** Schematics of the TOC combustion columns. Original (A) versus modified (B) layouts.

When a large batch of samples was analysed, water vapour resulting from the combustion seemed to pass through the TOC analyser Peltier cooler. Although no signal alterations were noticed in the NDIR detector, a high water background (peak intensity >4000 mV) was recorded by the IRMS (m/z 18). In order to effectively dehumidify the gas stream, a magnesium perchlorate (ACS grade, Sigma Aldrich) water trap was inserted just prior the infrared detector.
A further modification was the introduction of a $CO_2$ trap consisting of sodium hydroxide on a silica support (0.8-1.6 mm granule size, Sigma Aldrich) just prior the NDIR reference gas inlet in order to deliver reference $CO_2$-free gas for accurate quantitative and isotopic DOC measurements.

#### 3.2.1.2 Mechanical modifications

Air constitutes one of the main sources of contamination for $\delta^{13}C_{DOC}$ analysis. In order to prevent atmospheric $CO_2$ invasion to the system, the polytetrafluoroethylene (PTFE) tubing of the TOC analyser was replaced with 1/8 inch outer diameter (o.d.) stainless steel tubing and appropriate fittings; it has been shown that PTFE material allows diffusion of air through the tubing walls (Huygens et al., 2005) thus increasing the risk of isotopic $CO_2$ signal alteration. Stainless steel is impermeable so no gas exchange can occur. The only tubing that was not replaced with stainless steel was the sample and waste tubing. The sample tube was substituted with new polyether ether ketone (PEEK) tubing in order to allow visual inspection during sample uptake and injection, while the waste tubing was replaced with a 1/8 inch o.d plastic tube from a 1/2 inch o.d. one in order to reduce the outflow of the $CO_2$ gas.

The carrier gas humidifier and high purity water trap were removed. The former was disconnected in order to decrease the water background while the latter was bypassed in order to prevent $CO_2$ dissolution and potential isotopic fractionation into the collected condensed water. The elimination of the humidifier and the water trap reduced the total volume of the flow path

and a change to peak retention times was observed. The installation of a longer tube (1.5 m) after the combustion tube and the reduction of the carrier gas flow rate from 150 ml/min to 100 ml/min resulted in a stable signal with a maximum peak measurement time between 190 and 210 seconds.

Although the main carrier gas used was high purity He (Grade 5.0, BOC Ltd), an oxygen pulse (30ml/min) was applied during every sample injection. The pulse was controlled mechanically by a Swagelok SS Poppet Check Valve connected to a rotameter. The oxygen gas was automatically introduced into the He carrier gas flow just before the combustion tube as a consequence of a drop in the carrier gas pressure due to the kinetics of water vaporization (Peterson et al., 2003) in the combustion chamber immediately following a sample injection.

Finally, an additional pressure regulator and a flow controller were added to the reference gas flow line immediately before the custom made $CO_2$ trap in order to deliver pure He at a constant flow rate (100ml/min) to the analyser NDIR reference inlet.

#### 3.2.1.3 Software modifications

The TOC analyser used in this study was controlled by TOC control 5000/5000A software (version 1.05.01, Shimadzu Corp., Japan) running under a Windows 95 (version 4.00.950 B) environment installed on a desktop computer. The first sensible step to take was the replacement of the obsolete desktop computer. Since the original disk files for a new installation were not available and the program was not compatible with higher operating system environments, a full disk image backup was undertaken. Acronis True Image 2014 (version 17.0.0.1646) was used to back up the entire computer including the operating system. In order to execute the program, the Oracle VM VirtualBox (version 4.3.20) cross-platform virtualisation application was installed on a Windows XP desktop computer where the IRMS software (Isodat 3.0) was running. Following appropriate COM port configuration settings, VirtualBox allowed the simultaneous control of the TOC analyser and all other peripheral devices (EA, GC, Conflo IV, CTC Combi-Pal autosampler, GasBench, PreCon) connected to the IRMS by using the main desktop computer only.

### 3.2.2 “Bridge” interface

The gas stream emerging from the TOC analyser was trapped at liquid nitrogen (LN) temperatures (-196 °C), focused and purified before the isotopic measurement. A PreCon (Thermo Finnigan) device connected to a GasBench II (Thermo Finnigan) was used as a bridge interface between the TOC analyser and the IRMS. The peripheral consists of a high flow part (20-25 ml/min) which carries the sample through a 1 mm i.d. stainless steel tube immersed in a LN bath where condensable gases ($CO_2$ and $N_2O$) can be trapped, and a low flow part (1-2 ml/min)

which transfers the trapped gases to a smaller trap (0.5 mm i.d. stainless steel tube) and subsequently to the 30 m long GC PoraPLOT Q column (Agilent) of a GasBench II device for gas separation. A chemical trap for $CO_2$ (ascarite) and a combustion reactor are placed just after the sample container (100 ml glass vessel).

Two major modifications were carried out on the original PreCon device in order to receive the $CO_2$ gas generated from the TOC analyser in a continuous flow mode: the substitution of the sample container with a custom made valve system connected to an extra trapping loop (60 cm long, 1/8 inch i.d. stainless steel tube filled with Ni wire for quantitative trapping) and the replacement of the $CO_2$ chemical trap with a water and halogen trap containing magnesium perchlorate and silvered cobaltous/ic oxide (Figure 3-3). Isodat PreCon software was modified in order to match the TOC analysis timing with the run sequence script (Appendix C, Isodat PreCon Script) controlling the entire process from the sequential trapping (3 separate traps) to the GC column sample transfer.

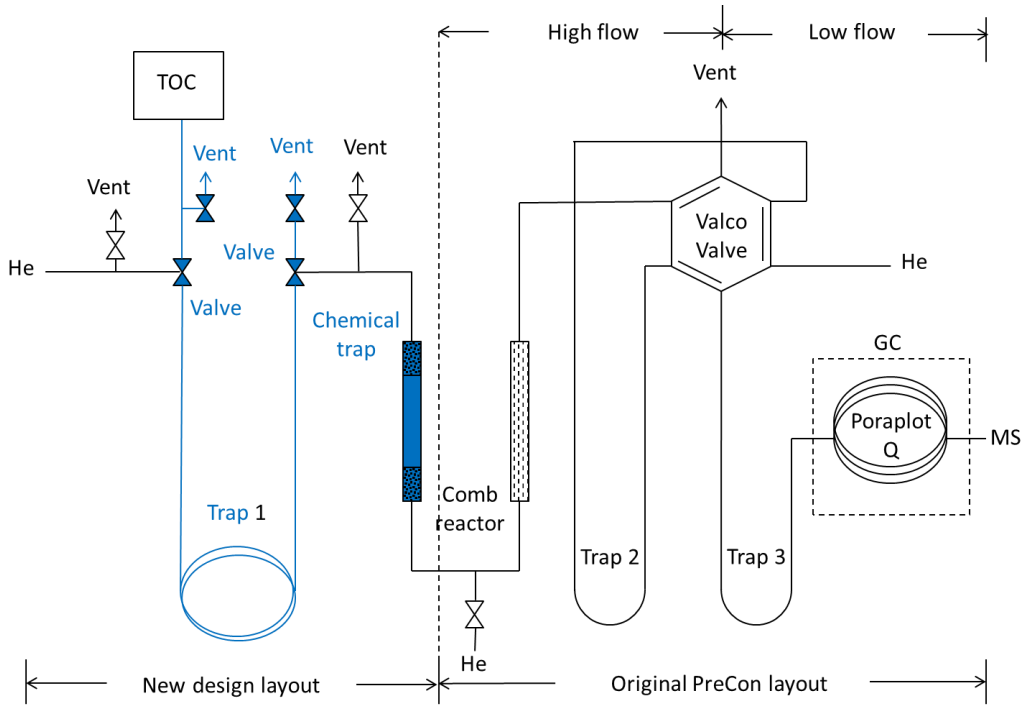


**Figure 3-3.** Schematic of the interface connection between the TOC and the IRMS. Modifications are marked in blue colour. Adapted from Brand (1995)

### 3.2.3 Isotope ratio mass spectrometer and pre-run checks

The IRMS instrument used in this work is a Thermo Scientific Delta V Advantage configured to receive reference and sample gases in continuous flow mode. All aspects of the mass spectrometer, including ion generation, mass separation, ion detection, ion source tuning, data evaluation and storage are controlled by Isodat 3.0 software. Prior to sample analysis, a series of checks was performed in order to minimise isotopic variations in the analytical procedures.

System conditions were kept constant (He= 1.2 bar, $CO_2$= 0.9 bar, Vacuum= $1.8*10^{-6}$ mbar, electron currents box/trap=0.7/0.8 mA) for all the runs and monitored before the start of any analysis. Periodic leak checks were performed by spraying argon gas on valves and connections while monitoring m/z 40. Water levels were monitored daily and analyses were performed only when the background signal was stable and within the acceptable limits (less than 3 V).

The stability and linearity of the system were further checked for both constant and variable signal intensities, respectively, through sequential isotopic measurements of the reference gas (Research grade $CO_2$ N5.0, BOC Ltd). Moreover, the peak intensities of the reference gas were finely tuned to match the expected sample intensities (m/z 44 =6900 mV, m/z 45 =8000 mV, m/z 46 =9700 mV) and the $\delta^{13}C$ values were recorded in order to check for any long term shifts in isotopic composition (gas-liquid fractionation in the working gas cylinder). The helium supply was checked for any $CO_2$ contamination by cryogenically trapping the carrier gas for 8-10 minutes and analysing it as a sample. Daily measurements of TOC background and ultrapure water (MilliQ, Millipore) injections were performed at the start and end of any run in order to quantify and characterise background signal as well as to detect any possible drift.

### 3.2.4 Reagents, standards preparation and calibration

No certified reference material for $\delta^{13}C_{DOC}$ measurements is currently available. In order to examine the accuracy and precision of the system, precisely weighed amounts of potassium hydrogen phthalate (ACS grade, Fisher Scientific), urea (analytical grade, IVA Analysentechnik), glutamic acid (99 %, Acros Organics) and sucrose (99+ %, Acros Organics) were dissolved in ultrapure water (Milli-Q water, resistivity 18.2 MΩ·cm). Potential traces of inorganic carbon were removed by acidifying the stock solutions to pH 2 using analytical reagent grade HCl (Fisher Scientific). A series of standard solutions (30-750 µM range) were prepared daily by dilution of variable volumes of the prepared stock solutions in pre-combusted (450 °C for at least 8h) borosilicate glass vials (24 ml). The effect of salt on the combustion efficiency was evaluated through the analysis of standards prepared in increasing sodium chloride (NaCl) concentrations (equivalent to salinities of 0, 15 and 35). The carbon content of the standards and samples was verified by periodic analysis of aliquots of oceanic deep seawater consensus reference material (Lot No 8-08, Hansell Laboratory, University of Miami), which is widely used as an inter-comparison material for quantitative DOC measurements. The isotopic composition of these compounds was also separately determined on an EA-IRMS (Flash EA 2000 Thermo Scientific) by repeat analysis of weighed amounts of each compound and calibration against international standard materials (IAEA-600, $\delta^{13}C$= -27.77 ‰; IAEA-CH-6, $\delta^{13}C$= -10.45 ‰; NBS-18, $\delta^{13}C$= -5.01 ‰) provided by The International Atomic Energy Agency (IAEA, Vienna). Several other carbon

containing substances were analysed in order to obtain a wide diversity of in house calibrated standards (Appendix B, EA Calibration). Sample concentrations were calculated from the linear relationship between the DOC areas versus standard solutions with known DOC concentrations, while the carbon content of the blank was determined from the y-intercept. The $\delta^{13}C$ values of the samples were expressed as parts per mil (‰) deviations from the international standard Vienna Pee Dee Belemnite (VPDB) and calculated from the best fit regression line derived by analysis of at least three different standards with known isotopic composition. Procedures for the determination of the background isotopic signal and mathematical corrections applied to the data are described in the following section.

## 3.3 Results and Discussion

### 3.3.1 Analytical performances

High temperature combustion TOC analysers employ repeated injections of a small amount (typically 100 µl) of sample. Each injection generates a peak that is proportional to the amount of carbon contained in a given sample. Heights and shapes of the peaks are determined by the concentration of the sample injected but also vary with system pressure and carrier gas flow rate. The unmodified Shimadzu 5000A TOC analyser is optimised to work at a pressure of 200 kPa and an $O_2$ carrier flow rate of 150 ml/min. After modifications and system combination, the internal volumes and analytical conditions changed and sample peak shapes became irregular, tailed and sometimes overlapped making integration difficult and reproducibility poor. The high flow rate negatively affected the combustion efficiency, probably as a consequence of the reduced combustion time for the sample within the column. The ideal Gaussian-like shape of a peak is therefore the consequence of sample concentration, system pressure and flow rate. With sample concentrations ranging between 30 and 750 µM, a working pressure of 300 kPa and carrier flow rate of 100 ml/min was applied in order to obtain acceptable peak shapes and maximal peak intensities. The higher pressure was necessary to push the gas through the trapping loop while the lower carrier gas flow rate increased the sensitivity and the sample retention time. Good reproducibility of the analysis was obtained and the variations among 4 to 5 repeated injections (over 2000 injections) of a water sample was always better than 2.5%.

The PreCon device was originally developed for the high precision isotope analysis of atmospheric gases (Brand, 1995). Based on sequential cryo-focusing, isotopic information about samples at nanomole to picomole levels was obtained. The addition of the extra trapping loop allowed receiving sample gas from the TOC analyser in a continuous flow without affecting quantitative measurements. Using sequential trapping gives the advantage of pre-concentrating

the small amount of the eluted $CO_2$ gas focusing it for a sharper IRMS peak shape. The efficiency of the added trapping loop was tested by trapping the gas for different time periods. Increasing the trapping time generated higher IRMS peak areas without affecting the isotopic ratios of the sample trapped (Figure 3-4).

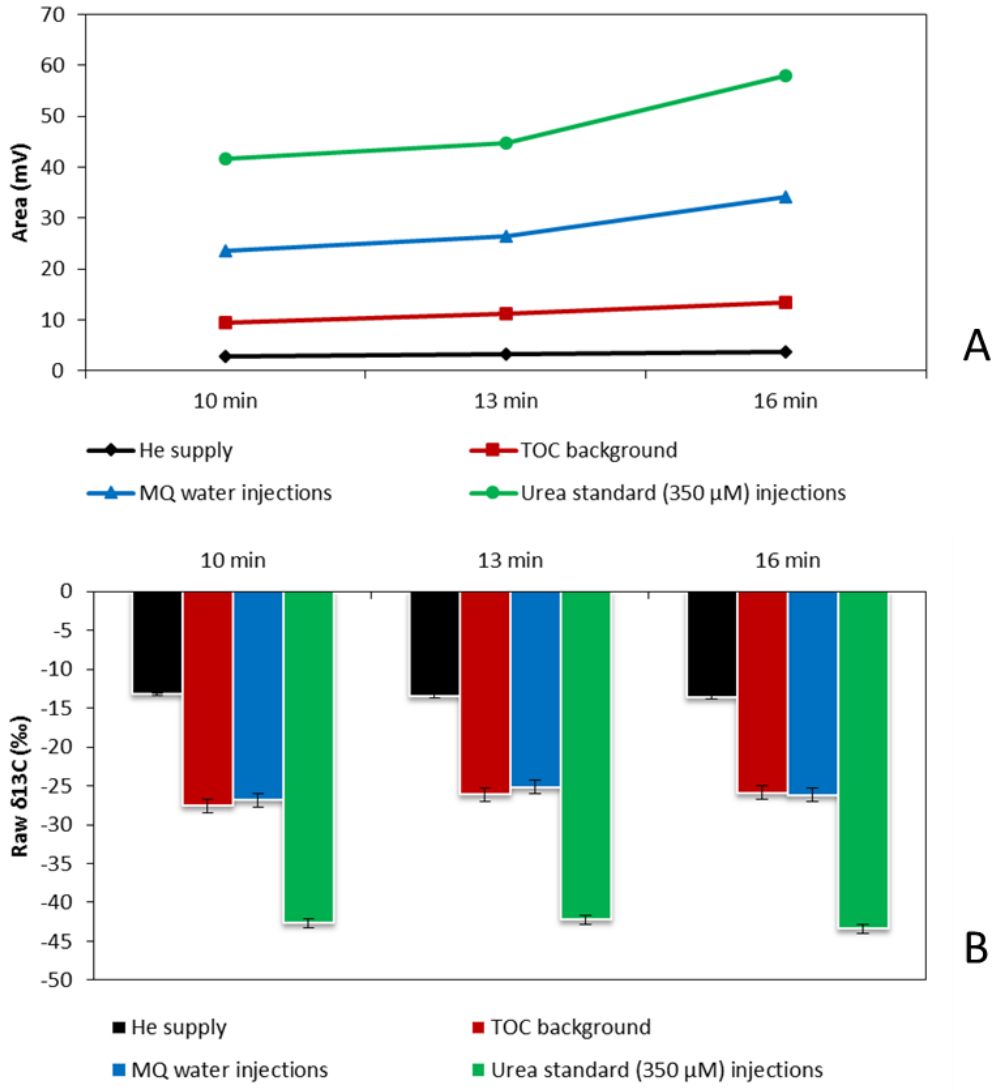


**Figure 3-4.** Efficiency of the trapping loop. Relationship between $CO_2$ trapping time (x-axis) with IRMS peak area (A) and isotopic signatures (B). Eluted $CO_2$ from the helium supply, TOC background, sequential injections (1 injection takes 3 minutes) of ultrapure water and urea standard was trapped in liquid nitrogen and analysed on IRMS.

The background $CO_2$ intensity is cumulative with time (2.5 ± 0.1 nmol of C/min), and therefore to keep sample to background ratio high, the trapping time was set to 10 min (three injections) for the majority of the samples. The magnitude of the TOC background and the relative contribution to the isotopic analysis is described below in a separate section. The test showed that 10 min trapping time was sufficient to obtain information on the $CO_2$ contamination in the He supply (bypassing the TOC analyser). The amount of $CO_2$ contained in two different He cylinders

was in general one third of the carbon TOC background and it exhibited a fairly constant isotopic signature ($\delta^{13}C$= -11.60 ± 1.45 ‰, n=22).

The analysis time of one sample ranged between 34 and 38 minutes but the sequences of sparging (8-12 min), trapping (10 min), cryo-focussing (6 min) and IRMS analysis (10 min) overlapped so that in a series of samples the analysis time was reduced to 16-18 min per sample. When a series of samples with different concentrations and isotopic signatures is analysed, cross contamination from mechanical components or un-combusted material can occur. In order to minimise potential sample carry-over, the sample syringe and tubing were flushed 4 times between injections and the sample trapping loop was flushed with high purity He (1 min at 25ml/min flow rate) after every sample. No carry-over effect both between injections and samples was noticed.

### 3.3.2 Oxidation efficiency and recovery test

Combustion efficiency and carbon recovery for DOC measurements were tested following the modifications of the TOC analyser. The use of helium as a carrier gas combined with the change of the combustion column packing material decreased the background $CO_2$ signal by an order of magnitude (from ≈2000 to ≈200 digital units) without affecting oxidation efficiency, as previously demonstrated for other similar system designs (Qian & Mopper, 1996; Panetta et al., 2008). Although the presence of water in the carrier gas is a source of reactive oxidising species that facilitate combustion (Chen et al., 2002), the removal of the humidifier was necessary in order to decrease the water background signal measured by the IRMS. Notwithstanding the use of a dry helium carrier gas, combustion efficiency appeared to be unaffected. The water contained in an injection together with the $O_2$ pulse might have been the factors providing adequate oxidation conditions.

The high dissolved salt concentration in seawater samples has historically been a problem for DOC analysis. The effects of salts include reduction of the efficiency of the catalyst (Skoog et al., 1997), memory effects due to random washing off of the salt deposits from the tube walls (Qian & Mopper, 1996) and clogging of the combustion tube (Panetta et al., 2008; Lalonde et al., 2014). The analysis of increasing concentrations (50 – 150 – 300 µM) of 5 compounds with different refractory properties (phthalate, caffeine, urea, glutamic acid and sucrose) dissolved at 3 different salinities (salinities of 0, 15 and 35) showed that the modifications carried out on the TOC analyser were not affecting either the combustion efficiency nor the recovery of carbon (Figure 3-5). Results from one-way ANOVA analyses showed no statistically significant salt effect on the combustion efficiency of all the different compounds ($p = 0.96$, 0.59 and 0.49 for salinity of 0, 15 and 35, respectively.

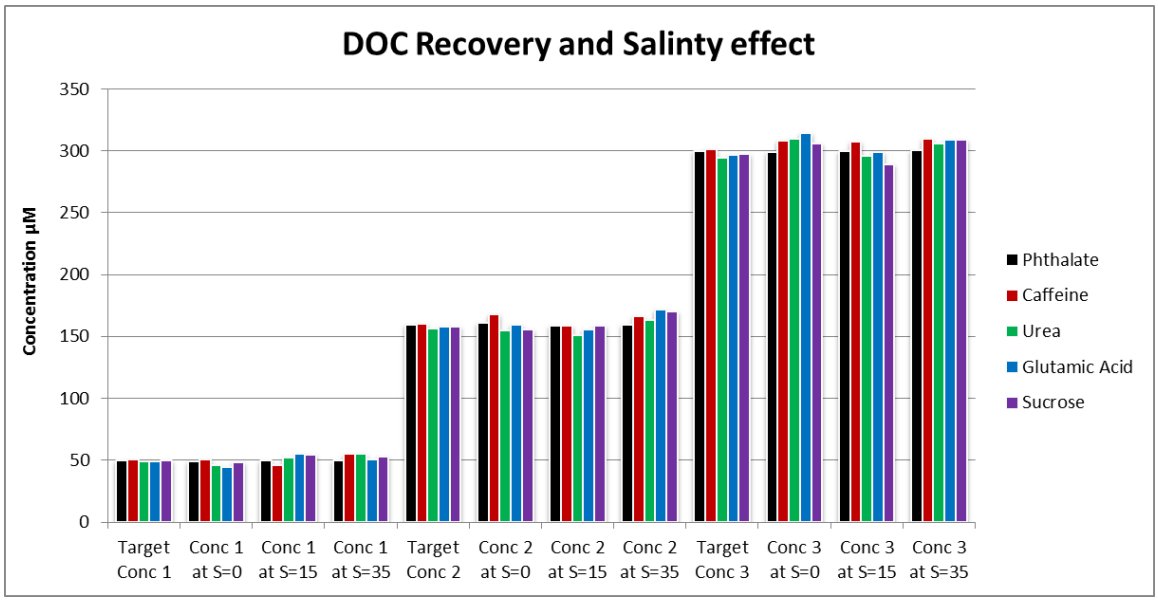


**Figure 3-5.** DOC recovery and salinity effect test. Analysis of three increasing concentration (50 – 150 – 300 µM) phthalate, caffeine, urea, glutamic acid and sucrose standards prepared in ultrapure water with increasing salinity values of 0, 15 and 35.

All components were measured accurately with good reproducibility among injections (variations less than 2%) and showed an average carbon recovery of 102 ± 3 % (relative to the phthalate standards) for the different components at the different concentrations and salinities. No direct effect of salt was noticed during the test however the number of injections of seawater samples is limited. After about 200 samples peaks started to appear with a delay and with a tailing end most likely because of carrier flow reduction due to the formation of a layer of salt in the combustion tube. In order to avoid overpressure and potential column breakage, columns were replaced after about 100 samples.

Halide gases generated from combusted salts negatively affect analytical precision, corrode system components and reduce the lifetime of the IRMS filament (Lalonde et al., 2014). Therefore, in addition to the TOC analyser halogen scrubber (Cu wire/wool) a silvered cobaltous/ic oxide layer was added before the $CO_2$ trapping loop and the PreCon furnace used to remove the residual volatile species. The effect of the chemical trap and the hot furnace (nickel wire at 200 °C) were clearly noticed when urea standards were run (higher N/C ratio compared to the other standards used). Prior to the installation of the trap and activation of the furnace, $N_2O$ was eluting from the GC column and detected by the IRMS. After the modifications, better precision was obtained and the $N_2O$ peaks were undetectable as nitrogen oxide gases were reduced to $N_2$ which could freely pass the cryogenic trap.

### 3.3.3 Blank assessment and background correction

In order to accurately measure DOC concentrations and stable isotopes, it is necessary to identify, quantify and correct for the blank contribution. In general two types of blanks can be identified: (1) a system blank which is associated to the carbon introduced by analytical gases, reagents and instrument itself and (2) a water blank deriving from the carbon contained in the water used to prepare analytical standards. The magnitude of the blank is relatively easy to quantify as it can be calculated from the TOC analyser response factor by injecting aliquots of "zero carbon" water. After column conditioning (over 200 injections of ultrapure water) blank values ranged between 4 and 10 µM of carbon, falling into similar previously reported concentrations range of 3 to 9 µM (Sharp et al., 2002; Peterson et al., 2003; Lalonde et al., 2014). The measured DOC blank values represent the sum of the system blank and the water blank. As the water used to prepare calibration standards and blanks was taken daily and directly from the ultrapure water purification unit (Merck Millipore Advantage), the carbon content was assumed to be insignificant. Therefore the $CO_2$ peaks generated by the injections of ultrapure water must be attributed to the system blank and deriving mainly from desorption of $CO_2$ from the combustion column packing material. As carbon adsorption and desorption rate varies with column characteristics and wait-time between injections (Peterson et al., 2003), analytical conditions were kept as constant as possible.

Although the presence of a low and constant intrinsic system blank was not affecting DOC measurements, the accuracy of stable carbon isotope analysis can be greatly affected if the signature of the eluted $CO_2$ gas is not accurately determined. Daily trapping of TOC analyser carrier gas and successive IRMS analysis showed that the system background was measurable (57-80 µg/l) with blank isotopic ratios ($\delta^{13}C_{blank}$) ranging between -29 and -26 ‰ over a period of 3 months and using 3 different combustion columns.

An alternative and commonly used method for the determination of the $\delta^{13}C_{blank}$ is however based on isotopic mass balance. The blank delta value is determined by measuring the isotopic signature of standard solutions at different concentrations and extrapolating the value from the regression line between delta values and the inverse of the peak areas (Fry & Brand, 1992; Panetta et al., 2008; Lalonde et al., 2014). In order to verify that sequential trapping of background $CO_2$ and regression analysis generate a similar isotopic signature of the system blank, 4 to 5 solutions with concentrations ranging between 60 and 500 µM of 4 different standard compounds (urea, sucrose, glutamic acid and phthalate) were analysed (Figure 3-6).

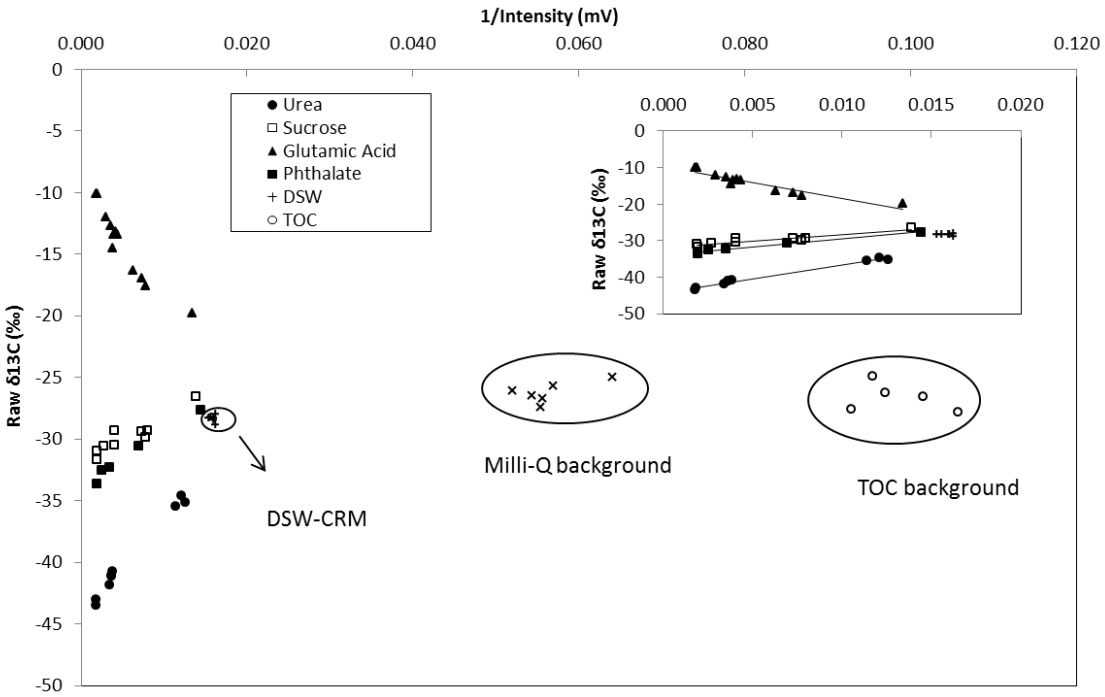


**Figure 3-6.** Comparison between direct and calculated determination of the system blank isotopic signature. Measured $\delta^{13}C$ values of urea, sucrose, glutamic acid and phthalate solutions with concentrations ranging between 50-500 µM are plotted versus the inverse of the signal intensity. The inset plot shows the linear functions used to calculate the isotopic composition of the blank. The circles highlight the intensities and $\delta^{13}C$ of trapped TOC background, ultrapure water and deep seawater reference material.

At the time of the test the isotopic signature of the blank determined directly from sequential trapping of TOC background was -26.6 ± 1.16 ‰ (n=5). The isotopic signature of the blank calculated from the slope of the urea standards was in good agreement ($\delta^{13}C_{blank}$ = -25.6 ‰) with the direct blank measurements, however, when the blank isotopic ratio was calculated from the average of the four standard compounds, more enriched values ($\delta^{13}C_{blank}$ = -21.3 ± 3.8 ‰) were produced. It must be pointed out that although the standards were prepared at the same time and using the same ultrapure water, the test was performed over 4 days, with urea being the first compound to be analysed (day 1) so that possible contamination of the other standards might have occurred. Nonetheless, variations towards more enriched blank values during a few days of regular use of properly conditioned combustion columns have been previously noticed, with reported values shifting by up to 8 ‰ (Panetta et al., 2008; Lalonde et al., 2014).

Although the magnitude of the blank can be reasonably stable, small variations in the isotopic signature appear to be still unavoidable. Daily measurements of the isotopic signature of the system blank at the beginning and at the end of any analysis are therefore essential in order to appropriately correct the sample values for the background contribution. The equation used for the background correction is based on isotopic mass balance and it is applied to every

measured sample as follow, once the blank isotopic ratio and signal intensity are determined (Panetta et al., 2008):

$$\delta^{13}C_{sample} = \frac{[(\delta^{13}C_{meas} * A_{meas}) - (\delta^{13}C_{blank} * A_{blank})]}{A_{meas} - A_{blank}} \quad (3.1)$$

In equation 3.1, $\delta^{13}C_{meas}$ and $\delta^{13}C_{blank}$ are the isotopic ratios of sample and blank, respectively, and $A_{meas}$ and $A_{blank}$ are the respective peak areas (signal intensities). All standards and samples were corrected for background contribution using the blank value measured on the specific day of the analysis.

### 3.3.4 $\delta^{13}C_{DOC}$ normalisation

During each individual analysis, the isotopic composition of the analyte gas was compared to the isotopic composition of the laboratory $CO_2$ reference gas in order to convert the raw $\delta$ values (measured) into the true $\delta$ value (international isotope reference scale). Three pulses of reference gas were injected into the IRMS just before the sample gas was eluted from the GC column and transferred into the ion source of the IRMS. Although the reference gas is expected to be clean and stable with respect to its isotopic composition, one point anchoring calibration is not ideal as it might introduce errors in the results that sometimes exceed the maximum total uncertainties often reported in the literature (Paul et al., 2007).

For best accuracy, a linear normalisation based on the best fit regression line generated by three or four reference standard materials (urea, glutamic acid, sucrose and/or phthalate) was used. The choice of the reference standards was determined by the isotopic composition of the samples to be analysed so that sample signatures fell within the range of the reference material isotopic signatures. Working standards were run at the beginning of each run and periodically within the run sequence. Moreover, varying concentrations of the standard compounds were used in order to obtain a proper estimate of the slope and correct for a concentration depended IRMS response. The normalisation applied consisted in plotting the certified $\delta$ values (true values) of the reference standards versus the measured $\delta$ values (Figure 3-7).

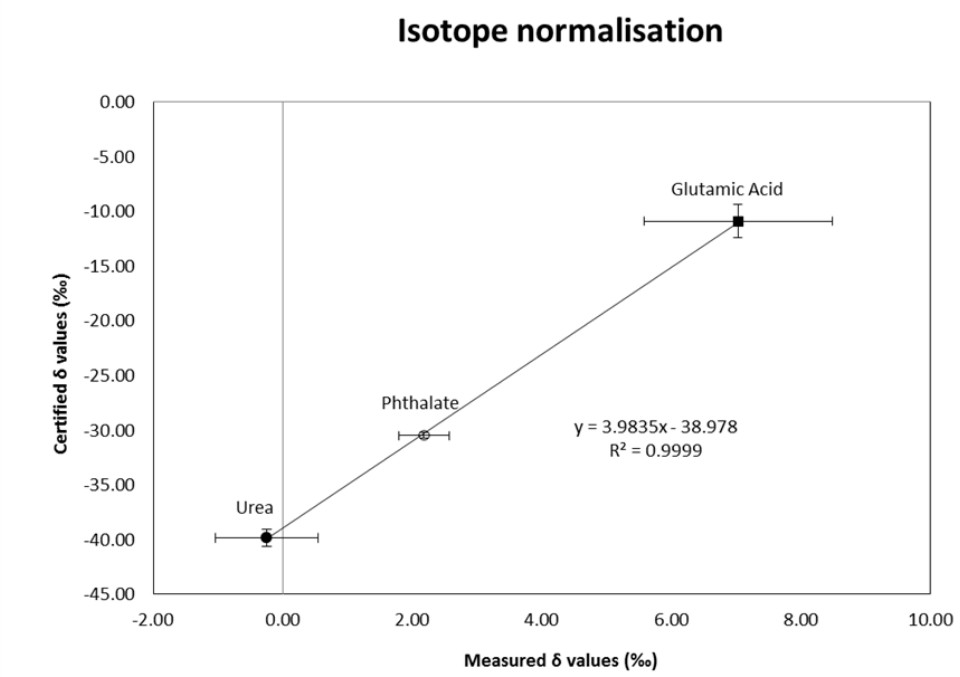


**Figure 3-7.** Three-point normalisation plot for the conversion of raw isotopic values of samples into true values (relative to the international isotope reference scale). Certified isotopic values of three standard reference materials (urea, phthalate and glutamic acid) are plotted versus measured values for the determination of the best fit regression line equation.

The determined regression line equation was expressed as follow:

$$\delta_{true} = m * \delta_{raw} + b \quad (3.2)$$

where $\delta_{true}$ is the internationally accepted value and $\delta_{raw}$ is the measured value. The slope m generally called "stretch factor", indicates the gradient of deviation of the measured values from the certified isotopic values of the three reference materials. The factor b is an "off-set" and should represent the true $\delta$ values of the working gas. The isotopic signature of the working gas determined by the three-point TOC-IRMS normalisation procedure was -38.97 ‰, however when compared to the true $\delta$ value calculated from the EA calibration curve (Appendix B) about a 2 ‰ depletion was noticed ($\delta^{13}C$= -36.69 ± 0.14 ‰). The resulting different isotopic compositions were due to the passage of the $CO_2$ reference gas through different analytical paths and components in the two systems. A similar difference has been noticed in a comparison study between multiple-point linear normalisation procedures applied to a GasBench device and an EA with reported $\delta$ values of the reference gas equal to -10.78 ‰ and -8.66 ‰, respectively (Paul et al., 2007). Notwithstanding the different calculated δ values of the reference gas, normalisation errors are reduced if analysis conditions are kept constant and multi-point calibration procedures are applied. Moreover with a three-point calibration it is possible to identify errors associated to analytical procedures. The good correlation ($r^2$ = 0.9999) calculated herein, indicates that the

analyser was performing well with efficient oxidation and successive trapping of the standard compound gases.

### 3.3.5 Accuracy of the isotopic measurements

The accuracy of the $\delta^{13}C_{DOC}$ measurements was tested by analysing a series of in-house standards (phthalate, urea, glutamic acid, sucrose and caffeine) calibrated against certified reference materials by EA-IRMS (Table 3-1).

| Substance | EA-IRMS | | | | TOC-IRMS | | | |
|---|---|---|---|---|---|---|---|---|
| | Weight (mg) | $\delta^{13}C$ (‰) | St Dev 1σ | n | Range mg/l | $\delta^{13}C$ (‰) | St Dev 1σ | n |
| **Phthalate** | 0.1-0.5 | -30.32 | 0.15 | 9 | 1.4-7.9 | -30.53 | 0.24 | 17 |
| **Urea** | 0.1-2.5 | -39.85 | 0.08 | 121 | 1.6-9.0 | -39.69 | 0.77 | 27 |
| **Glutamic Acid** | 0.1-2.0 | -10.75 | 0.21 | 40 | 0.4-6.6 | -10.68 | 1.52 | 27 |
| **Sucrose** | 0.1-1.5 | -26.63 | 0.14 | 38 | 1.4-5.4 | -26.50 | 0.80 | 15 |
| **Caffeine** | 0.1-0.5 | -33.31 | 0.08 | 33 | 1.0-1.9 | -33.77 | 0.89 | 2 |
| **DSW-CRM** | - | - | - | - | 0.52 | -19.12 | 0.36 | 6 |

**Table 3-1.** Comparison of $\delta^{13}C$ values of in-house standard materials determined by EA-IRMS and TOC-IRMS.

Small amounts (0.1 – 2.5 mg) of the pure compounds were weighed and analysed directly by EA-IRMS while standard solutions with concentrations ranging between 0.4 and 9 mg/l were prepared in ultrapure water and analysed by TOC-IRMS. All the standards were run as unknown samples both for the EA-IRMS and the TOC-IRMS systems. Comparable isotopic signatures were obtained although the precision of the $\delta^{13}C_{DOC}$ measurements from the TOC-IRMS analyser was slightly lower than that from the EA-IRMS system, particularly for low concentration standards. The isotopic signature of the glutamic acid was more variable for both systems ($\sigma$ = 0.21 and 1.52 ‰ from EA and TOC analysis, respectively). The low precision could be explained by inhomogeneity of the compound more than by analytical or instrument errors. However, the good correlation ($r^2$ = 0.9994) between the TOC-IRMS and the EA-IRMS analysis (Figure 3-8) indicate that no isotopic fractionation was occurring during the analysis.

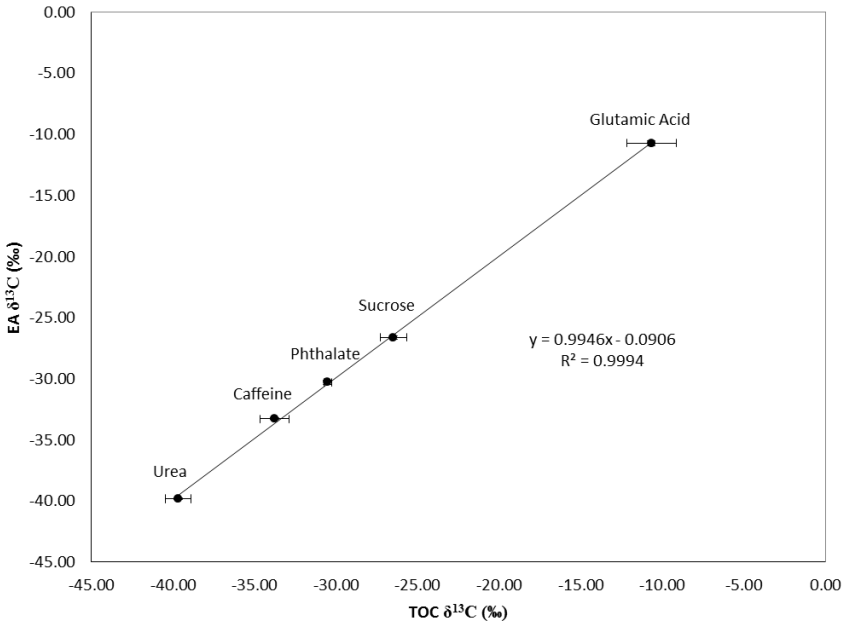


**Figure 3-8**. Correlation between isotopic measurements of standard materials determined by EA-IRMS versus TOC-IRMS analysis.

The regression line derived by plotting $\delta^{13}C$ measurements from the EA-IRMS versus the one calculated from the TOC-IRMS had a slope value of 0.9946 and an intercept value of -0.0906 showing agreement with the ideal values of 1 and 0, respectively which would indicate perfect combustion of the analysed compounds and no isotopic fractionation effects due to any analytical procedure (e.g. cryogenic trapping of $CO_2$ gas). A t-test was used to compare the slopes of the regression lines derived by plotting certified versus measured $\delta^{13}C$ values and no statistically significant differences ($r_1$, $p = 0.54$; $r_2$, $p = 0.52$) were found between isotopic measurements of the standards determined by EA and TOC systems. The average measured $\delta^{13}C$ value of each standard was always within ± 0.3 ‰ of the calibrated value.

Although oxidation efficiency and recovery tests performed on the combined TOC-IRMS system showed no combustion related problems or detrimental salt effects, all the compounds used for the preparation of the standards were simple soluble organic molecules that might have not fully represented the DOC structure of natural seawater samples. An international reference material for $\delta^{13}C_{DOC}$ measurements is currently not available however in order to prove the feasibility of using the instrument for analysis of natural seawater samples, deep seawater consensus reference material distributed from the laboratory of D. Hansell (University of Miami) and routinely used to validate quantitative DOC analysis, was run. The blank corrected isotopic signature of the consensus reference material (CRM Batch 8-2008) was -19.12 ± 0.36 ‰ (n = 6). During subsequent analysis, CRMs were periodically run in order to validate both quantitative measurements and day-to day variability of the isotopic measurements. The $\delta^{13}C$ values were on

average -19.49 ± 0.57 ‰ and they were similar to previously reported values of -19.5 ± 0.4 ‰ (Bouillon et al., 2006) and -19.9 ± 0.4 ‰ (Lalonde et al., 2014).

### 3.3.6 Application to natural seawater samples

The $\delta^{13}C$ signature of marine organic matter is set during photosynthetic fixation of $CO_2$ by phytoplankton (Bauer, 2002). The preferential uptake of the light carbon isotope ($^{12}C$) during photosynthesis (O'leary, 1981) causes the resulting organic compounds to be isotopically depleted in $^{13}C$ compared to the source. In marine environments carbon assimilation hence the isotopic fractionation depends on environmental factors such as temperature, light and nutrients (Fontugne & Duplessy, 1981), planktonic community structure (Falkowski, 1991; Burkhardt et al., 1999) and $CO_2$ concentrations (Kukert & Riebesell, 1998). However in the above mentioned studies fractionation rates were assessed through isotopic measurements of particulate carbon only and no $\delta^{13}C_{DOC}$ measurements were reported. In light of the recent focus on global change and the related uncertainties on the effects of the increased $pCO_2$ on marine ecosystems and biogeochemical processes, three long term mesocosm experiments (Sweden 2013, Gran Canaria 2014 and Norway 2015) were carried out in order to assess plankton community responses under future $CO_2$ level (up to 2000 ppm) scenarios. Using the modified combined TOC-IRMS system, measurements of $\delta^{13}C_{DOC}$ were performed. When samples were run in duplicate, precisions of ± 1 µM and ± 0.5 ‰ for DOC and $\delta^{13}C_{DOC}$ measurements, respectively were obtained. The $\delta^{13}C_{DOC}$ measurements of the fjord waters during the Swedish and Norwegian mesocosm experiments (-27 ‰ to -19 ‰ and -25 ‰ to -21 ‰, respectively) are comparable to previously reported $\delta^{13}C_{DOC}$ values between -28‰ and -24 ‰ in the northern Baltic sea during an annual cycle (Rolff, 2000) and $\delta^{13}C$ values of organic aggregates between -32‰ and -19 ‰ during an early spring fractionation study in the Gullmar fjord (Waite et al., 2005). No clear $CO_2$ response was observed in our study (Figure 3-9), however the approach was suitable for measurements of both DOC concentrations and stable carbon isotopes. About two months were required to analyse circa 200 samples with an average of 16 analyses per day including trapping of He carrier gas, TOC background, ultrapure water and 3 to 4 standards. Daily regeneration of the copper oxide was performed overnight by increasing the $O_2$ flow rate to 120 ml/min and reducing the He flow to 10 ml/min in order to keep optimal oxidation efficiency. When variations between multiple injections were consistently higher than 3%, high salt deposition or catalyst degeneration was presumed and therefore the combustion column material was replaced. Notwithstanding some unavoidable maintenance operations and/or daily environmental variations, the TOC-IRMS system is able to produce consistent data as long as the analytical conditions are monitored and keep constant.

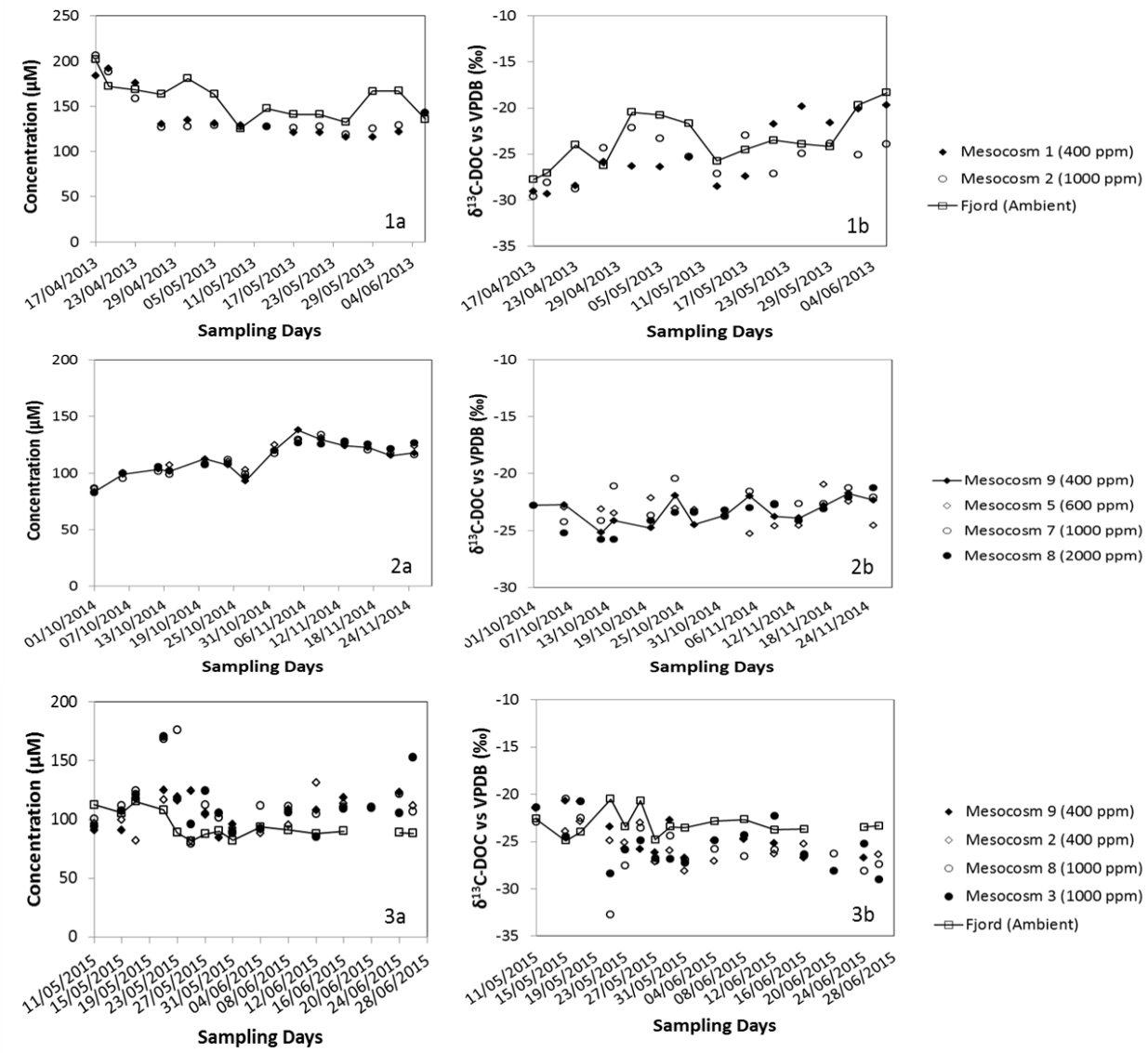


**Figure 3-9.** Measurements of dissolved organic carbon concentrations (a) and stable isotopes (b) from mesocosm experiments carried out in the Gullmar fjord, Sweden 2013 (1), in Gando Bay, Gran Canaria 2014 (2) and in Raunefjord, Norway 2015 (3). The lines show the control values as measured in the surrounding waters or in untreated mesocosms.

## 3.4 Conclusion and Recommendations

Measurements of the stable isotopic composition of DOC in seawater have historically been problematic and to date analyses are still quite laborious and challenging. Based on recent direct combinations of HTC-TOC analysers with IRMS instruments (Lang et al., 2007; Panetta et al., 2008; Lalonde et al., 2014), a new analytical set-up was assembled. The in-house TOC-IRMS system is able to perform parallel measurements of DOC concentrations and stable carbon isotopes with a precision of ± 1 µM and ± 0.5 ‰ for DOC and $\delta^{13}C_{DOC}$, respectively, in less than 20 min per sample when a sequence is analysed. The use of sequential trapping and cryo-focusing allowed $\delta^{13}C_{DOC}$

measurements of samples with concentrations as low as 40 µM (typical of deep seawater samples) with a precision of ± 0.4 – 0.6 ‰. The mechanical and chemical modifications performed on the system considerably reduced the background signal, however, small variations in the isotopic composition of the blank were still unavoidable although less variable (3 ‰ differences) than previously reported differences of up to 8 ‰ (Panetta et al., 2008; Lalonde et al., 2014). Appropriate blank correction procedures were therefore necessary in order to obtain accurate isotopic measurements. The system did not appear to be affected by sample salt content however oxidation efficiency loss must be monitored and combustion column should be replaced after about 100 samples. A multi-point calibration with standards of known isotopic signatures bracketing the range of the samples to be analysed was preferred to a single or two-point anchoring normalisation, in order to convert the raw isotopic values into true values (relative to the international VPDB scale). The strong correlation ($r^2 = 0.9999$) between the $\delta$ values showed optimal analyser performances. Results indicate that the system is able to oxidise and measure the $\delta^{13}C$ signature of different components (calibrated by EA-IRMS) with an average accuracy of ± 0.3 ‰ (relative to the pure compounds). However until the distribution of an internationally certified standard that represents the complex structure of seawater samples, $\delta^{13}C_{DOC}$ accuracy is still not assessed in an ideal manner; the regular use of deep seawater consensus reference material (Hansell lab, Miami) is recommended and although not isotopically certified, measurements showed good agreement with values reported in the literature.

## 3.5 Future Directions

The present combined TOC-IRMS system is able to perform $\delta^{13}C_{DOC}$ measurements of seawater samples with precision and accuracy comparable to the only two other similar set-ups reported in the literature (Panetta et al., 2008; Lalonde et al., 2014) however, few developments are being undertaken in order to potentially improve the analytical outputs. As blank contribution is still a problem for low concentration samples, future work will be addressed at increasing the sample to background ratio. A viable option could be to increase the volume of the injections to the point that a single injection is sufficient to generate a measureable signal. With one single trap the analysis time will be shorter (hence reduced trapping of background gas) and more replicate isotopic measurements would be allowed. However combustion efficiency and/or column resistance to breakage must be investigated and the possibility of new designs is not excluded. A direct consequence of an increased injection volume is the formation of a larger quantity of water vapour, installation of a more efficient dehumidification unit could be required.
Another potential improvement will consist of the installation of an autosampler which would undoubtedly reduce sample contamination and human error. A specific software program

controlling start/stop functions and trapping operations is being investigated. The advantage will be a potential increase in data reproducibility and sample throughput as a result of full automation of the hyphenated system.

The system's capability to deal with solutions containing high carbon concentrations (> 1 mM) was not investigated. However, considering that the TOC analyser is able to perform quantitative measurements of dissolved inorganic carbon (typically around 2 mM for seawater samples), the use of the combined TOC-IRMS system for the determination of the stable carbon isotope signature of DIC samples is not excluded. To solve the problem of an excessively large signal, only a single or no trapping of the $CO_2$ sample gas might be needed. The activation of the sample dilutor of the GasBench device which allows dilutions of up to 50 % of the analyte gas with pure He, could also be required.

As a final stage, potential future application of the TOC-IRMS system may include combined isotope carbon-nitrogen ($\delta^{13}C_{DOC}$ and $\delta^{15}N_{DON}$) measurements. Theoretically, only a few adjustments are needed to the system to perform dual analysis: the PreCon combustion furnace should be placed after the cryogenic trapping in order to retain $N_2$ gas, the carrier gas flow rate to the GC column should be set to an appropriate value to allow net chromatographic separation of the $N_2$ and $CO_2$ peaks, and the IRMS should be carefully configured to switch between masses (magnetic jump) for correct ion collection. The feasibility of using the combined TOC-IRMS system for dual isotope analysis must be further explored however, the development of an appropriate method and a successful configuration would certainly open the door to new approaches for investigation of organic matter sources and cycling in marine ecosystems.

# Chapter 4: Measurements of stable carbon isotopes in North Atlantic fjord waters: two mesocosm studies

## 4.1 Introduction

In the ocean interior, the abundance of carbon species is mainly determined by physico-chemical (solubility pump) and biological (soft tissue pump) processes. These mechanisms affect the transport and storage of both inorganic and organic carbon species. The flux of carbon from one compartment to another is currently being altered by human activities at an unprecedented rate of atmospheric $CO_2$ emissions. Measurements of stable carbon isotopes can contribute towards the quantification of the impact of increased atmospheric $CO_2$ concentrations on the marine carbon cycle. Various authors (Sonnerup et al., 1999; Quay et al., 2007) have used isotopic measurements of dissolved inorganic carbon ($\delta^{13}C_{DIC}$) to determine and quantify the spatial distribution of anthropogenic $CO_2$ in the ocean by assessing long term changes in oceanic $\delta^{13}C$. Others have used stable carbon isotope measurements to interconnect organic matter pools identifying sources and sinks in estuaries (Simenstad & Wissmar, 1985; Finlay & Kendall, 2007; Michener & Kaufman, 2007) and in a full-depth Arctic Ocean profile (Griffith et al., 2012). Several other studies have been carried out to assess the effects of $CO_2$ uptake on the stable carbon isotope composition of marine phytoplankton (Rau et al., 1996; Burkhardt et al., 1999; Hoins et al., 2016). Stable carbon isotope analyses of marine sediments have been widely used in the paleo community to infer past climate changes and they proved to be an effective tool to assess oceanic paleo-productivity and ancient preservation patterns (Berner et al., 1983; Veizer et al., 1999; Ghosh & Brand, 2003).

More recently, mesocosm experiments have offered the opportunity to compare parallel ecosystems and investigate the effects of increasing $CO_2$ concentrations on phytoplankton communities (Delille et al., 2005; Riebesell et al., 2007; Schulz et al., 2008; de Kluijver et al., 2010). However, no mesocosm study has been carried out so far to directly assess the effect of increasing $CO_2$ concentrations on the distribution of stable carbon isotopes in the marine system. In mesocosm systems, the isotopic signal can be followed through the various carbon compartments, from the dissolved inorganic pool to the settled particulate portion and the mechanisms driving its distribution can be linked to biogeochemical processes such as photosynthesis, organic matter oxidation and/or deposition. In order to assess the impact of human perturbation on the isotopic signature of carbon species, it is important to first have a full characterisation of the current carbon species profile. Subsequently, variations in the isotopic

signature of any carbon species due to different $CO_2$ conditions can be identified. Here, for the first time, the isotopic composition of inorganic and organic dissolved and particulate carbon was determined in two long-term mesocosm experiments (Sweden, 2013 and Norway, 2015). Sample collection and analytical procedures are described in this chapter. For each type of sample, raw data processing and calibration procedures are outlined with the aim of providing accurate and inter-comparable measurements. In addition, the effect of high $CO_2$ levels on the isotopic distribution of carbon species in the mesocosm systems was determined. The current stable carbon isotope measurements were combined in an attempt to show and explain carbon dynamics in a marine ecosystem under different $CO_2$ conditions. Additionally, the new isotopic datasets and the related biogeochemical outcomes could be beneficial for the validation and potential new parameterisation of model simulations used to estimate $CO_2$ changes in geological past. With a better understanding of the factors driving isotopic variations in the marine carbon compartments, the number of assumptions generally made to infer past climate changes could be reduced and paleoclimatic interpretations can be more effectively asserted.

## 4.2 Materials and method

### 4.2.1 Mesocosms locations and experimental set-ups

Two Kiel Off-Shore Mesocosms for Future Ocean Simulations (KOSMOS) experiments were carried out in North Atlantic fjord waters. The first study was conducted in 2013 off the west coast of Sweden while the second was carried out in 2015 off the west coast of Norway (Figure 4-1).

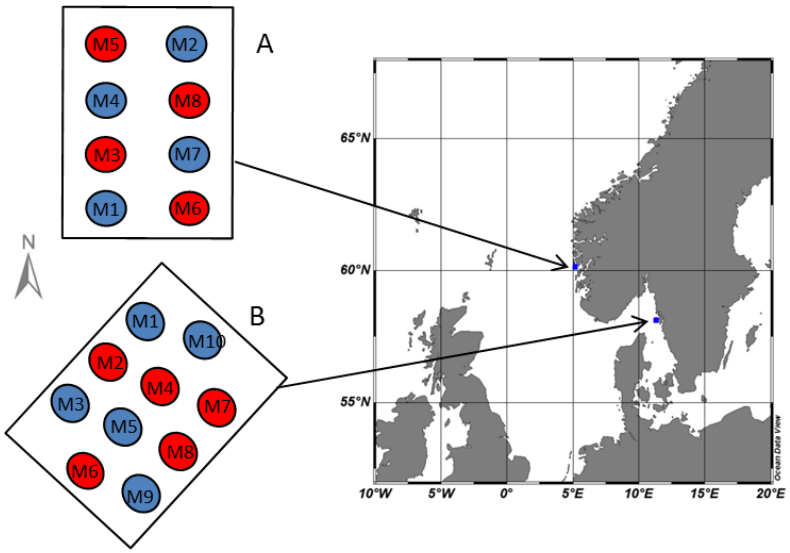


**Figure 4-1.** Experiments location (map) and geographical arrangements (A-B). Raunefjord, Bergen (Norway) 2015 (A) and Gullmar fjord, Kristineberg (Sweden) 2013 (B). Numbers show mesocosm arrangement while blue and red colours represent ambient and high $CO_2$ replicates, respectively.

The two studies employed the same mesocosm units (Figure 4-2) and similar experimental set-ups.

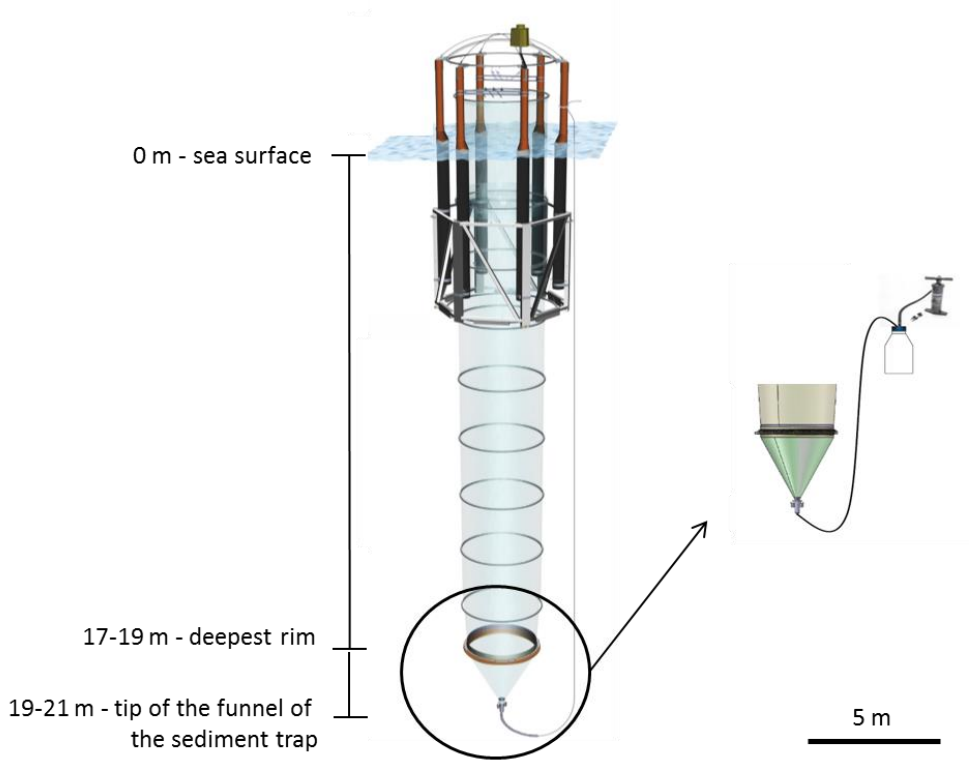


**Figure 4-2.** Schematic of mesocosm unit. The sediment trap (circled) is shown on the right. Sampling of settled matter is achieved via silicone tubing connected to 5 l flask and a hand-operated vacuum pump.

*Sweden mesocosm study*

A detailed description of the Swedish mesocosm experiment is reported in Bach et al. (2016). Briefly, ten mesocosms, were deployed in the Gullmar Fjord (58° 16' N, 11° 28' E), approximately 100 km north of Gothenburg on the Swedish west coast, from March to June 2013 (Figure 4-1). The water depth at the deployment site was between 60 and 80 m. The bottom ends of the mesocosms bags were lowered to a depth of 19 m below the surface enclosing a volume of about 55 $m^3$ of fjord water each. Two different $CO_2$ levels were established: five of the ten mesocosms (M1, M3, M5, M9 and M10) were untreated controls with $CO_2$ levels ~360 ppm while the other five (M2, M4, M6, M7 and M8) were manipulated to achieve $CO_2$ levels of about 1000 ppm. During the course of the experiment five $CO_2$ adjustments were performed in order to compensate $CO_2$ loss due to outgassing and biological uptake.

*Norway mesocosm study*

On the 11$^{th}$ of May 2015, eight KOSMOS were deployed and moored in the Raunefjord at 60° 15' N 5° 12' E, near Bergen in the Southern Norway (Figure 4-1) at a water depth between 55 and 65 m. The cylindrical mesocosm bags (2 m diameter) were lowered to a depth of 21 m (including

sediment trap) enclosing a volume of about 60 $m^3$ of fjord water each. Two different $CO_2$ levels were established: four of the eight mesocosms (M1, M2, M4 and M7) were untreated controls at ambient $CO_2$ levels (~370 ppm) while the other four (M3, M5, M6, M8) were manipulated by adding aliquots of $CO_2$ enriched water in order to reach $CO_2$ concentration of about 2200 ppm. On a regular basis, $CO_2$ levels were adjusted to reach the target concentrations through five further $CO_2$ additions. The experiment ran for about two months ending on the 30$^{th}$ of June 2015.

In both experiments $CO_2$ manipulation was performed by pumping $CO_2$-saturated seawater into the mesocosm bags. The $CO_2$-saturated seawater was prepared by aerating about 1500 L of filtered (20 µm) fjord waters with pure $CO_2$ gas for at least one hour to reach pH value of ~4. Both experiments start at day t0 which denotes the day of the first $CO_2$ manipulation. Several regular operations were performed in both experiments such as weekly cleaning of the inside (with a specific cleaning ring (Riebesell et al., 2013)) and outside (with brushes) walls, seeding (addition of planktonic organisms) and fish eggs and/or larva addition.

### 4.2.2 Sampling strategy

Sampling for stable carbon isotopes analysis was carried out every fourth and second day for the Swedish and Norwegian mesocosm experiments, respectively. While a full experiment sampling was carried out during the Norwegian study, sampling from the Swedish mesocosm experiment started only after t39 due to unavailability of sampling bottles. Isotopic measurements are therefore limited to the second half of the experiment only. Duplicate samples from M1, M2 and fjord water were collected during the Swedish mesocosm campaign while two controls (M1 and M2), two treated mesocosms (M3 and M8) and the fjord water were sampled during the Norwegian mesocosm experiment. Fjord water sampling was performed as an additional control on mesocosms performance. Mesocosms were sampled in the morning (9 a.m.) starting from the sediment traps using a vacuum system as described in Boxhammer et al. (2015) and proceeding to the water column and surrounding fjord waters using a 5 L depth integrating water sampler (IWS, Hydro-Bios).

To minimise gas exchange, dissolved inorganic carbon was always the first sample to be drawn from the IWS. Samples for stable isotopic analyses of dissolved inorganic carbon ($\delta^{13}C_{DIC}$) were collected into 100 ml Pyrex borosilicate glass bottles with a glass stopper. Sample preservation was performed by spiking the samples with 20 µl of saturated mercuric chloride solution (Dickson et al. 2007). For analyses of stable isotopes of dissolved organic carbon ($\delta^{13}C_{DOC}$), samples were collected into 30 ml acid-washed (HCl, 10 %) and pre-combusted (at 450 °C for 12h) TOC glass vials after filtration through a pre-combusted 25 mm carbon cleaned GF/F filter (Dickson et al. 2007). Samples were fixed by addition of 100 µl of 4 M hydrochloric acid solution

and stored refrigerated in the dark. Samples for the isotopic analysis of particulate organic carbon ($\delta^{13}C_{POC}$) were collected on 0.7 µm pore size pre-combusted (at 450 °C for 12 h) glass-fibre filters (GF/F) by filtration of 0.5 L of seawater drawn directly from the IWS into 1 L narrow mouth sampling bottles (high density polyethylene (HDPE), Nalgene, PK 50). For the stable carbon isotope analysis of the sediment material ($\delta^{13}C_{Sed}$), 3 ml of sediment trap supernatant were vacuum filtered on pre-combusted 25 mm, 0.7 µm pore size glass microfiber Whatman GF/F filters. Filters for $\delta^{13}C_{POC}$ and $\delta^{13}C_{Sed}$ were stored frozen (-20 °) immediately after sampling into clean Eppendorf Safe-Lock tubes (1.5 ml) for later analysis.

### 4.2.3 Ancillary data

Every sampling day, after sediment and water column sampling, a CTD60M (Sea & Sun Technologies) was used to determine vertical profiles of salinity, temperature, pH, chlorophyll, and photosynthetically active radiation (PAR) in every mesocosm and in the surrounding water.

### 4.2.4 Sample analysis

#### 4.2.4.1 Expression of isotopic composition

Stable isotope data were expressed in the delta notation ($\delta^{13}C$) relative to the international V-PDB standard according to equation 4.1

$$\delta^{13}C = \frac{R_{sample} - R_{standard}}{R_{standard}} \times 1000\ ‰ \quad (4.1)$$

where R represents the ratio of the heavy carbon isotope ($^{13}C$) to the light carbon isotope ($^{12}C$).

#### 4.2.4.2 Analysis procedure

Measurements of the stable carbon isotopic composition of the collected samples were performed on a Delta V Advantage isotope ratio mass spectrometer (Thermo Fisher Scientific). Samples and standards were delivered to the ion source by using three different inter-calibrated sample preparation devices connected to specific peripherals in a continuous flow approach (Figure 4-3).

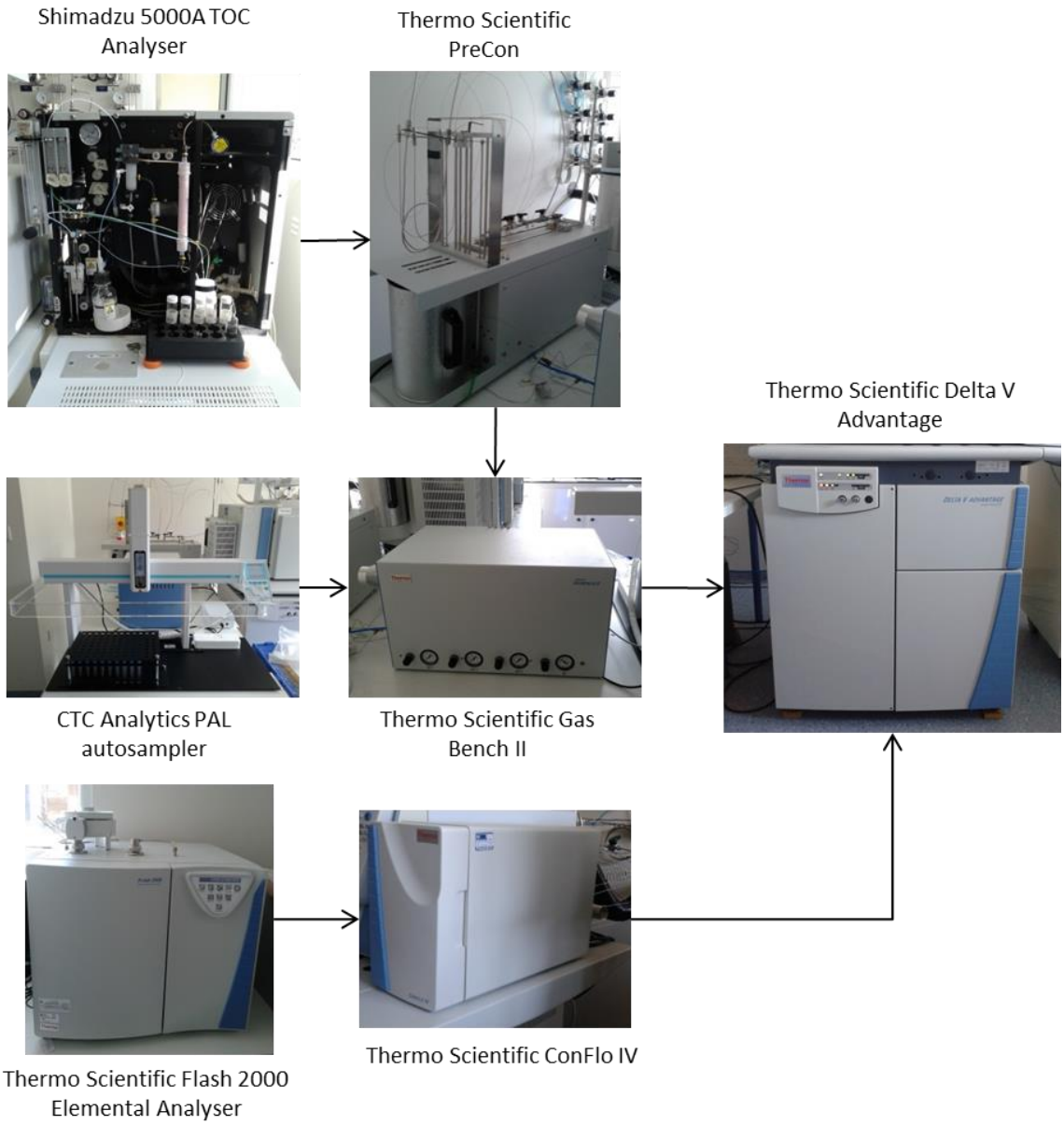


**Figure 4-3.** Instruments used to perform stable isotope analysis of dissolved and particulate organic and inorganic carbon for the samples collected during the mesocosm campaigns in Sweden (2013) and Norway (2015).

A total of 241 samples for $\delta^{13}C_{DIC}$ measurements were analysed using a GasBench II preparation device (Thermo Scientific) over 6 runs. Each run was arranged in batches consisting of 31 vials for calibrants (MAB, NA, NS and CA, Appendix A) and quality controls plus a variable set of samples (typically around 50). Samples from the mesocosm experiment in Sweden were run in duplicate while only technical replicates (repeated measurements of the same sampling vial) were carried out for the $\delta^{13}C_{DIC}$ samples collected during the mesocosm study in Norway.

A total of 122 samples for $\delta^{13}C_{DOC}$ measurements were analysed using an in-house combined TOC-IRMS system (Figure 4-3). Six consecutive sequences were employed for the Swedish mesocosm experiment while samples from the Norwegian mesocosm experiment were

analysed over nine consecutive runs. Each analytical sequence consisted of initial background and ultrapure water blank measurements, followed by calibration standards (phthalate, urea, glutamic acid and sucrose) and a variable set of fjord water samples (typically 8 per day). Standards were prepared by dilution of the organic material in ultrapure water (Milli-Q water, resistivity 18.2 MΩ·cm) obtaining a concentration range of 30 - 600 µM of C used both for isotopic calibration and sample concentration determination. Deep seawater consensus reference material (Lot No 8-08) distributed from the laboratory of D. Hansell (University of Miami) and urea or sucrose standards were analysed between samples for analytical consistency and instrument drift checks.

Samples for $\delta^{13}C_{POC}$ and $\delta^{13}C_{Sed}$ measurements were analysed using a Flash 2000 Elemental Analyser (Figure 4-3). In total 312 samples (178 and 134 for $\delta^{13}C_{POC}$ and $\delta^{13}C_{Sed}$, respectively) were analysed over 29 analytical runs, each consisting of 3 initial blank measurements (tin capsule and pre-combusted blank filters), triplicate measurements of calibrated urea, caffeine, sucrose, glutamic acid and CM standards (Appendix B) followed by a set of mesocosm samples (typically 10). When sediment samples were analysed, dilution (50 or 78 % helium) was activated in order to avoid IRMS signal intensity saturation. Standard materials were accurately weighed (readability of 0.01 mg) and used both for isotopic calibration and sample concentration determination.

### 4.2.5 Measurement processing

All raw $\delta^{13}C$ results were processed following a common procedure: removal of anomalous measurements, linearity correction ($\delta^{13}C_{DIC}$ only), blank correction, calibration to V-PDB and averaging.

#### 4.2.5.1 $\delta^{13}C_{DIC}$ processing

Anomalous $\delta^{13}C_{DIC}$ measurements were removed based on the peak area shape and height. Out of six technical replicates, the best five (based on those with the lowest standard deviation) were chosen and if peak areas of more than three replicates were falling outside of the calibration range (peak area between 10 and 130 mV s) the entire sample was discarded.

The amplitude of the peak area and $\delta^{13}C$ value of each of the technical replicates declines with time as a consequence of sample dilution during analysis. A linear relationship exists between the two and the following formula was applied to convert the results:

$$\delta_{lin\_corr} = \delta_{DIC\_raw} - m_{lin} * A_r \quad (4.2)$$

where $\delta_{lin\_corr}$ is the linearity-corrected $\delta^{13}C$, $\delta_{DIC\_raw}$ is the raw $\delta^{13}C$ measurements, $m_{lin}$ is the correction gradient determined by averaging the linearity slopes of increasing concentration of standard materials and $A_r$ is the peak area for the technical replicate.

A series of blank measurements consisting of 2 clean empty vials, 2 vials containing only phosphoric acid (80 µl) and 2 vials containing phosphoric acid plus 1 ml of ultrapure water (Milli-Q water, resistivity 18.2 MΩ·cm) were included at the beginning of each run. A mean peak area ($A_{DIC\_blank}$) of 0.247 ± 0.015 mV s and a mean $\delta^{13}C$ value ($\delta_{DIC\_blank}$) of -18.68 ± 0.89 ‰ were determined from the analysis of the ultrapure water plus phosphoric acid vials. The values were used to make blank correction to all the standards prepared in ultrapure water according to:

$$\delta^{13}C_{DIC_{blank\_corr}} = \left[\frac{\left(\delta_{lin\_corr} * A_r\right) - \left(\delta_{DIC\_blank} * A_{DIC\_blank}\right)}{\left(A_r - A_{DIC\_blank}\right)}\right] \quad (4.3)$$

Calibration of the samples to the V-PDB international standards was performed by three- and four-point linear regression fits for the Swedish and Norwegian $\delta^{13}C_{DIC}$ measurements, respectively, according to:

$$\delta^{13}C_{DIC} = m_{DIC} * \delta^{13}C_{DIC_{blank\_corr}} + b \quad (4.4)$$

where $m_{DIC}$ is the gradient derived by plotting the mean peak area corrected MAB, NA, NS and CA values against the corresponding certified values relative to V-PDB and b is the off-set.

After calibration, the final dataset was compiled. Final $\delta^{13}C_{DIC}$ values were reported as the average between two duplicate measurements (when available) and when a data point had to be removed (outlier) the mean of nearby points was used to replace the missing value.

#### 4.2.5.2 $\delta^{13}C_{DOC}$ processing

Anomalous $\delta^{13}C_{DOC}$ measurements were removed when DOC concentrations were more than one order of magnitude higher than the average sample concentrations. In such cases, sample contamination was assumed.

A blank correction was applied to all the raw data. Samples were corrected for the instrument blank background according to

$$\delta^{13}C_{DOC_{blank\_corr}} = \left[\frac{\left(\delta_{DOC_{raw}} * A_{DOC}\right) - \left(\delta_{DOC_{blank}} * A_{blank}\right)}{\left(A_{DOC} - A_{blank}\right)}\right] \quad (4.5)$$

where $\delta_{DOC\_raw}$ is the measured isotopic ratio of the sample, $\delta_{DOC\_blank}$ is the average isotopic ratio of the instrument background determined by sequential trapping and successive analysis of TOC baseline, $A_{DOC}$ and $A_{blank}$ are the mean peak area of sample and background, respectively. A water blank correction was applied to the calibration standards using the mean intensity and $\delta^{13}C$ value of sequential trapping of ultrapure water.

A three-point calibration curve was used to normalise the samples to the V-PDB standards according to:

$$\delta^{13}C_{DOC} = m_{DOC} * \delta^{13}C_{DOC_{blank\_corr}} + c \quad (4.6)$$

where $m_{DOC}$ defines the slope calculated by plotting the mean blank corrected $\delta^{13}C$ value of the standards against the corresponding certified values relative to the V-PDB and c is the intercept. Two regression lines were determined as the phthalate standard used in the analysis of the Swedish samples was substituted by a sucrose standard during the analysis of the samples from the Norwegian mesocosm experiment. In addition two different $CO_2$ reference gas tanks were used for the two separate mesocosm datasets.

The average $\delta^{13}C_{DOC}$ value between two duplicate measurements (when available) was reported as final value. Missing data were compiled by averaging two nearby points of the time-series data.

#### 4.2.5.3 $\delta^{13}C_{POC}$ and $\delta^{13}C_{Sed}$ processing

All the $\delta^{13}C_{POC}$ and $\delta^{13}C_{Sed}$ measurements were inspected for outliers based on atypical peak shape and height. A blank correction was applied to all the raw data. Two blank values (one for the standards and one for the samples) were determined for each dataset. As standard material was weighed directly into tin capsules and samples were placed on filters, the mean peak area ($A_{POC}$) and mean $\delta^{13}C$ value ($\delta_{POC\ blank}$) of empty tin capsules and empty pre-combusted GF/F filters inserted at the beginning of every run were used to make a blank correction for the standards and samples, respectively. The following equation was used:

$$\delta^{13}C_{POC_{blank\_corr}} = \left[\frac{\left(\delta_{POC_{raw}} * A_{POC}\right) - \left(\delta_{POC_{blank}} * A_{blank}\right)}{\left(A_{POC} - A_{blank}\right)}\right] \quad (4.7)$$

where $\delta_{POC\_raw}$ and $A_{POC}$ are the measured isotopic ratio and peak area, respectively, of the standard material (or sample).

A five-point calibration curve was used for each dataset in order to normalise the samples to the V-PDB standards according to:

$$\delta^{13}C_{POC} = m_{POC} * \delta^{13}C_{POC_{blank\_corr}} + d \quad (4.8)$$

where $m_{POC}$ defines the slope calculated by plotting the mean blank corrected $\delta^{13}C$ value of the standards against the corresponding certified values relative to the V-PDB and d is the off-set. Urea, glutamic acid, sucrose, caffeine and calcite (MAB) standards were used to determine the two regression lines.

### 4.2.6 Precision of the measurements

Analytical precision (1σ) was calculated from repeated measurements of the same sample. To evaluate the error introduced by the sampling procedure, duplicate samples (collected only during the Swedish mesocosm experiment) were analysed. The mean absolute difference between two duplicate $\delta^{13}C$ samples was divided by $2/\sqrt{\pi}$ in order to assess and quantify the sampling related uncertainty (Thompson & Howarth, 1973; Humphreys et al., 2016). The analytical error was instead estimated from repeated measurements of standards and reference materials spread throughout the analytical sequences.

### 4.2.7 Statistical treatment of the datasets

After measurement processing, the quality controlled isotopic datasets were tested for normality distribution using the online Grubbs test (http://graphpad.com/quickcalcs/grubbs1/). To determine potential $CO_2$ addition effects on the $\delta^{13}C$ distribution in the mesocosms water column and sediment material, several t-tests were performed. Using the data analysis tool in MS Excel, each set of carbon isotope data was tested for equality of means between paired samples. Ambient versus treated, ambient versus ambient and treated versus treated mesocosms were equally tested. A significant difference was accepted for p-values < 0.05.

### 4.2.8 Data analysis

The isotopic incorporation of $CO_2$-saturated water into the mesocosms was calculated as a relative change of the isotopic signature in carbon samples according to:

$$\Delta\delta^{13}C = \delta^{13}C_{sample} - \delta^{13}C_{initial} \quad (4.9)$$

where $\delta^{13}C_{sample}$ and $\delta^{13}C_{initial}$ are the isotopic signature of the samples and the background (before any $CO_2$ addition), respectively.

Photosynthetic isotope fractionation ($\varepsilon_{phyto}$) between the inorganic carbon and phytoplankton following the uptake process was calculated as:

$$\varepsilon_{phyto} = \frac{\delta^{13}C_{DIC} - \delta^{13}C_{POC}}{1 + \delta^{13}C_{POC}/10^3} \quad (4.10)$$

with the assumption that DIC represented the substrate (source) and POC (product) represented the active phytoplankton. Although the use of $\delta^{13}C$ signatures of DIC and POC is not the most appropriate, the equation can still give an approximate indication of isotopic discrimination during photosynthetic carbon uptake.

The exchange between POC and DOC during the post bloom phase was estimated through the isotopic relationship between the two pools in terms of isotope effect ($\varepsilon_{POC \rightarrow DOC}$) calculated according to Hayes (2004) as:

$$\varepsilon_{POC \rightarrow DOC} = \alpha_{DOC/POC} - 1 \quad (4.11)$$

where $\alpha_{DOC/POC}$ is the fractionation factor determined using the following equation:

$$\alpha_{DOC/POC} = \frac{\delta^{13}C_{DOC} + 1}{\delta^{13}C_{POC} + 1} \quad (4.12)$$

Isotopic measurements of sediment material were used to calculate the fraction of settled material derived from the POC in the upper layer. The following mixing equation (Fry, 2006) was used:

$$f_{settled} = \frac{(\delta^{13}C_{Sed} - \delta^{13}C_{control})}{(\delta^{13}C_{POC} - \delta^{13}C_{control})} \quad (4.13)$$

where $\delta^{13}C_{control}$ is the average isotopic signature of particulate organic carbon at day 0 before any $CO_2$ additions. The equation was applied on the treated mesocosms and it was used as a measure of sinking of freshly produced material.

## 4.3 Results and Discussion

### 4.3.1 General observations and considerations

The great efforts and determination of the many scientists, engineers and technical staff involved in the two mesocosm campaigns, allowed the continuous sampling and the achievement of successful experiments notwithstanding the multiple technical difficulties (mesocosm buoyancy, holes) and adverse environmental conditions (sea ice, strong currents) encountered. In both studies, $CO_2$ manipulation was effective with $pCO_2$ concentrations reaching the target values (1000 ppm and 2000 ppm for Sweden and Norway, respectively). A wide range of oceanographic measurements was performed during the two studies however at the present time many data are still not finalised or available (particularly for the Bergen mesocosm experiment) as they need processing or further quality control checks; therefore only an early stage biogeochemical assessment can be made. Moreover, it must be pointed out that the sampling of the second part only (from t39 onwards) and the lower sampling frequency (every four days) carried out during the Swedish mesocosm study potentially limits the use of this dataset for solid biogeochemical interpretations. However, these isotopic measurements will be part of a larger dataset containing a wide range of biological and oceanographic data. The combination of the numerous data types

will contribute to explain biogeochemical variations, and the integration of the isotopic data with supplementary measurements will help to fill the existing gaps in understanding the anthropogenic $CO_2$ induced effects on marine ecosystems. The $\delta^{13}C$ measurements described here will be available to the KOSMOS team for download from https://ftp.geomar.de/downloads/Kristineberg2013 server and from the "KOSMOS 2015 Bergen" GEOMAR shared folder, for the Swedish and Norwegian mesocosm experiment datasets, respectively.

### 4.3.2 $\delta^{13}C_{DIC}$ measurements

Only 3 mesocosm $\delta^{13}C_{DIC}$ measurements were considered anomalous, however the values of the respective duplicate samples were acceptable and therefore used as final values. Measured $\delta^{13}C_{DIC}$ values for $CO_2$-enriched water were on average -10.42 ± 0.03 ‰ (n=2) and -36.82 ± 0.46 ‰ (n= 13 from 6 different batches), for the Swedish and Norwegian mesocosm experiment samples, respectively. The different values were possibly due to the sampling strategies employed more than to actual isotopic composition of the employed $CO_2$ gas: samples from the Bergen experiment were drawn directly from the 1500 L $CO_2$-rich seawater tank while during the Swedish study samples were prepared by bubbling ultrapure water with pure $CO_2$ gas (the same as the one used for the preparation of the seawater batch) for about 20 min. However, it is possible that the bubbling time was not enough to enrich the ultrapure water with $CO_2$ or that the fast purging was aiding degassing rather than a $CO_2$ increase in the solution. The DIC concentrations of $CO_2$-enriched water were between 30 and 50 mM (values calculated using the CO2SYS Excel spreadsheet v2.1) and while half sample aliquots (0.5 ml instead of usual 1 ml) and GasBench dilution were necessary in order to avoid saturation of the MS signal during the analysis of $CO_2$-enriched water samples from Norway, analysis of the ultrapure $CO_2$-enriched water did not required any dilution and showed a relatively weak $CO_2$ signal. The $\delta^{13}C_{DIC}$ values measured for the Swedish mesocosm ultrapure $CO_2$-enriched water might not have represented the true isotopic value of the added $CO_2$-enriched water and they were therefore flagged.

The linearity slopes for calibration standards and samples were determined for every analytical sequence. The relationship between peak area and $\delta^{13}C$ of each standard was similar among all the runs with variation among average slopes better than 1.1 % (Table 4-1). However each analytical run was corrected using the corresponding (daily) linearity equations.

| *Name* | *Chemical Composition* | *Number of sequences* | *Average linearity slope* | *Variation (%)* |
|---|---|---|---|---|
| MAB | $CaCO_3$ | 6 | $-1.77 * 10^{-3}$ | 0.6 |
| NA | $NaHCO_3$ | 6 | $-0.26 * 10^{-3}$ | 1.1 |
| NS | $Na_2CO_3$ | 6 | $7.22 * 10^{-3}$ | 0.1 |
| CA | $CaCO_3$ | 4 | $-5.34 * 10^{-3}$ | 0.3 |
| Samples | Seawater | 6 | $0.59 * 10^{-3}$ | 0.8 |

**Table 4-1.** Variation of standards and samples correction gradients among different analytical sequences

Analysis of blank vials did not yield a clear signal for sample isotopic correction. However, the isotopic value ($\delta^{13}C$ = -18.68) of ultrapure water was used to correct NS and NF samples that were prepared in ultrapure water (NSL, NFL).

After blank correction, a separate equation for each batch was determined for the sample V-PDB calibration. Variations of the slope and intercept among all the analysis calibration equations were 0.8 and 0.5 %, respectively. In order to test if application of a single equation to all the data would have affected the final results, the mean value of each standard (calculated for each analysis) was compared to the one calculated using the single equation and tested for equality using paired t-tests (Table 4-2).

| | *Multiple equations* | | | *Single equation* | | |
|---|---|---|---|---|---|---|
| Name | Mean $\delta^{13}C$ value (‰) | St. Dev within analysis | St. Dev among analysis | Mean $\delta^{13}C$ value (‰) | St. Dev among analysis | Significantly different for $p < 0.05$ |
| MAB | 2.48 | 0.07 | 0.09 | 2.48 | 0.27 | 0.986 |
| NA | -4.71 | 0.06 | 0.18 | -4.70 | 0.15 | 0.615 |
| NS | -39.87 | 0.08 | 0.13 | -39.85 | 0.25 | 0.155 |
| CA | -24.18 | 0.14 | 0.35 | -24.10 | 0.37 | < 0.05 |
| NFL | -8.94 | 0.07 | 0.05 | -9.17 | 0.15 | < 0.05 |
| NSL | -39.68 | 0.07 | 0.09 | -39.57 | 0.20 | < 0.05 |
| CRM | 1.07 | 0.07 | 0.09 | 1.06 | 0.27 | 0.803 |

**Table 4-2.** Differences among standard values calculated using multiple (daily) V-PDB calibration curves and a single V-PDB equation.

Using a single equation compared to day by day calibration would have not significantly affected the final results however, precision would have decreased. The reported lower precision

might be due to the combination of unavoidable changes in IRMS settings (reference gas intensity, focusing), to possible errors during preparation of the standards and to the 3-month gap between the two mesocosm dataset analyses.

Repeated measurements of the same sample had a standard deviation (SD) of 0.04 ‰. When the same sample was collected into different bottles, precision decreased to 0.24 ‰. Similar values were reported in previous studies where duplicate pair SD were 0.23 and 0.17 ‰ for Griffith et al. (2012) and Humphreys et al. (2015), respectively. Although sample uncertainty was 0.04 ‰, final precision of the $\delta^{13}C_{DIC}$ measurements was established as 0.09 ‰, based on the highest variance (1σ) calculated between replicate measurements of all the liquid standards among all the analysis in compliance with the principle of identical treatment (Werner & Brand, 2001).

In this study, the seawater reference material (CRM) bottles were from batch 144 (http://cdiac.ornl.gov/oceans/Dickson_CRM/batches.html) and although an internationally certified $\delta^{13}C_{DIC}$ value is not available, Humphreys et al. (2016) reported a carbon isotopic signature of 1.27 ‰ for the same batch. However when they applied a drift correction to their analysis a $\delta^{13}C_{DIC}$ value of 1.22 ± 0.17 ‰ was produced. Although slightly isotopically enriched, both values are within the analytical uncertainty of the ones measured in the present study (Table 4-2).

### 4.3.3 $\delta^{13}C_{DOC}$ measurements

In total, eight measurements were removed from the $\delta^{13}C_{DOC}$ datasets based on obvious contamination (open vials, leaks and concentrations outside calibration range). Three of the removed data points were substituted by the corresponding duplicate while the remaining five were interpolated.

The quantification and isotopic characterisation of the TOC instrument background and ultrapure water blank signal is one of the main steps to undertake in order to appropriately correct the sample values for the background contribution and obtain accurate $\delta^{13}C_{DOC}$ measurements. The average background signal was -29.02 ± 0.58 ‰ and -26.04 ± 0.39 ‰ for the Swedish and Norwegian mesocosm datasets, respectively. The 3 ‰ enrichment was probably due to the change of the combustion column packing material. Analysis of ultrapure water blanks yielded similar isotopic values (Table 4-3) confirming the minor contribution of the water blank compared to the system blank (Lang et al., 2007).

| | *Sweden mesocosm* | *Norway mesocosm* |
|---|---|---|
| TOC system background | $\delta^{13}C$ = -29.02 ± 0.58 ‰ | $\delta^{13}C$ = -26.04 ± 0.39 ‰ |
| Ultrapure water signal | $\delta^{13}C$ = -28.58 ± 0.25 ‰ | $\delta^{13}C$ = -26.78 ± 0.61 ‰ |
| Water blank intensity | 7.13 µM of C | 11.26 µM of C |

**Table 4-3.** System and water blank values determined during the analysis of $\delta^{13}C_{DOC}$ samples from the Swedish and Norwegian mesocosm campaigns.

Two separate equations were determined for the samples V-PBD calibration, one for each mesocosm campaign. This was reasonable as the analysis of samples from each campaign was performed over consecutive days, however, it was necessary to apply different calibration curves to the two datasets as a different set of standards (Table 4-4) was used. Additionally, there was a 6-week gap between the two sets of analysis and two different $CO_2$ reference gases were employed. The $\delta^{13}C_{DOC}$ uncertainty determined as sampling related calculated from duplicate samples, was 0.60 ‰. Better precision (σ = 0.48 ‰) was obtained when replicate measurements of the same sample (same vial) were performed. The precision of the calibration standards was however always worse (except for phthalate) than that of the samples (Table 4-4). The poor precision of the standards might be associated with standard preparation itself. A standard stock solution for each compound was prepared at the beginning of each set of analyses however standard solutions (calibrants) were prepared daily by dilution of variable volumes of the prepared stock solutions. Although standard containers (24 ml pre-combusted borosilicate glass vials) and ultrapure water are expected to be carbon-free, minor carbon isotopic contamination during standard preparation procedures could have not been excluded. The higher susceptibility to contamination of the calibration standards due to preparation procedures is confirmed by the better precision (σ = 0.38 and 0.58 ‰) of $\delta^{13}C_{DOC}$ measurements of seawater reference material (CRM). All the CRMs bottles used were from the same batch (Lot No 8-08, http://yyy.rsmas.miami.edu/groups/biogeochem/Table1.htm) so that it can be assumed that they were subjected to the same preparation and storage procedures. The CRM samples had an average carbon concentration of 42.61 ± 1.13 µM and a mean $\delta^{13}C_{DOC}$ value of -19.60 ± 0.62 ‰. As CRMs were placed randomly throughout analytical sequences and across the runs, the SD (σ = 0.62 ‰) of their measurements is highly representative of the precision and reproducibility of the current $\delta^{13}C_{DOC}$ analysis. Although the isotopic value for these CRMs is unknown, the measured $\delta^{13}C_{DOC}$ value (-19.60 ‰) was similar to previously reported results (Bouillon et al., 2006; Lalonde et al., 2014).

| | | | *Sweden mesocosm* | | | *Norway mesocosm* | | |
|---|---|---|---|---|---|---|---|---|
| Standard Compound | Concentration range (µM of C) | Certified $\delta^{13}C$ value (‰) | Measured $\delta^{13}C$ value (‰) | St. Dev (1σ) | n | Measured $\delta^{13}C$ value (‰) | St. Dev (1σ) | n |
| Urea | 100-700 | -39.84 | -39.96 | 0.79 | 11 | -39.36 | 0.72 | 10 |
| Phthalate | 100-600 | -30.49 | -30.31 | 0.39 | 11 | - | - | - |
| Sucrose | 100-400 | -26.45 | - | - | - | -26.37 | 0.97 | 8 |
| Glu-Acid | 100-550 | -10.91 | -10.97 | 1.45 | 8 | -10.56 | 0.71 | 10 |
| CRM | 41-43 | - | -20.11 | 0.38 | 4 | -19.34 | 0.58 | 8 |

**Table 4-4.** Set of standards used for the V-PDB calibration of $\delta^{13}C_{DOC}$ samples from the Swedish and Norwegian mesocosm experiments.

### 4.3.4 $\delta^{13}C_{POC}$ and $\delta^{13}C_{Sed}$ measurements

No outliers where detected among the $\delta^{13}C_{POC}$ and $\delta^{13}C_{Sed}$ measurements for both mesocosm experiments samples. However, 5 samples were lost and the missing data points were replaced by averaging two nearby points.

The areas of the blank measurements (empty tin capsules) were on average 5.72 and 5.38 mV s with corresponding $\delta^{13}C$ values of -30.32 and -29.96 ‰ for the Swedish and Norwegian mesocosm datasets, respectively. Combustion of empty glass fiber filters yielded larger peak areas (71.7 and 34.2 mV s) however similar $\delta^{13}C$ values (-29.92 and -29.46 ‰) were obtained, suggesting the carbon present both on tin capsules and filters was of the same nature and possibly derived from laboratory tools and/or environment.

Following blank correction, samples were normalised to the V-PDB reference standards. Two separate equations were determined and although regression lines were produced by different reference materials (Table 4-5) the variations between the two slopes and intercept values were 0.002 and 0.092 %, respectively. The application of a single calibration equation across all analysis batches instead of a separate one for each datasets did not yield a statistically significant difference ($p > 0.05$ for both mesocosm datasets) in the final results. However it was more appropriate to apply a separate equation for each dataset as samples from the two experiments were analysed approximately 10 weeks apart by using two different sets of standards.

Replicate measurements were not possible as no $\delta^{13}C_{POC}$ or $\delta^{13}C_{Sed}$ duplicate samples were collected for the two mesocosm experiments. Based on the highest SD of replicate measurements (n = 55) of the most used calibration standard (urea), the precision (1 σ) of the $\delta^{13}C_{POC}$ and $\delta^{13}C_{Sed}$ datasets was established as 0.12 ‰ (Table 4-5).

| | | | *Sweden mesocosm* | | | *Norway mesocosm* | | |
|---|---|---|---|---|---|---|---|---|
| Standard Compound | Weight range (mg of C) | Certified $\delta^{13}C$ value (‰) | Measured $\delta^{13}C$ value (‰) | St. Dev (1σ) | n | Measured $\delta^{13}C$ value (‰) | St. Dev (1σ) | n |
| Urea | 0.05-0.50 | -39.84 | -39.95 | 0.11 | 18 | -39.91 | 0.12 | 37 |
| Glu-Ac | 0.05-0.15 | -13.45 | -13.31 | 0.44 | 4 | - | - | - |
| Glu-Ac HCl | 0.05-0.60 | -10.91 | - | - | - | -10.85 | 0.33 | 11 |
| MAB | 0.10-0.50 | +2.48 | 2.30 | 0.17 | 3 | - | - | - |
| CM | 0.10-0.50 | +2.22 | - | - | - | 2.18 | 0.08 | 10 |
| Caffeine | 0.05-0.30 | -33.40 | - | - | - | -33.29 | 0.16 | 13 |
| Sucrose | 0.05-0.60 | -26.45 | - | - | - | -26.51 | 0.41 | 12 |
| IAEA-CH-6 | 0.10-0.20 | -10.45 | -10.32 | 0.08 | 2 | - | - | - |
| IAEA-600 | 0.10-0.20 | -27.77 | -27.75 | 0.06 | 2 | - | - | - |

**Table 4-5.** Set of standards used for the V-PDB calibration of $\delta^{13}C_{POC}$ and $\delta^{13}C_{Sed}$ samples from the Swedish and Norwegian mesocosm experiments.

### 4.3.5 Environmental conditions and mesocosm phases

During each experiment, the variations in environmental properties of the water mass enclosed in the different mesocosm bags were similar. The temperature as well as salinity development in the mesocosms was quasi-identical within each experiment during both studies (Figure 4-4 and Figure 4-5 A and B). The average salinity during the sampling period was 29.21 ± 0.09 and 31.89 ± 0.12 for the Swedish and Norwegian mesocosm experiments, respectively. Temperatures were on average colder (8.65°C) during the mesocosm experiment in Sweden, varying from 4.20 to 14.30°C compared to an average temperature of 9.48°C (ranging between 8.48 and 10.35°C) observed during the Norwegian mesocosm study. After t37, i.e. when sampling for carbon isotopes commenced, a thermocline established in the Swedish experiment mesocosms (Figure 4-4, C) and prevailed until the end of the study (Bach et al., 2016). From raw CTD data, a comparable stratification was observed in the water mass of the mesocosms deployed at the Norwegian fjord (Figure 4-5, C).

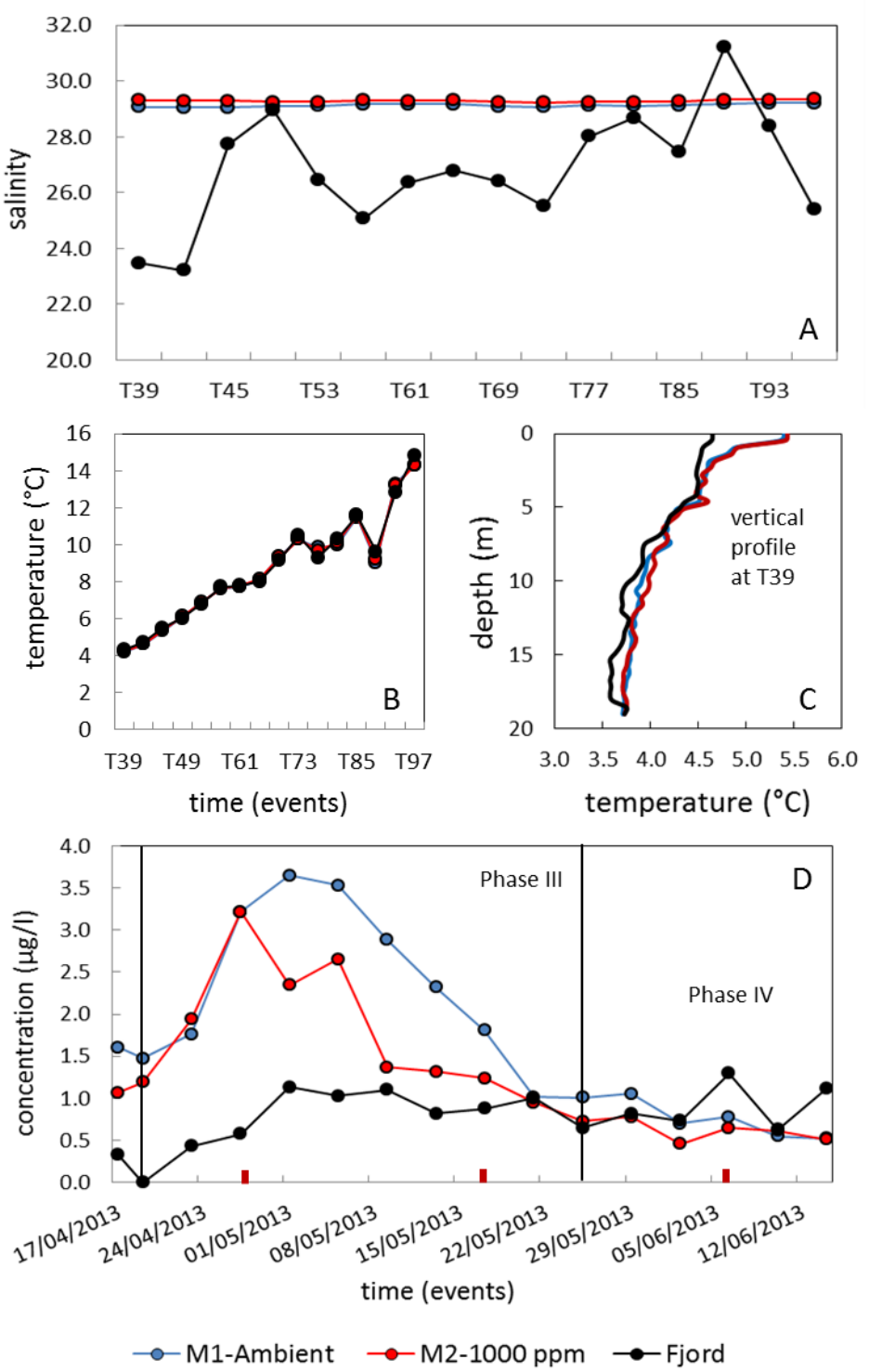


**Figure 4-4.** Salinity (A), temperature (B and C) and chlorophyll *a* concentration (D) plots during the Swedish mesocosm campaign (Gullmar fjord, 2013). Red marks on the x-axis indicate $CO_2$ addition events while black vertical lines define the different experimental phases.

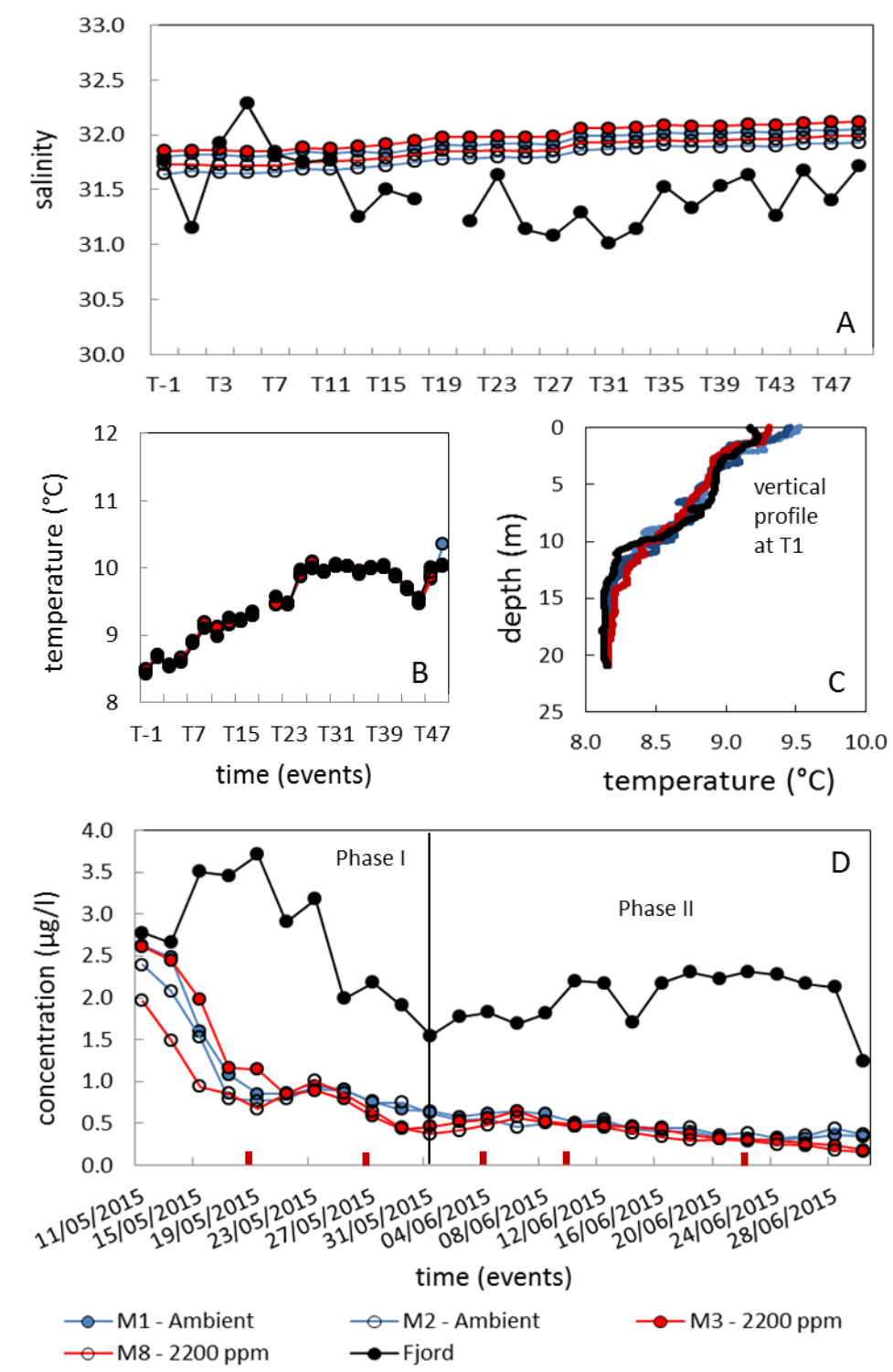


**Figure 4-5.** Salinity (A), temperature (B and C) and chlorophyll *a* concentration (D) during the Norwegian mesocosm campaign (Raunefjord, 2015). Red marks on the x-axis indicate $CO_2$ addition events while black vertical lines define the different experimental phases

Measurements of chl *a* were used to define mesocosm experimental phases (Figure 4-4, D and Figure 4-5, D). For the Swedish experiment, only the defined (Bach et al., 2016) phase III (from t41 to t77) and phase IV (from t77 until the end) will be considered in this chapter due to a lack of

stable carbon isotope measurements prior to these periods. Phase III was characterised by an increase in chl *a* to values up to 3.65 µg/l and it represented the development of a second phytoplankton bloom. The magnitude of the bloom was on average lower than the first bloom (phase II chl *a* maximum values of 5.61 µg/l) and it was considered to be fuelled by remineralised nutrients that were directly transferred into phytoplankton biomass (Bach et al., 2016). Phase IV started after the decline of the second bloom and covered the post bloom period.

A detailed characterisation of the phases encountered during the Norwegian mesocosm experiment is not yet available however, based on raw chl *a* measurements, a broad separation into two main phases can be made. Phase I lasted from the beginning of the experiment until t19 and included chl *a* maximum (2.62 µg/l) and decline. Phase II covered the post bloom period in which chl *a* concentrations remained constantly lower than 0.65 µg/l.

### 4.3.6 Mesocosm stable carbon isotopes distribution

The measured $\delta^{13}C$ values during both mesocosm experiments are presented in Figure 4-6 and Figure 4-7. The $\delta^{13}C_{DIC}$ measurements of control mesocosms and fjord waters were comparable, with average values of -0.87 ± 0.46 ‰ and -0.49 ± 0.70 ‰, respectively, for the Swedish mesocosm experiment and average values of 0.55 ± 0.18 ‰ and 0.72 ± 0.17, respectively for the Norwegian mesocosm campaign. The results are in general agreement with previously reported values between -2 and -1.5 ‰ (at depths below 40 m) and 0.75 to 1.6 ‰ (during a phytoplankton bloom) for the Gullmar Fjord (Nordberg et al., 2009) and for a Norwegian fjord near Bergen (Kukert & Riebesell, 1998), respectively. Similarly, the carbon isotopic signatures of the dissolved and particulate organic material in the control mesocosms and fjord water samples were comparable for both experiments. The $\delta^{13}C_{DOC}$ values were on average -24.96 ± 3.62 ‰ and -23.47 ± 2.93 ‰ for control and Gullmar fjord water, respectively, while average values of -25.11 ± 1.87 ‰ and -23.24 ± 0.97 ‰ were measured for the control mesocosms and Raunefjord water samples, respectively. In general the $\delta^{13}C_{POC}$, were similar to the isotopic pattern of the dissolved organic carbon with average $\delta^{13}C_{POC}$ values of -24.17 ± 1.52 ‰ and -25.52 ± 1.66 ‰ for control mesocosms and Gullmar fjord samples, respectively, and mean values of -22.39 ± 0.82 ‰ and -22.70 ± 1.04 ‰ for control mesocosms and Raunefjord samples, respectively. The isotopic trend of sediment trap measurements was mostly mirroring the carbon isotopic pattern of the $\delta^{13}C_{POC}$ in the corresponding water column suggesting sinking of freshly produced material was occurring. Carbon isotopic signatures of particulate material, both in the water column and in the sediment trap, were in general similar to previously reported values between -26 and -19 ‰ for geographically proximate areas (Kukert & Riebesell, 1998; Rolff, 2000; Waite et al., 2005).

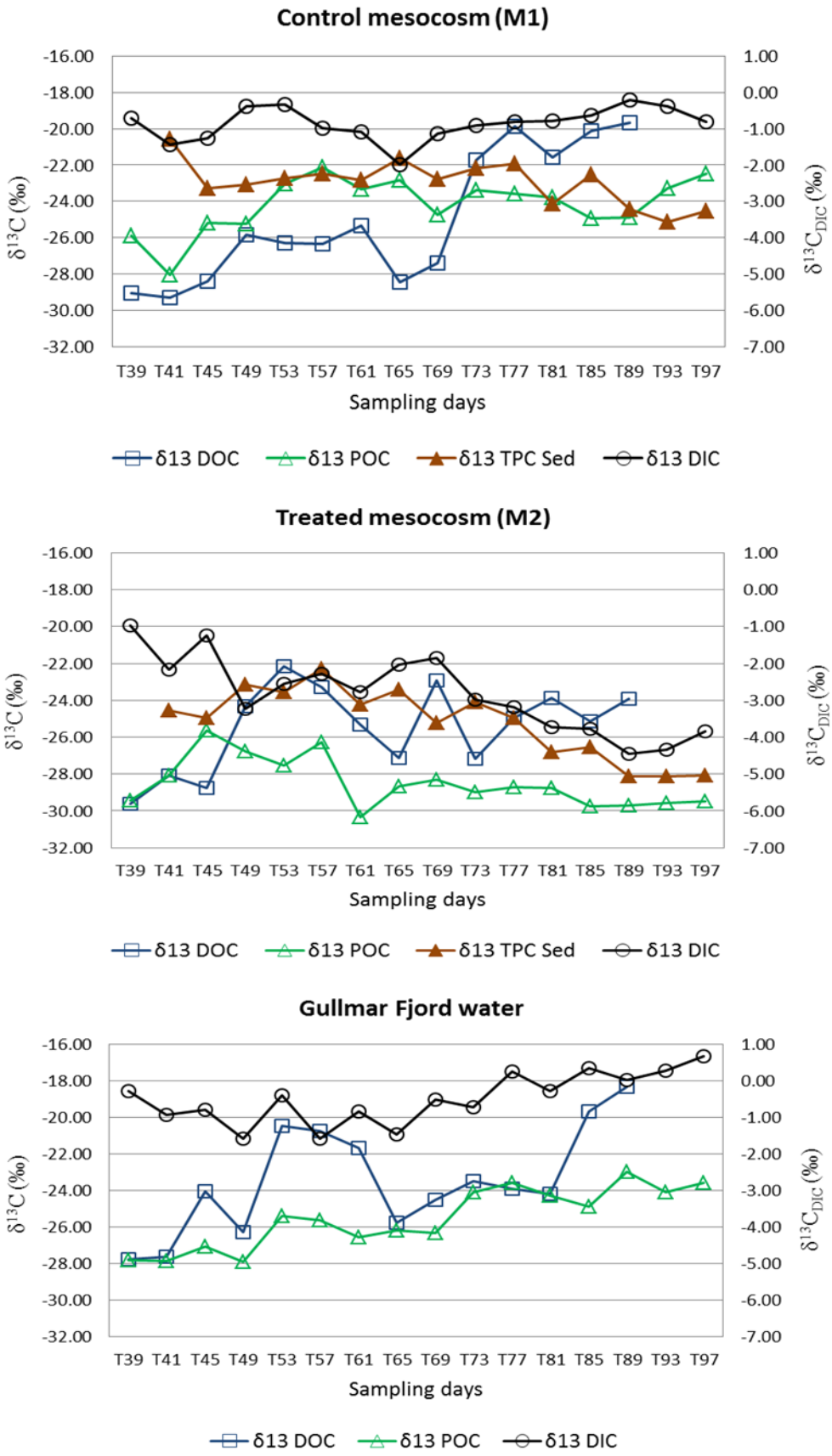


**Figure 4-6.** Temporal development of stable carbon isotopes during the Gullmar fjord (Sweden) mesocosm campaign in 2013. Treated mesocosms were $CO_2$ manipulated in order to reach $pCO_2$ concentrations of 1000 ppm.

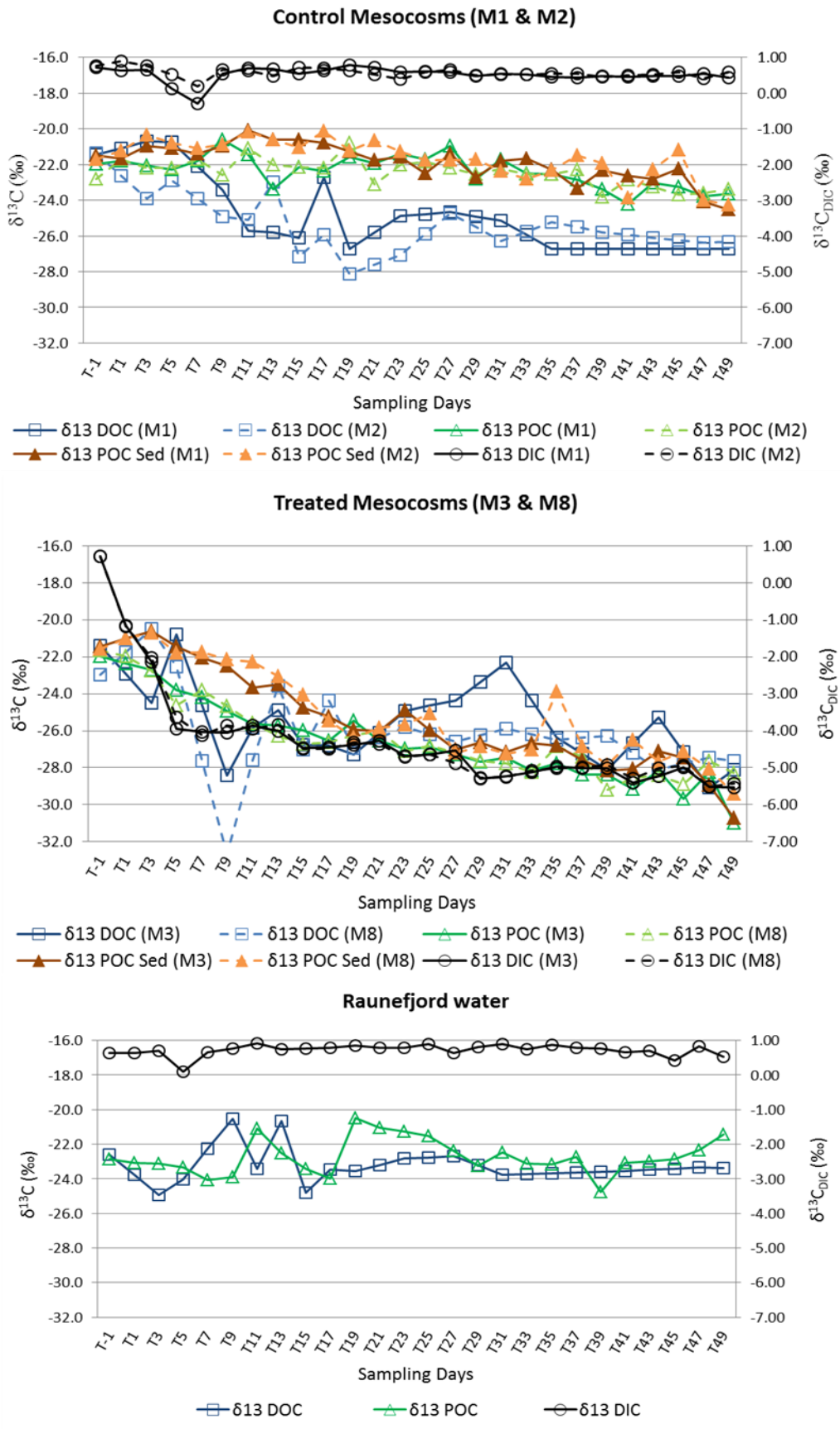


**Figure 4-7**. Temporal development of stable carbon isotopes during the Raunefjord (Norway) mesocosm campaign in 2015. Treated mesocosms were $CO_2$ manipulated in order to reach $pCO_2$ concentrations of 2000 ppm.

#### 4.3.6.1 $CO_2$ addition effects

Additions of isotopically light $CO_2$ into the mesocosm bags decreased the $\delta^{13}C$ of DIC to values as low as -4.46 ‰ and -5.53 ‰ for the Swedish and Norwegian mesocosm experiment, respectively. As a consequence of preferential uptake of light carbon isotope ($^{12}C$) during photosynthetic fixation (O'leary, 1981), the $\delta^{13}C$ of particulate organic material decreased to values of -30.33 ‰ and -30.97 ‰ for the Swedish and Norwegian treated mesocosm samples, respectively. Comparable isotopic signatures were obtained for the sediment trap samples with lowest $\delta^{13}C_{Sed}$ values of -28.11 ‰ for the Gullmar fjord and -30.73 ‰ for the Raunefjord experiment. Although less pronounced, the stable isotopic signature of DOC followed the general isotopic pattern of the particulate counterpart (Figure 4-6 and Figure 4-7). Paired sample t-tests were conducted to compare the consistency of equal mesocosm treatments and the effects of $CO_2$ additions on the isotopic distribution of carbon species (Table 4-6).

| | **Swedish experiment** | | | | **Norwegian experiment** | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Control M1 vs Treated M2** | | | | **Control M1 vs Control M2** | | | | **Treated M3 vs Treated M8** | | | | **Controls M1 & M2 vs Treated M3 & M8** | | | |
| | $\delta^{13}C$ DIC | $\delta^{13}C$ DOC | $\delta^{13}C$ POC | $\delta^{13}C$ Sed | $\delta^{13}C$ DIC | $\delta^{13}C$ DOC | $\delta^{13}C$ POC | $\delta^{13}C$ Sed | $\delta^{13}C$ DIC | $\delta^{13}C$ DOC | $\delta^{13}C$ POC | $\delta^{13}C$ Sed | $\delta^{13}C$ DIC | $\delta^{13}C$ DOC | $\delta^{13}C$ POC | $\delta^{13}C$ Sed |
| t | 5.7 | 0.5 | 8.2 | 6.4 | 1.6 | 1.6 | 0.7 | 1.7 | 0.9 | 1.5 | 1.1 | 2.2 | 25.6 | 2.5 | 15.9 | 12.5 |
| df | 15 | 13 | 15 | 14 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 25 | 51 | 51 | 51 | 51 |
| p | **<.01** | 0.58 | **<.01** | **<.01** | 0.11 | 0.11 | 0.48 | 0.09 | 0.36 | 0.13 | 0.28 | **0.04** | **<.01** | **0.02** | **<.01** | **<.01** |

**Table 4-6.** Results of paired t-tests to assess the statistical significance of $CO_2$ treatments on mesocosm stable carbon isotopes. The two control and treatment mesocosms sampled during the Norwegian experiment were also tested.

There was a highly significant difference (p-values < 0.01) in the isotopic signatures of dissolved inorganic and particulate organic carbon between control and treated mesocosms during both experiments. However no statistical significant (p = 0.58) difference in the $\delta^{13}C$ distribution of dissolved organic carbon was found between control and treated mesocosm during the Swedish experiment. During the Norwegian mesocosm experiment, $CO_2$ manipulation had instead a statistical significant (p = 0.02) effect on $\delta^{13}C_{DOC}$ distribution although smaller compared to the other carbon species (Table 4-6). Comparisons between various control mesocosms, and also treated mesocosms, showed no statistical significant difference on the $\delta^{13}C$ distribution for most of the carbon species suggesting that environmental and experimental conditions in the mesocosm units were constant and replicable. Only the isotopic signature of particulate carbon sediment material between the two treated mesocosms was significantly different (although a p-

value of 0.041 could be tentatively rejected). The observed difference was most likely due to biological factors as a consequence of different export rates, presence of phytoplankton species with different isotopic fractionation or different microbial remineralisation rates between the two treated mesocosms.

### 4.3.7 Isotopic exchange among carbon compartments

The effect of each $CO_2$ manipulation on the isotopic signature of DIC is shown in Figure 4-8 as relative incorporation of the isotopically light carbon into mesocosm waters DIC. Initial stable carbon isotope measurements of DIC during the Swedish experiment were not performed, therefore the average $\delta^{13}C_{DIC}$ of the control mesocosm (M1) calculated from the final phase was chosen as background signal. Although the use of such a background signal could lead to a disputable accuracy of the isotopic incorporation, the resulting $\Delta\delta^{13}C_{DIC}$ pattern remains unaffected.

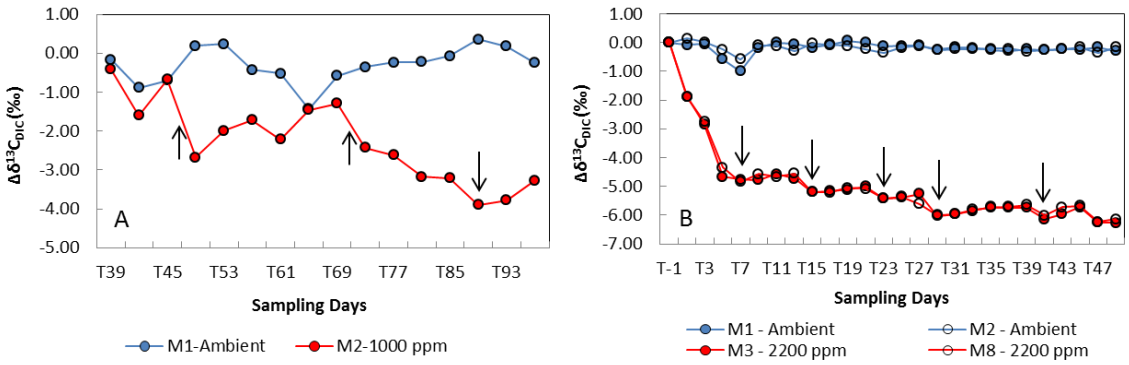


**Figure 4-8.** Temporal change of isotope ratios of DIC during the Swedish (A) and Norwegian (B) mesocosm campaigns in 2013 and 2015, respectively. Black arrows indicate the $CO_2$ enrichment events.

In both experiments, each addition was contributing to a further decrease of the $\delta^{13}C_{DIC}$ signature and any phytoplankton activity or $CO_2$ diffusion into the atmosphere could counterbalance the isotopic decrease of mesocosm DIC waters.

The initial accumulation of the added $CO_2$ into POC could not be determined for the Swedish mesocosm experiment as the first isotopic measurements were performed when a phytoplankton bloom had already occurred. However, incorporation of light carbon was observed after the second bloom and remained relatively constant until the end of the experiment (Figure 4-9 A). The incorporation of the added $CO_2$ into POC during the Norwegian mesocosm experiment was gradual and increased until the end of the experiment without however reaching a stable point (Figure 4-9 B).

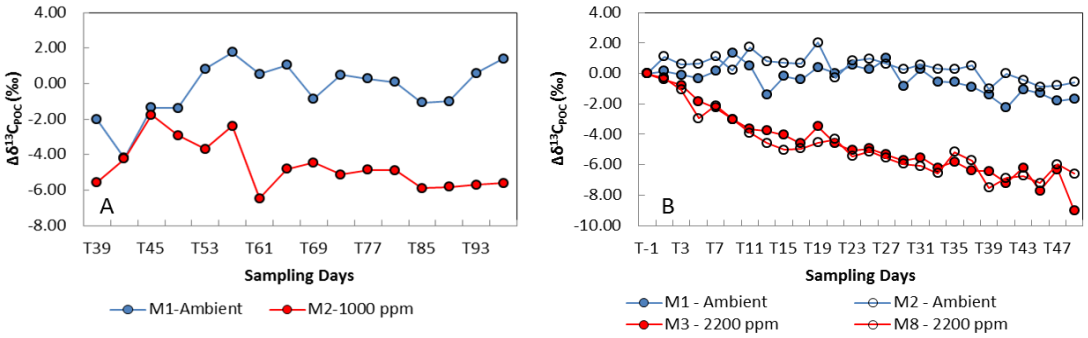


**Figure 4-9.** Temporal change of isotope ratios of POC during the Swedish (A) and Norwegian (B) mesocosm campaigns in 2013 and 2015, respectively.

The different intensity of the phytoplankton blooms between the two experiments might have contributed to determine the observed $\Delta\delta^{13}C_{POC}$ patterns. In addition, the presence of different phytoplankton groups is likely to have influenced both the rate and the nature of carbon uptake. A potential different phytoplankton composition between experiments could have explained the different phytoplankton fractionation ($\varepsilon_{phyto}$) observed in response to high $CO_2$ levels during the two experiments (Figure 4-10).

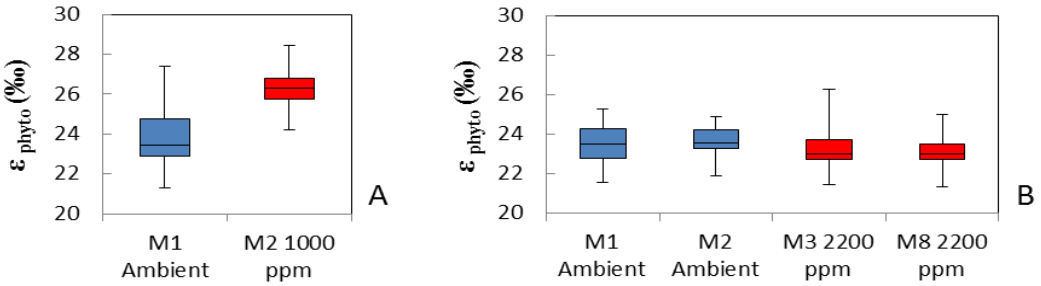


**Figure 4-10.** Box whisker plots of phytoplankton fractionation values during the Sweden, 2013 (A) and Norwegian, 2015 (B) mesocosm experiments.

While high $CO_2$ concentrations increased $\varepsilon_{phyto}$ by almost 3 ‰ (from 23.7 to 26.2 ‰) during the Swedish mesocosm experiment, no clear change in phytoplankton fractionation was observed for the Norwegian experiment and $\varepsilon_{phyto}$ values were on average 23.3 ± 0.2 ‰ for all the sampled mesocosms. The observed change in fractionation corroborates the work by Bach et al. (2016) where a positive $CO_2$ effect on the abundance of smaller planktonic species (picoeukaryotes) during Phase III of the Swedish mesocosm experiment was described. However the current unavailability of planktonic species composition in the Norwegian mesocosms cannot exclude the establishment of a similar or different $CO_2$ effect during the latter experiment.

Assuming a direct relationship between DOC and POC, the calculated $\varepsilon_{POC \rightarrow DOC}$ can give indication on the $^{13}C$ exchange between the two pools (equation 4.12). Additions of isotopically light carbon into mesocosm waters and subsequent incorporation into POC yielded, as a

consequence, higher $\varepsilon_{POC \rightarrow DOC}$ values in ambient $CO_2$ level mesocosms compared to the treated ones during both the experiments (Figure 4-11).

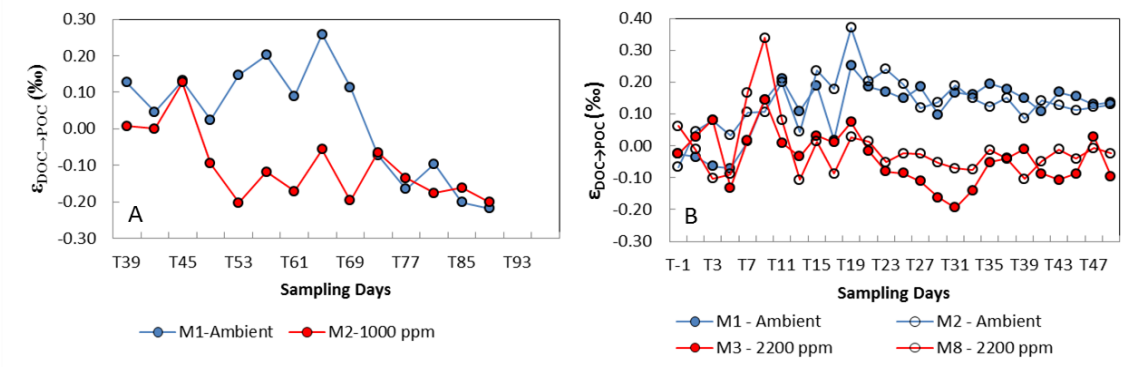


**Figure 4-11.** Isotope effect between POC and DOC pools during the Swedish, 2013 (A) and Norwegian, 2015 (B) mesocosm experiments.

Although the magnitude of the isotope effect between the two pools was relatively small (-0.2 < $\varepsilon_{POC \rightarrow DOC}$ < 0.2 ‰), a net separation could be observed after the decline of the phytoplankton bloom during both experiments. The observed patterns could be explained by potentially similar bacterial activity and temporal development in the mesocosm bags during the two studies. The combination of isotopic data with measurements of bacterial production could help to quantify the degree of coupling between phytoplankton and bacteria. However, the current unavailability of those measurements can make us formulate only early stage hypotheses.

Isotope mixing between the water column and sediment traps gave indication of sinking of freshly produced material (equation 4.13). The fraction of the settled material derived from the added $CO_2$ incorporated into POC increased following the phytoplankton bloom and stabilised to values of 80 to 100 % soon after the decline, during both mesocosm experiments (Figure 4-12).

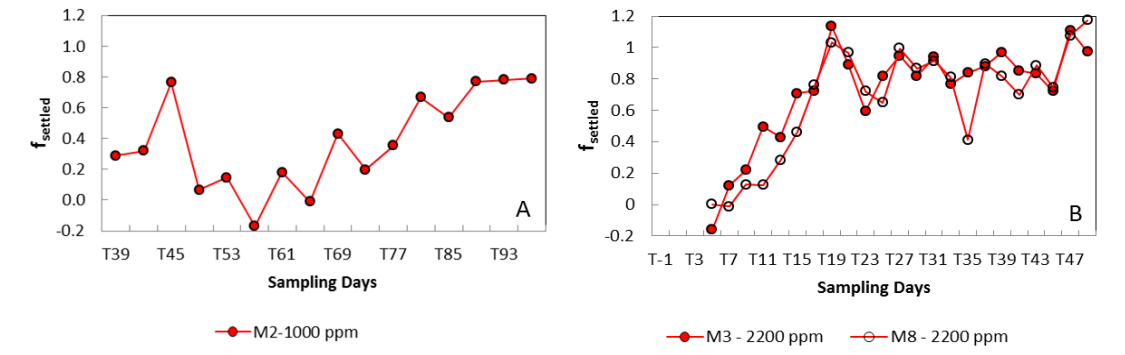


**Figure 4-12.** Fraction of added $CO_2$ settled in the sediment traps derived from the water column particulate carbon during the Swedish, 2013 (A) and Norwegian, 2015 (B) mesocosm experiments.

As isotopic measurements of POC before the first $CO_2$ addition during the Swedish mesocosm experiment were not performed, in order to calculate the fraction settled, the average

$\delta^{13}C_{POC}$ of the control mesocosm during the post bloom phase was used as background. During the Swedish mesocosm experiment, the exported isotopically light carbon observed before t49 was probably deriving from the previous phytoplankton bloom (Phase II). During the second bloom settling appeared to decrease, however the trend could have been a direct consequence of a further change in the POC signature, compared to the initial bloom, due to new carbon uptake. Nevertheless isotope mixing gave clear indication of sinking and both experiments confirmed a rapid and full export of the added isotopically light carbon derived from the water column particulate matter.

### 4.3.8 Assumptions, caveats and future directions

Measurements of stable carbon isotopes in samples collected during the two mesocosm experiments suggested that interactions among all the carbon pools followed a similar pattern and additions of $CO_2$ had comparable effects on the isotopic distributions of all the carbon species within the mesocosms. The statistical analysis performed on the two datasets confirmed that mesocosm responses to similar treatments were highly comparable and the replicated approach was useful to justify the outputs. However the statistical analyses applied were based on the assumption that biogeochemical processes inside the mesocosms were following parallel temporal development.

Mesocosms are defined as experimental enclosures designed to mimic natural conditions (Riebesell et al., 2013), therefore the degree of environmental realism can be disputed. Natural systems might be subjected to a range of external factors that mesocosms do not experience (water currents, marine community change and random variability) therefore evoking potentially biased responses. However, although slightly variable, in both studies the similarity of stable carbon isotope distribution between control mesocosms and surrounding fjord waters suggested a general agreement in ecosystem behaviour.

The current unavailability of many non-isotopic measurements precluded an in-depth assessment of carbon dynamics in the mesocosm systems. However, assessment of isotopic interactions among compartments suggested that the transfer of carbon in general followed a similar trend during both experiments although some local responses to $CO_2$ levels (phytoplankton fractionation) could be detected. In the near future the current stable carbon isotope measurements will be combined with the other physical, chemical and biological observations from the mesocosm experiments in order to better understand the effects of increasing $CO_2$ concentrations on marine ecosystems.

Additionally, the combination of quantitative and isotopic analysis of all the carbon system species will be useful to identify and quantify the carbon fluxes among each compartment within

mesocosms. Specific responses of primary producers to human perturbation could be investigated and assessment of the dynamics and the rates by which carbon is taken up, stored in the underlying sediments and marine biota or exported and recycled in the surrounding environment could be made. The use of suitable biogeochemical models could provide additional information on understanding the driving mechanisms and forecast the consequences of $CO_2$ addition on organisms and marine communities highlighting the sensitivity of key components. These isotopic datasets, providing a holistic view of the carbon cycle from the atmosphere, water column and sediment material, can be used to improve the precision of climate models and represent a new baseline for the reconstruction of historical $CO_2$ trends.

## 4.4 Conclusion

The stable carbon isotope composition of water samples from two North Atlantic fjords was successfully determined. Isotopic measurements were performed on dissolved and particulate inorganic and organic carbon samples collected during the Sweden mesocosm experiment in 2013 and during the Norwegian mesocosm experiment in 2015. Isotopic precisions ($1\sigma$) of 0.09 ‰, 0.62 ‰ and 0.12 ‰ were established for the $\delta^{13}C_{DIC}$, $\delta^{13}C_{DOC}$ and $\delta^{13}C_{POC}$ datasets, respectively. Although analyses were performed over several months no systematic offsets or biases were encountered either within datasets or within analytical sequences. Inter-calibration of the instruments employed through cross analysis of multiple standards calibrated versus V-PDB assured accuracy and consistency of the results. The analysis of reference materials obtained from A. G. Dickson (Scripps Institute of Oceanography, USA) and from the Hansell Laboratory (Rosenstiel School of Marine and Atmospheric Science, Miami, USA) although not internationally isotopically certified, yielded results comparable to previously reported values confirming both quality and internal consistency of the $\delta^{13}C_{DIC}$ and $\delta^{13}C_{DOC}$ measurements, respectively.

Stable carbon isotope measurements from fjord waters and mesocosm control samples were in general comparable to previously reported values from geographically proximate areas. Additions of $CO_2$ yielded significant effects on the isotopic distribution of all the carbon species. The incorporation of isotopically light carbon into the inorganic pool was proportionally transferred to the organic compartments through photosynthesis and bacterial activity. Additionally, the correlation between isotopic ratios of sediment trap material and water column particles suggested rapid sinking and showed that the nature of the suspended material was directly reflected in the settled counterpart. However ancillary data and/or integration with biogeochemical models are still necessary for an in-depth assessment of ecosystem responses to future high $CO_2$ concentrations and only early stage speculations have been made here.

# Chapter 5: Application of stable carbon isotopes in a mesocosm study

Mario Esposito, Eric P. Achterberg, Lennart T. Bach, Douglas Connelly, Ulf Riebesell, Jan Taucher. In preparation, to be submitted to *Frontiers in Marine Biogeochemistry* as part of the collection "Impacts of $CO_2$ Perturbation on the Ecology and Biogeochemistry of Plankton Communities during a simulated upwelling event: A mesocosm experiment in oligotrophic subtropical waters".

## 5.1 Introduction

Since the beginning of the industrial revolution the concentration of carbon dioxide ($CO_2$) in the atmosphere has increased by circa 40% from about 280 ppm to values above 400 ppm (http://www.esrl.noaa.gov). Atmospheric levels of $CO_2$ are expected to rise further and if no mitigation measures are taken, they could reach near 800 ppm by the end of this century (Prentice et al., 2001). The global ocean is attenuating this increase by absorbing about 25 % of the $CO_2$ emitted by humans (Canadell et al., 2007). However the current rate of $CO_2$ uptake is leading to alteration in the chemistry of the ocean causing disequilibrium in the carbonate system (ocean acidification) with potential adverse consequences on marine ecosystems. The flux of $CO_2$ between the atmosphere and the ocean is mainly controlled by physical processes; however biological processes (primary production, community respiration and export), also affect the $CO_2$ transfer between air and sea. Phytoplankton assimilate the inorganic carbon dissolved in the water and convert it into organic forms. As a consequence, surface water $CO_2$ concentrations decrease promoting further $CO_2$ transfer from the atmosphere. In general, primary production and export (sinking) contribute to increase ocean $CO_2$ sequestration from the atmosphere while community respiration tends to decrease it.

A wide range of research and development activities are underway in order to improve our understanding of the effects of rising $CO_2$ on carbon fixation and cycling in the marine environment. Mesocosm experiments have been carried out to investigate community dynamics, measure stocks and fluxes of reactive elements and compounds of interest and study biogeochemical processes under controlled experimental conditions (such as simulating high atmospheric $CO_2$ level scenarios). Mesocosms offer an intermediate scale between laboratory and natural conditions representing near natural ecosystems in which environmental factors can be manipulated and closely monitored (Riebesell et al., 2013). Over the last few years, several mesocosm studies aimed at investigating the effects of elevated $CO_2$ on marine ecosystems have been conducted. They have provided comprehensive datasets, although interpretation has been

highly complex and in some cases has led to conflicting results. One of the first mesocosm experiments was carried out in the subpolar waters of the Raunefjord (Norway, 2001) and no $CO_2$ effect on primary production was detected during the peak or the decline of a nutrient addition induced phytoplankton bloom (Delille et al., 2005). However, high $CO_2$ concentrations enhanced the production of transparent exopolymer particles (TEP) and potentially increased microbial activity (Engel et al., 2004). Four years later (2005) another mesocosm experiment carried out in the same area showed that high $CO_2$ levels enhanced inorganic carbon consumption relative to nutrient uptake (Riebesell et al., 2007), however no increased levels of organic material were observed in the water column pool suggesting a rapid export to the deeper layer (Schulz et al., 2008). Interestingly, in the same experiment, labelled $^{13}C$ measurements showed increased biomass of green algae and diatoms under high $CO_2$ levels, but no indication of enhanced sinking during the bloom phase (de Kluijver et al., 2010). In order to gain further insights on carbon transfer mechanisms, an additional mesocosm study was carried out in the polar region at Ny Ålesund (Svalbard, 2010) and through the use of labelled $^{13}C$, carbon fluxes were assessed. Elevated $CO_2$ concentrations had no direct effect on primary production and bacterial activity, however an increase in sedimentation of fresh organic material was observed (de Kluijver et al., 2013).

The use of a $^{13}C$ labelling technique in the past mesocosms experiments has given indications on the direct coupling between phytoplankton and bacteria. However tracer addition experiments are limited when the product is saturated with the labelled $^{13}C$ as further incorporation of substrate would not change the signature of the product. In the case of phytoplankton and bacteria, the fast turnover rates of 2 to 6 days (Field et al., 1998) limit the use of the labelled $^{13}C$ incorporation method for a long term assessment of carbon fluxes. Moreover, as carbon cycling in the marine system involves active exchanges between not only the phytoplankton and bacteria compartments, interactions among all of the carbon pools must be assessed. Here, the feasibility of using stable carbon isotope analysis as a tool for tracing the natural carbon component through the marine system in a long term mesocosm study is explored. The main research objective was to investigate how $CO_2$ additions alter the isotopic composition of carbon in the marine system. Without the use of labelled bicarbonate that could lead to phytoplankton saturation and represent a bias for carbon fluxes interpretation, the processes that control the distribution of stable carbon isotopes within the water column under different $CO_2$ level were identified and quantified. To the best of our knowledge this is the first time that this approach is used to directly assess fluxes among the carbon system compartments and determine the effects and fate of natural carbon in a phytoplankton succession.

## 5.2 Materials and Methods

### 5.2.1 The study site and mesocosm experiment description

The study was conducted in Gran Canaria (Spain) between the 22nd of September and the 27th of November 2014. The Canary Islands area represents a transitional zone between the northwest African coastal upwelling region and the open ocean oligotrophic waters of the subtropical gyre (González-Dávila et al., 2003). The islands are characterised by a relatively weak surface current (Canary Current) flowing southwestwards and driven by north-easterly trade winds. The experiment was carried out in Gando Bay (Figure 5-1) in order to ensure wind and wave protection. The experimental setup and mesocosm characteristics are described in detail in Taucher et al. (2017). Briefly, nine Kiel Off-Shore Mesocosms for Future Ocean Simulation (KOSMOS) were deployed and moored in clusters of three at 27° 55' N, 15° 21' W about 4.5 nautical miles (nmi) from Taliarte harbour. The water depth at the deployment site was between 18 and 22 m. The bottom ends of the mesocosm bags were lowered to a depth of 15 m below the surface enclosing a volume of about 35 $m^3$ of seawater. Before the closure of the mesocosm bags a 3 mm size mesh was attached to the top and bottom of the bags in order to exclude large organisms. The bags were closed on the 27th of September 2014 defined as time t-4, with t0 marking the day of the initial $CO_2$ manipulation. The main $CO_2$ addition was performed in 4 steps over 6 days by adding calculated amounts of $CO_2$-saturated seawater to each mesocosm as described by Riebesell et al. (2013) in order to yield a gradient of 370 - 2000 ppm. Two further $CO_2$ additions (t21 and t38) were performed during the course of the experiment to account for $CO_2$ loss related to outgassing.

At day t24, aliquots of deep water obtained from a depth of 650 m were added to each mesocosm to promote a phytoplankton bloom. The experiment was terminated about 15 days after the decline of the bloom at t55. The whole experiment was divided in three phases based on chlorophyll a (chl *a*) and nutrient dynamics: an oligotrophic phase I (t0 to t23), a phytoplankton bloom phase II (t25 to t35) and a post bloom phase III (t37 to t55).

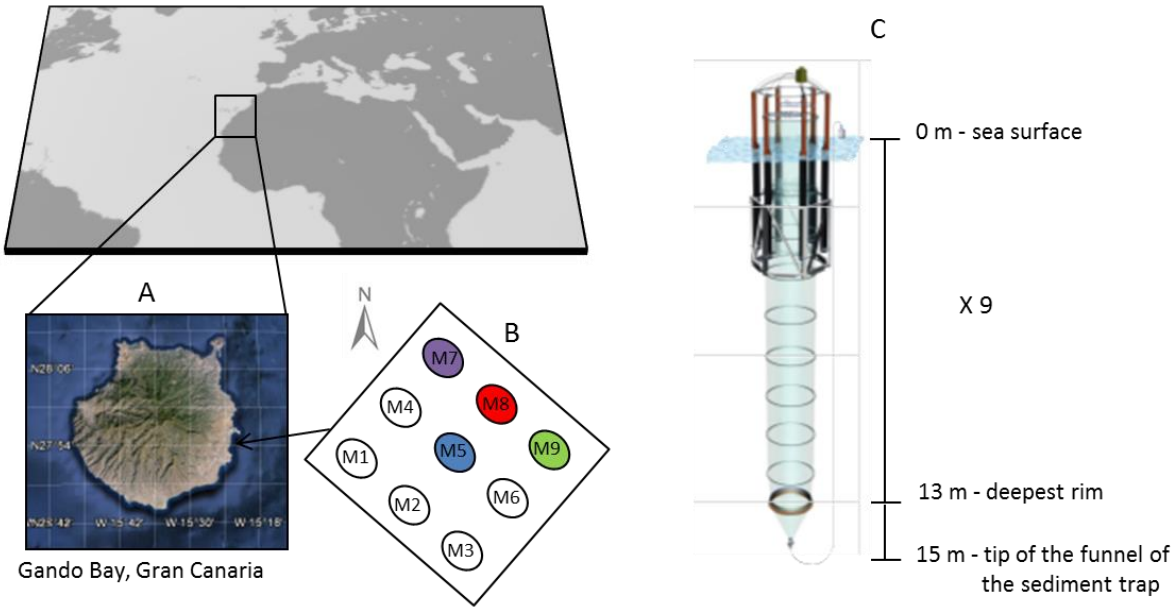


**Figure 5-1.** Study site (A) with mesocosm positioning (B) and characteristics (C), September-November 2014. Numbers show mesocosm arrangement while colours indicate the sampled mesocosms with different $CO_2$ concentrations (green= control at ambient $pCO_2$, blue= $pCO_2 \approx 450$ ppm, purple= $pCO_2 \approx 700$ ppm and red= $pCO_2 \approx 1000$ ppm).

### 5.2.2 Sampling strategy

Sampling was carried out every two days. Before water column sampling, the particles that settled in the mesocosm sediment traps were vacuum-pumped into a sampling flask following the procedure described in Boxhammer et al. (2015) in order to avoid possible resuspension of the settled material in the mesocosm water column. Subsamples for stable carbon isotope analysis of the sediment material ($\delta^{13}C_{Sed}$) were collected every other sampling day (every four days) from five mesocosms (M5 to M9). Sampling was conducted by gentle vacuum (< 200 mbar) filtration of 3 ml of homogenised sediment particle suspension on pre-combusted (450 °C for 12 h) 25 mm, 0.7 µm pore size glass microfiber Whatman GF/F filters. The filters were stored frozen (-20 °) immediately after sampling for later analysis.

Water column samples were collected every sampling day using a depth integrating water sampler (IWS, Hydro-Bios) that is able to evenly collect 5 litres of seawater over the entire depth (13 m) of the mesocosm. Separate sampling bottles were used to withdraw precise aliquots of seawater from the IWS for specific analysis types. These included samples for inorganic nutrients (nitrate, silicate, phosphate and ammonium), pH, dissolved inorganic and organic carbon (DIC and DOC, respectively) and pigments. Every other sampling day, the chosen 5 mesocosms were sampled for isotope analysis in the forms of dissolved and particulate inorganic and organic carbon (Figure 5-2).

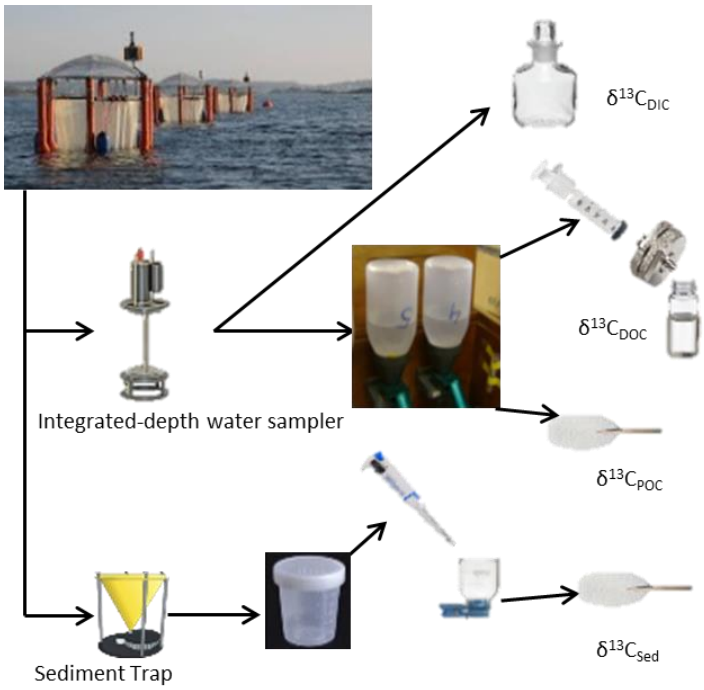


**Figure 5-2.** Sampling strategy for stable carbon isotope analysis for Gran Canaria 2014 mesocosm experiment.

Samples for stable isotopic analyses of dissolved inorganic carbon ($\delta^{13}C_{DIC}$) were collected into 100 ml Pyrex borosilicate glass bottles with a glass stopper. Sample preservation was performed by spiking the samples with 20 µl of saturated mercuric chloride solution (Dickson et al. 2007). For analyses of stable isotopes of dissolved organic carbon ($\delta^{13}C_{DOC}$), samples were collected into 30 ml acid-washed (HCl, 10 %) and pre-combusted (at 450 °C for 12h) TOC glass vials after filtration through a pre-combusted 25 mm carbon cleaned GF/F filter (Dickson et al. 2007). Samples were acidified with 100 µl of 4M hydrochloric acid solution for preservation. Samples for isotopic analysis of particulate organic carbon ($\delta^{13}C_{POC}$) were collected on 0.7 µm pore size pre-combusted (at 450 °C for 12h) glass-fiber filters (GF/F) by filtration of 1 L of seawater collected into 1 L narrow mouth nalgene (HDPE, PK 50) sampling bottles. The filters were stored frozen (-20 °) immediately after sampling for later analysis.

Every sampling day after sediment and water column sampling, a CTD60M (Sea & Sun Technologies) was used to determine vertical profiles of salinity, temperature, pH, chl *a*, and photosynthetically active radiation (PAR) in every mesocosm and in the surrounding water.

### 5.2.3 Analytical methods

In most cases, analysis of the collected samples followed standard procedures. Inorganic nutrients were filtered (0.45 µm cellulose acetate filters, Whatman) directly after sampling and analyzed on the same day to avoid concentration changes due to biological growth or decay. Measurements were performed using a SEAL Analytical QuAAtro AutoAnalyzer connected to a

SEAL Analytical XY2 autosampler according to Hansen and Grasshoff (1983) for the determination of nitrate and silicate and according to Murphy and Riley (1962) for the determination of phosphate. Carbonate chemistry samples (DIC and pH) were sterile-filtered (0.2 µm) through a syringe and stored at 4°C in the dark for a maximum of three days until colorimetric titration (Johnson et al., 1987) and spectrophotometric analysis (Clayton & Byrne, 1993) for the determination of DIC and pH, respectively. Phytoplankton pigments were extracted in acetone (90 %) to determine chl *a* concentrations using reverse phase high performance liquid chromatography (HPLC) as described by Paul et al. (2015).

#### 5.2.3.1 Stable carbon isotope analysis

Stable carbon isotope measurements of DIC ($\delta^{13}C_{DIC}$) were performed on a GasBench II preparation device connected to a Delta V Advantage isotope ratio mass spectrometer (both Thermo Fisher Scientific). Samples were analysed in 2 batches consisting of 88 and 79 measurements each. A total of 27 Exetainer vials per batch were used for calibration and quality control standards, while the rest were used for seawater samples (52 plus 4 $CO_2$-saturated seawater). Three in-house calibration standards MAB, NA and NS (Appendix A) were run at the beginning and at the end of the analytical sequence, while quality control samples (blanks, NFL, NSL and Dickson CRMs) were placed every 12-14 seawater samples. All the samples were run in duplicates.

Measurements of $\delta^{13}C_{POC}$ and $\delta^{13}C_{Sed}$ were performed on a Flash 2000 Elemental Analyser (EA) connected to a Delta V Advantage IRMS through a ConFlo IV interface device (all Thermo Fisher Scientific). A total of 104 samples (52 from the water column and 52 from the sediment traps) were analysed in duplicate over 10 analytical runs. Each run consisted of 3 initial blank measurements (tin capsule and pre-combusted blank filters), duplicate measurements of calibrated urea, caffeine and sucrose standards (Appendix B), followed by a set of mesocosm samples. In the case of sediment samples, dilution (78 % helium) was activated in order to avoid IRMS signal intensity saturation. Standard materials were accurately weighed (readability of 0.01 mg) between 0.1 and 1.0 mg in order to bracket the marine particulate carbon concentration range and used both for isotopic calibration and sample concentration determination.

Measurements of $\delta^{13}C_{DOC}$ were carried out using an in-house combined TOC-IRMS system (refer to Chapter 3 for system details and standard material calibration). A total of 52 samples were analysed over 5 runs. Each analytical sequence consisted of initial background and ultrapure water blank measurements, followed by calibration standards (phthalate, urea, glutamic acid and sucrose) and a variable set of seawater samples. Standards were accurately prepared with concentration ranging between 30 and 600 µM of carbon and used both for isotopic calibration and sample concentration determination. Deep seawater consensus reference material (Lot No 8-

08) distributed from the laboratory of D. Hansell (University of Miami) and additional urea or sucrose standards were analysed between samples in order to check for accuracy, consistency and correct for potential instrument drifts.

#### 5.2.3.2 Data analysis

Stable isotope data were expressed in the delta notation ($\delta^{13}C$) relative to the VPDB standard according to

$$\delta^{13}C = \frac{R_{sample} - R_{standard}}{R_{standard}} \times 1000\ ‰ \quad (5.1)$$

where R represents the ratio of the heavy carbon isotope ($^{13}C$) to the light isotope ($^{12}C$). The isotopic incorporation of $CO_2$-saturated water into mesocosm systems was calculated as relative and absolute changes in the $^{13}C$ fraction of the samples. Relative changes were calculated as a delta difference ($\Delta\delta^{13}C$) between the isotopic signature of the samples and the initial signatures (before $CO_2$ additions). The absolute concentration of the $^{13}C$ fraction was calculated as

$$^{13}C = \Delta^{13}r \times concentration\ (\mu M) \quad (5.2)$$

with $\Delta^{13}r$ representing the difference between the $^{13}r$ of the samples and the $^{13}r$ measured before any $CO_2$ addition. The fractional ratio $^{13}r$ corresponds to

$$^{13}r = \frac{[^{13}C]}{[^{12}C + {}^{13}C]} \quad (5.3)$$

and it is derived from the delta notation.

Air sea gas exchange was calculated both as total flux ($F_C$) and as outgassing of the $^{13}C$ fraction ($F_{13C}$). The total flux was determined as

$$F_C = -k(DIC_{sample} - DIC_{sat}) \quad (5.4)$$

where $DIC_{sample}$ is the dissolved inorganic carbon concentration of the samples, $DIC_{sat}$ is the dissolved inorganic carbon saturation concentration corresponding to an atmospheric $CO_2$ partial pressure of 394 ppm (ftp://aftp.cmdl.noaa.gov/data/trace_gases/co2/flask/surface) The parameter k is the piston velocity depending on wind speed and sea surface temperature and it was calculated according to

$$k = -k_0 u^2 \left(\frac{Sc}{660}\right)^{-0.5} \quad (5.5)$$

where $k_0$ is a constant ($k_0 = 0.253$), u is the wind speed ($u = 2$ m/s) and Sc is the sea surface temperature (T) dependent Schmidt number, $Sc = 2073.1 - 125.62 \cdot T + 3.6276 \cdot T^2 - 0.043219 \cdot T^3$ for

$CO_2$ (Schmittner et al., 2013). The flux $F_{13C}$ was calculated according to Zhang et al. (1995) by using the following equation:

$$F_{13C} = -k\alpha_k\alpha_{aq\leftarrow g}\left(\frac{R_{DIC}}{\alpha_{DIC\leftarrow g}}\,DIC_{sample} - R_A DIC_{sat}\right) \quad (5.6)$$

where k is the piston velocity in m/s, $\alpha_k$ is a constant kinetic fractionation factor ($\alpha_k$ = 0.99915), $\alpha_{aq\leftarrow g}$ and $\alpha_{DIC\leftarrow g}$ are the temperature dependent isotopic fractionation factors from gaseous to aqueous $CO_2$ and from gaseous $CO_2$ to DIC, respectively. $R_{DIC}$ and $R_A$ correspond to the heavy to total isotope ratios of DIC and atmospheric $CO_2$, respectively and they were determined from $^{13}C$ fractions as $R_{DIC} = {}^{13}C_{DIC}/({}^{12}C_{DIC} + {}^{13}C_{DIC})$ and $R_A = {}^{13}C_A/({}^{12}C_A + {}^{13}C_A)$. The $R_A$ was derived from the isotopic signature of the atmospheric $CO_2$ which was assumed to have a fixed $\delta^{13}C$ value of -8.2 ‰ according to the reported monthly average isotopic air measurements for the Canary Islands area (ftp://aftp.cmdl.noaa.gov/data/trace_gases/co2c13/flask/).

The effect of air-sea flux on $\delta^{13}C_{DIC}$ distribution was calculated as a difference ($\Delta F_{13C}$) between the $F_{13C}$ (divided by isotope ratio of the standard, $R_{standard}$ = 0.0112) and the total carbon flux according to Schmittner et al. (2013) by using the following equation:

$$\Delta F_{13C} = \frac{F_{13C}}{R_{standard}} - F_C \quad (5.7)$$

Photosynthetic isotope fractionation ($\varepsilon$) between $CO_2$ and phytoplankton during the uptake process was calculated as

$$\varepsilon_{CO_2-phyto} = \frac{\delta^{13}C_{CO_2} - \delta^{13}C_{phyto}}{1 + \delta^{13}C_{phyto}/10^3} \quad (5.8)$$

where $\delta^{13}C_{CO2}$ is the isotopic signature of aqueous $CO_2$ calculated from $\delta^{13}C_{DIC}$ values using the equilibrium fractionation factors between DIC and $CO_{2(aq)}$ according to Zhang et al. (1995), and the isotope ratio of phytoplankton ($\delta^{13}C_{phyto}$) was calculated from a mass balance equation assuming that phytoplankton is represented by the particulate carbon fraction in the water column (POC) minus the exported fraction (TPC in the sediment traps). It was assumed that dissolved $CO_2$ was the main form of carbon assimilated by phytoplankton during growth due to its low energy costs associated with passive intracellular transport (Burkhardt et al., 1999; Marty & Planas, 2008) and to its generally high availability found in geographically proximate areas during past similar periods of the year (Santana-Casiano et al., 2001; González-Dávila et al., 2003).

Phytoplankton biomass was derived from chl *a* measurements assuming a constant C:Chl *a* ratio of 60 as recommended by Strickland (1960) for warm and nutrient poor waters. Carbon uptake by phytoplankton was calculated from the daily change in DIC concentrations corrected for air-sea carbon transfer during the bloom period. The contribution of the particulate carbon to

the dissolved organic carbon pool was assessed as isotopic fractional transfer between the two pools using a mass balance mixing equation. Similarly, stable isotope data from sediment traps were used to calculate the amount of $^{13}C$ in the settled material derived from the POC as an indication of sinking by using the following isotope mass balance mixing equation (Fry, 2006):

$$f_{settled} = \frac{(POC * \delta^{13}C_{Sed})}{(POC * \delta^{13}C_{POC} + POC_{Initial} * \delta^{13}C_{Initial})} \quad (5.9)$$

where $\delta^{13}C_{Initial}$ and $POC_{Initial}$ are the average isotope ratio and concentration (µmol/l) of particulate organic carbon, respectively at the beginning of the experiment before any $CO_2$ addition.

#### 5.2.3.3 Statistical analysis

The isotopic data reported are average values between duplicate samples with standard deviations (1σ) within the analytical error. The dataset was tested for normality distribution using the online Grubbs test (http://graphpad.com/quickcalcs/grubbs1/) and eventual outliers were removed and/or mathematical interpolated. Linear regression analyses were used to determine the relationship between $pCO_2$ and average response of the variables to each $CO_2$ addition phase. Significance was accepted for p-values < 0.05. The potential effect of the added $CO_2$ on the various parameters was also calculated by subtracting observations of the control mesocosm from the treated mesocosms on the specific sampling day.

## 5.3 Results

### 5.3.1 Mesocosm performance and mesocosm phases

Although 9 mesocosms were deployed at Gando bay, only 5 of them were sampled for stable carbon isotope analysis. A very strong eastward current event (t25 - t27) damaged mesocosm M6 and no more sampling was performed from this unit until the end of experiment. Data from mesocosm M6 were therefore excluded from the analyses and only mesocosm M5, M7, M8 and M9 will be considered herein. The closure of the mesocosm bags was conducted when similar conditions between the various mesocosms were established. The timing was assessed based on the lack of detectable differences in salinity, temperature, density, chl *a* and nutrient concentrations between mesocosms. Average initial salinity in the mesocosms was 37.08 ± 0.01 and gradually increased throughout the experiment to reach final values of 38.03 ± 0.02. The reason for the change was mostly due to evaporation although other environmental (rain, waves) and experimental (deep water addition) events might have contributed to the salinity variation. Temperatures decreased gradually from 24.31 ± 0.02 °C to 22.22 ± 0.01 °C during the

course of the experiment. According to CTD profiles, no halocline or thermocline developed during the experiment and all mesocosms parameters were relatively homogeneous through the water column. Increasing injections of $CO_2$-saturated seawater into the designated mesocosms formed a concentration gradient with average $pCO_2$ values of 473.9 ± 22.6 µatm (M5), 776.8 ± 42.8 µatm (M7) and 1311.7 ± 105.3 µatm (M8) determined after each $CO_2$ addition (t5, t23 and t39). Mesocosm M9 was not treated (control) and exhibited average $pCO_2$ concentrations of 364.4 ± 64.0 µatm measured over the same days. Although $CO_2$ treatments did not reach target $pCO_2$ values a linear concentration gradient ($r^2$ = 0.995) was established (Figure 5-3).

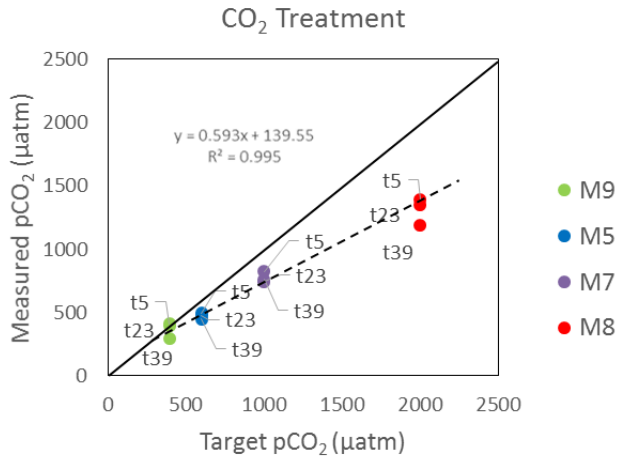


**Figure 5-3.** Measured mesocosm $pCO_2$ concentrations versus target $pCO_2$ concentrations determined on the days of $CO_2$-saturated seawater addition (t5, t23 and t39).

Additions of $CO_2$-saturated seawater increased mesocosm DIC concentrations proportionally and linear regression analysis confirmed the significant differences ($p<0.05$) among treatments. However no significant differences (Table 5-1) among mesocosms were observed overall for other physical parameters or nutrient data when $CO_2$ treatments were compared.

| | Parameter | P value | Multiple $R^2$ | F statistic | Parameter | P value | Multiple $R^2$ | F statistic |
|---|---|---|---|---|---|---|---|---|
| **Phase I** | $NO_3^- + NO_2^-$ | 0.183 | 0.816 | 3.997 | $PO_4^3$ | 0.899 | 0.100 | 0.020 |
| **Phase II** | | 0.972 | 0.027 | 0.002 | | 0.224 | 0.753 | 3.015 |
| **Phase III** | | 0.772 | 0.228 | 0.110 | | 0.125 | 0.874 | 6.507 |
| **Phase I** | $SiO_4^4$ | 0.089 | 0.910 | 9.733 | Chl *a* | 0.291 | 0.708 | 2.017 |
| **Phase II** | | **0.015** | **0.984** | **63.446** | | 0.911 | 0.088 | 0.015 |
| **Phase III** | | 0.795 | 0.204 | 0.081 | | 0.079 | 0.920 | 11.126 |

**Table 5-1.** Results of linear regression analyses testing for statistical significance of $pCO_2$ effect on mesocosm nutrients and chlorophyll *a* for each experimental phase. The statistically significant ($p < 0.05$) effects of $CO_2$ are reported in bold.

Nutrient concentrations as well as chl *a* variations and $CO_2$ manipulation were used to define the different experimental phases (Figure 5-4). Initial concentrations of nitrate plus nitrite ($NO_3^-$ + $NO_2^-$) were between 0.06 and 0.15 µM, phosphate ($PO_4^{3-}$) ranged between 0.01 and 0.07 µM and silicate ($SiO_4^{4-}$) was on average 0.23 ± 0.054 µM for all mesocosms until the day of deep water addition. On day t24 about 8 $m^3$ of deep seawater were added per mesocosm bringing nutrient concentrations to 3.19 ± 0.02 µM, 0.17 ± 0.01 µM and 1.61± 0.11 µM for $NO_3^-$ + $NO_2^-$, $PO_4^{3-}$ and $SiO_4^{4-}$, respectively. The addition triggered a phytoplankton bloom that lasted circa 10 days after which inorganic nutrients stabilised back to initial concentration levels (Figure 5-4). Measurements of chl *a* confirmed the progression of the three phases (Figure 5-4). During the initial oligotrophic phase, chl *a* values were between 0.18 and 0.24 µg/l. With the development of the bloom phase chl *a* values increased and on t31 the average concentration was 2.95 ± 0.67 µg/l. The maximum value (5.62 µg/l) was recorded for mesocosm M9 on t28. In the post bloom phase chl *a* concentrations dropped although they were on average higher (0.57 < chl *a* < 1.10 µg/l) than the initial concentrations. No statistically significant differences in chl *a* concentrations were observed between $pCO_2$ treatments (Table 5-1).

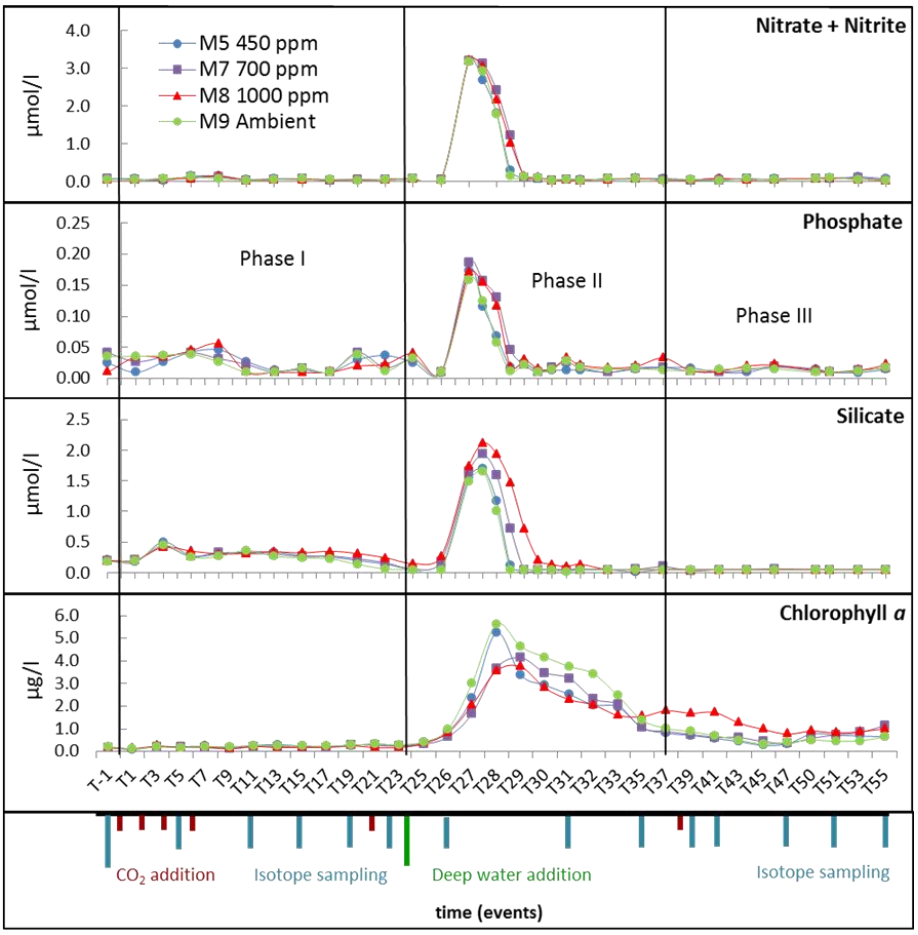


**Figure 5-4**. Concentrations of inorganic nutrients and chlorophyll *a* during the Gran Canaria mesocosm experiment (2014). Sampling days (T-days) and main experimental manipulations are shown on the x-axis. Vertical lines define the experimental phases.

### 5.3.2 Stable carbon isotopes measurement processing

The three different instrument configurations used to perform stable carbon isotope analysis were calibrated by cross check measurements of the same standard compounds. Raw $\delta^{13}C$ results were processed following the same procedure: removal of anomalous measurements, linearity correction, blank correction, calibration to V-PDB and average of duplicates. Anomalies were assessed based on peak area height and when the intensity of the signal fell outside of the calibration range the data point was removed. Only 0.8% of the total measurement was eliminated. Linearity correction was performed by quantification of the relationship between the $\delta$ values of repeated measurements of standards with increasing concentrations versus the respective signal intensities. The mean gradient for each standard was calculated and used for linearity correction. Variation among gradients was 0.0083 ‰. Instrument background, reagent blank intensities and isotopic ratios were stable for all the analytical methods with average total variation of about 2 ± 1.2 %. Calibration of the samples to the V-PDB international standards was performed by three-point linear regression fits using standards of known isotopic ratios. Average $r^2$ was 0.9998 ± 0.00017. Final $\delta^{13}C$ values were reported as the average of both analytical replicates and sample duplicates when available. Calculated uncertainties (1σ) were 0.02, 0.17 and 0.71 ‰ for $\delta^{13}C_{DIC}$, $\delta^{13}C_{POC}$ and $\delta^{13}C_{DOC}$ measurements, respectively.

### 5.3.3 Mesocosm carbon dynamics: concentrations and stable isotopes

Carbon dynamics during the mesocosm experiment can be categorised into three phases based on the three main $CO_2$ additions (Figure 5-5). In most of the cases, carbon trends agreed well with the phases defined by nutrients and chl *a* dynamics: oligotrophic phase I, phytoplankton bloom phase II and post bloom phase III. Before any $CO_2$ addition, on t-1, the $\delta^{13}C_{DIC}$ of the water was 1.06 ‰. Analysis of aliquots of $CO_2$-saturated seawater showed that the isotopic signature ($\delta^{13}C_{DIC}$) of the added seawater was -35.64 ± 0.43 ‰ (n = 10) for the first batch and -36.28 ± 0.06 ‰ (n = 12) for the second batch. Samples from the third batch were not taken therefore no measurements are available. The effects of the added $CO_2$ on mesocosm carbon species are summarised in Table 5-2 where statistical significance of $pCO_2$ treatments on each of the carbon system parameters for each experimental phase is reported.

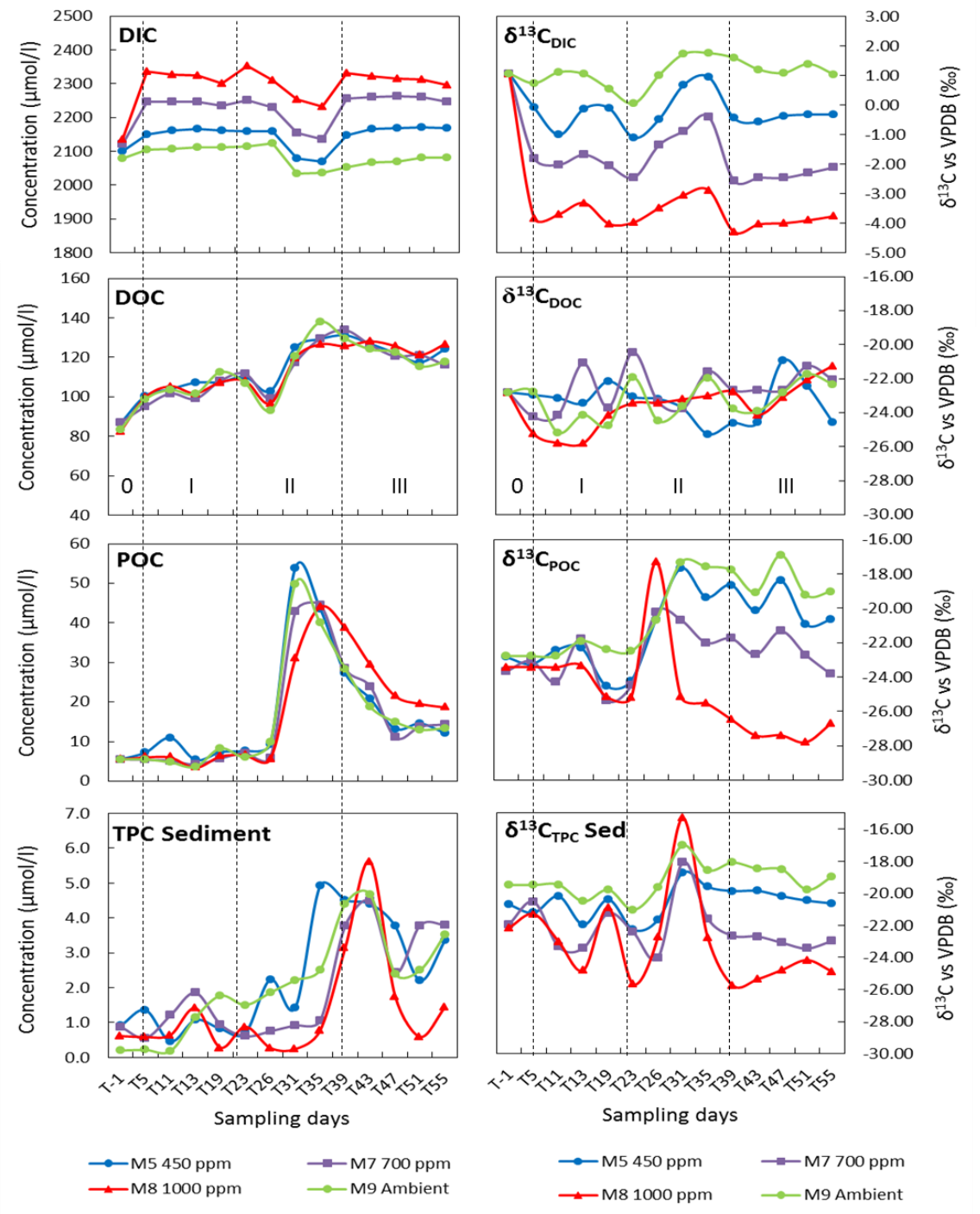


**Figure 5-5.** Temporal development of concentrations and stable isotopes of carbon during the mesocosm experiment in Gran Canaria 2014. The vertical dashed lines denote the timing of $CO_2$ additions and 0, I, II and III represent initial, oligotrophic, phytoplankton bloom and post bloom phases, respectively. DIC = dissolved inorganic carbon, DOC = dissolved organic carbon, POC = particulate organic carbon and TPC = total particulate carbon.

*Oligotrophic Phase I*

Following the first $CO_2$ addition, the $\delta^{13}C_{DIC}$ of the mesocosms decreased significantly (Table 5-2) in proportion to the added amount with average $^{13}C$ depletion of 1.4 ‰, 2.9 ‰ and 4.8 ‰ for mesocosm M5, M7 and M8, respectively, throughout the whole oligotrophic phase. A slight isotopic enrichment (0.2 ‰) combined with a decrease in DIC concentrations over time was observed in the treated mesocosms. This trend was mostly driven by outgassing rather than biological activity as indicated by the low levels of particulate organic carbon in the water column (mean POC = 6.1 ± 1.90 µmol/l) and consequently in the sediment traps (mean $TPC_{Sed}$ = 0.9 ± 0.46 µmol/l) during the entire oligotrophic phase. The isotopic signature of the particulate carbon in the water column was on average -23.5 ± 1.11 ‰ while higher isotopic variation was found in the sediment TPC samples with values ranging between -24.80 and -20.18 ‰. The variation was possibly due to the contribution of the particulate inorganic portion which tended to decrease the carbon isotopic signature in the high $CO_2$ treatment mesocosms. Based on the measured low chl *a* values during the first phase (Figure 5-4), no evidence of strong biological activity was observed, however dissolved organic carbon concentrations gradually increased in all mesocosms from an average initial value of 98 ± 2.4 µmol/l on t5 to a mean value of 108 ± 0.4 µmol/l on t19. The $\delta^{13}C$ of the DOC pool was highly variable with measured ratios between -25.8 and -21.1 ‰. At first glance the $CO_2$ treatments appeared to lower the $^{13}C$ component, however isotopic ratios of DOC in mesocosm M9 (control) were similar to the high treatment mesocosm with an average $\delta^{13}C_{DOC}$ value of -23.9 ± 1.06 ‰. Linear regression analysis confirmed there was no significant effect of $pCO_2$ treatment on $\delta^{13}C_{DOC}$ dynamics (Table 5-2).

*Phytoplankton bloom Phase II*

The second $CO_2$ addition (t21) in general tended to stabilise DIC concentrations to target levels without having any major effect on the isotopic signature of the inorganic carbon pool. The addition of deep water stimulated a phytoplankton bloom which characterises the second phase (t25-t35). With the development of the bloom DIC concentrations decreased on average by 5 % with a corresponding isotopic enrichment of about 2 ‰ for all mesocosms. The DOC concentrations decreased soon after the addition (average DOC = 98 ± 4.2 µmol/l at t26) possibly due to the mixing with organic carbon depleted deep water but rapidly increased by about 25 % reaching a mean value of 131 ± 4.9 µmol/l for all mesocosms. Measurements of $\delta^{13}C_{DOC}$ samples showed reduced variation among mesocosms during the bloom period with an average value of -23.4 ± 0.49 ‰. Particulate organic carbon in general mirrored chl *a* dynamics showing a rapid increase in all mesocosms to concentrations up to 53.9 µmol/l (M5 at t31). The sediment counterpart showed a slight delay with $TPC_{Sed}$ concentrations starting to increase at the end of phase II. Maximum concentrations of sediment particulate organic carbon (between 4.5 and 5.6

µmol/l) were reached only in the post bloom phase on t43. The particulate carbon in the water column showed a rapid isotopic enrichment (about 4 ‰) following deep water addition, however with the development of the phytoplankton bloom, the isotopic ratios of POC in individual mesocosms tended to significantly diverge (Table 5-2) according to the added $CO_2$ levels (lower ratios for higher $pCO_2$ treatments). A similar trend was observed for $\delta^{13}C_{Sed}$ samples although the corresponding isotopic response was detected on the successive sampling day (temporal shift).

*Post bloom Phase III*

The last $CO_2$ addition on t38 brought concentrations and isotopic ratios of DIC to pre-bloom levels. Over the entire post bloom phase, DIC concentrations in mesocosm M8 and M7 decreased by 1.5 and 0.5 %, respectively while an increase of 0.9 and 1.5 % was observed in mesocosms M5 and M9, respectively. This trend suggested that air sea gas exchange was driving seawater $pCO_2$ towards the equilibrium with the overlaying atmosphere. The slow gas exchange had little effect on the $\delta^{13}C_{DIC}$ distribution with isotopic variations within mesocosms lower than 0.2 ‰ for the duration of the entire phase III. DOC concentrations remained stable with average concentrations of 123.8 ± 5.02 µmol/l and the $\delta^{13}C_{DOC}$ showed no significant differences (Table 5-2) among treatments. However, an isotopic enrichment (1.5 ‰) was observed in the $\delta^{13}C_{DOC}$ for the high $CO_2$ treatment mesocosm M8. In the water column, particulate carbon concentrations dropped by about 51 % for all mesocosms. In the sediment traps, maximum concentrations (4.5 ± 0.14 µmol/l) were recorded at the beginning of the phase on t43 as a consequence of the phytoplankton bloom decline and subsequent settling, until they stabilised to final TPC values of 3.6 ± 0.23 µmol/l. Compared to the other mesocosms, at the end of the experiment, M8 had higher POC concentrations (18.6 µmol/l) in the water column and consequently lower TPC values (1.4 µmol/l) in the sediment trap. Isotopic signatures of the particulate carbon reflected the isotopic separation of $\delta^{13}C_{DIC}$ both in the water column and in the sediment traps showing significant correlations (Table 5-2) with the $pCO_2$ treatments.

| | Parameter | P value | Multiple $R^2$ | F statistic | Parameter | P value | Multiple $R^2$ | F statistic |
|---|---|---|---|---|---|---|---|---|
| **Phase I** | DIC | **0.021** | **0.987** | **43.718** | $\delta^{13}C_{DIC}$ | **0.019** | **0.981** | **51.213** |
| **Phase II** | | **0.016** | **0.983** | **59.050** | | **0.038** | **0.962** | **24.796** |
| **Phase III** | | **0.026** | **0.974** | **36.902** | | **0.009** | **0.990** | **101.072** |
| **Phase I** | DOC | 0.711 | 0.286 | 0.711 | $\delta^{13}C_{DOC}$ | 0.329 | 0.670 | 1.632 |
| **Phase II** | | 0.224 | 0.775 | 3.021 | | 0.930 | 0.068 | 0.001 |
| **Phase III** | | 0.190 | 0.809 | 3.808 | | 0.456 | 0.543 | 0.838 |
| **Phase I** | POC | 0.558 | 0.441 | 0.484 | $\delta^{13}C_{POC}$ | 0.126 | 0.873 | 6.431 |
| **Phase II** | | 0.083 | 0.916 | 10.528 | | **0.002** | **0.998** | **509.683** |
| **Phase III** | | 0.133 | 0.866 | 6.023 | | **0.008** | **0.991** | **112.841** |
| **Phase I** | $TPC_{Sed}$ | 0.242 | 0.757 | 2.698 | $\delta^{13}C_{Sed}$ | 0.112 | 0.888 | 7.439 |
| **Phase II** | | 0.131 | 0.868 | 6.141 | | **0.037** | **0.962** | **24.950** |
| **Phase III** | | 0.228 | 0.771 | 2.945 | | **0.008** | **0.992** | **120.018** |

**Table 5-2.** Results of linear regression analyses testing for statistical significance of $pCO_2$ effect on mesocosm carbon system parameters for each experimental phase. The statistically significant ($p < 0.05$) effects of $CO_2$ are reported in bold. DIC = dissolved inorganic carbon, DOC = dissolved organic carbon, POC = particulate organic carbon and $TPC_{Sed}$ = total particulate carbon in sediment traps.

### 5.3.4 Carbon fluxes

The flux of the added isotopically light $CO_2$ due to gas exchange between water and air was calculated for phase I and III only, when biomass was low (0.03 ± 0.003 mg C/l, Chl *a* < 0.3 µg/l). The effect of air-sea gas exchange on mesocosm water $\delta^{13}C_{DIC}$ was proportional to $pCO_2$ enrichments. Increasing positive $\Delta F_{13C}$ values were observed for the treated mesocosm (M5 = 1.3 ± 0.4, M7 = 6.6 ± 1.1 and M8 = 11.7 ± 0.3) suggesting outgassing, with greater tendency to isotopic enrichment of surface water DIC for higher $CO_2$ level mesocosms. Net in-gassing ($\Delta F_{13C}$ = -2.57 ± 0.4) was instead observed for the ambient $pCO_2$ mesocosm control (M9) which tended to lower surface $\delta^{13}C_{DIC}$ signature during both phase I and III. Average daily $CO_2$ influx was 1.4 ± 0.72 µmol/l per day in mesocosm M9 while a $CO_2$ efflux of 0.7 ± 0.02 µmol/l and 2.2 ± 0.03 µmol/l per day was calculated for mesocosm M7 and M8, respectively. Mesocosm M5 exhibited variable daily fluxes with overall outgassing (0.05 µmol/l per day) throughout phase I and a general $CO_2$ influx (0.06 µmol/l per day) during the post bloom phase III. The transfer from DIC to phytoplankton during phase II was rapid as $\delta^{13}C_{POC}$ signatures changed the day after the addition of nutrient-rich deep water. The incorporation of the added carbon into phytoplankton coincided with the relative changes in the $^{13}C_{DIC}$ fraction (Figure 5-6).

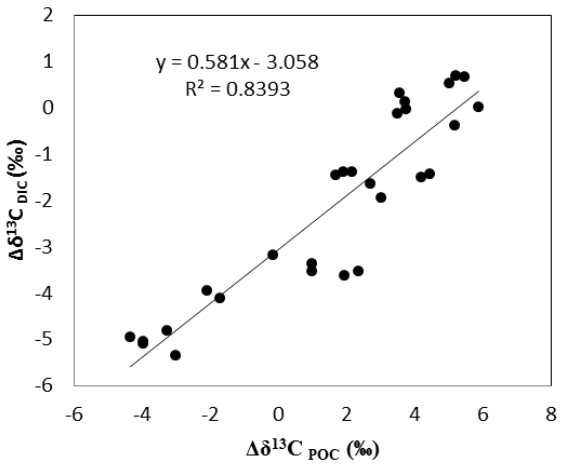


**Figure 5-6.** Indication of $^{13}C$ incorporation into phytoplankton. Relative $\delta^{13}C_{DIC}$ changes versus $\delta^{13}C_{POC}$ changes during bloom and post bloom phases.

Carbon uptake by phytoplankton was on average 7.1 ± 0.99 µmol/l per day during the growing period. Phytoplankton biomass during the bloom phase was similar for all mesocosms and ranged from 0.11 to 0.34 mg C/l. The carbon isotopic composition of phytoplankton $\delta^{13}C_{phyto}$ was positively correlated ($r^2$=0.82, p<0.001; Figure 5-7 A) with the $\delta^{13}C$ of aqueous $CO_2$ and negatively correlated ($r^2$=0.81, p<0.001; Figure 5-7 B) with the concentration of aqueous $CO_2$. The influence of the different concentration and isotopic values of aqueous $CO_2$ was also reflected in phytoplankton fractionation (Figure 5-7 C). The $\varepsilon_{CO2\text{-}phyto}$ ranged between 9.6 and 16.5 ‰ with higher values observed in the high $CO_2$ treatment mesocosms (mean ε = 15.8 ‰ in mesocosm M8 and 12.7 ‰ in M7). Average fractionation values of 11.8 and 12.0 ‰ were calculated for mesocosm M5 and M9, respectively.

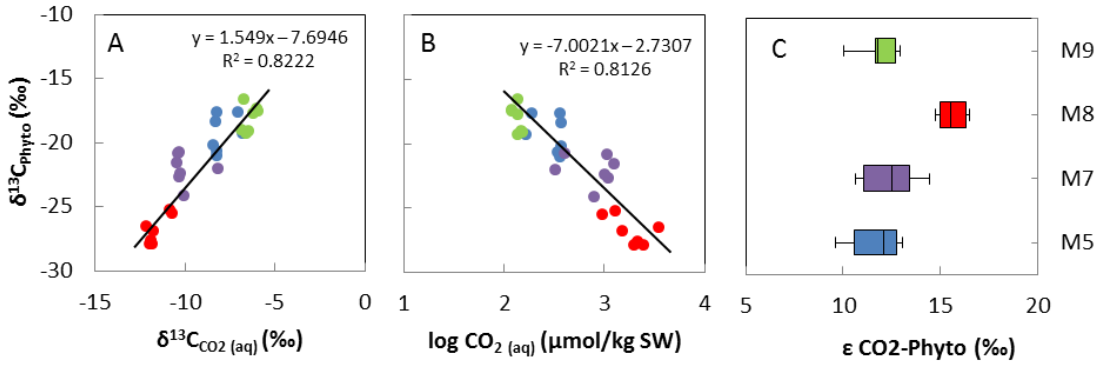


**Figure 5-7.** Relation of $\delta^{13}C_{phyto}$ to isotopic signatures (A) and concentrations (B) of aqueous $CO_2$ in mesocosm experiment during phase II and III. Panel C presents a box whisker plot of phytoplankton fractionation values for the four mesocosms. M5 (blue) = 450 ppm, M7 (purple) = 700 ppm and M8 (red) = 1000 ppm, M9 (green) = control mesocosm at ambient $pCO_2$ concentrations.

The transfer of freshly produced particulate organic carbon into the dissolved organic carbon pool was estimated from the daily changes in the relative incorporation of the $^{13}C$ fraction within each pool. The transfer was variable for each phase and mesocosm: during the oligotrophic phase little (0.01 µmol/l of C per day) or no transfer between the two pools was observed. With the development of the phytoplankton bloom, the $\delta^{13}C$ of DOC in the high $CO_2$ treatment mesocosms (M7 and M8) tended to increase suggesting that light carbon was rapidly consumed and was not accumulating in the DOC pool. The transfer of the $^{13}C$ fraction derived from the POC to the DOC pool was 1.05 and 0.71 % for M7 and M8, respectively during the period following the bloom. No clear changes were observed in the ambient and low $CO_2$ treatment mesocosms (M5 and M9, respectively) and the contribution of the produced POC fraction channelled into DOC was difficult to constrain; small contributions of 0.1 and 0.2 % were estimated.

The average carbon flow into the sediment traps during Phase I was 0.19 ± 0.023 µmol/l per day for all mesocosms. The slow settling process was confirmed by the low fraction ($f_{settled} < 0.2$) in the traps deriving from the overlaying POC (Figure 5-8). With the development of the phytoplankton bloom, the overall particulate carbon export, based on the amount of carbon settled, was between 38.8 and 45.8 % of primary production. Export was not related to $CO_2$ levels ($r = 0.25$, $p > 0.05$). The fraction settled in the traps derived from the overlaying POC increased with the onset of the phytoplankton bloom until the first part of the post bloom phase. Sinking of phytoplankton derived organic carbon continued until the end of the experiment although decreased after t47 with in general lower levels observed in the high $CO_2$ treatment mesocosms (Figure 5-8).

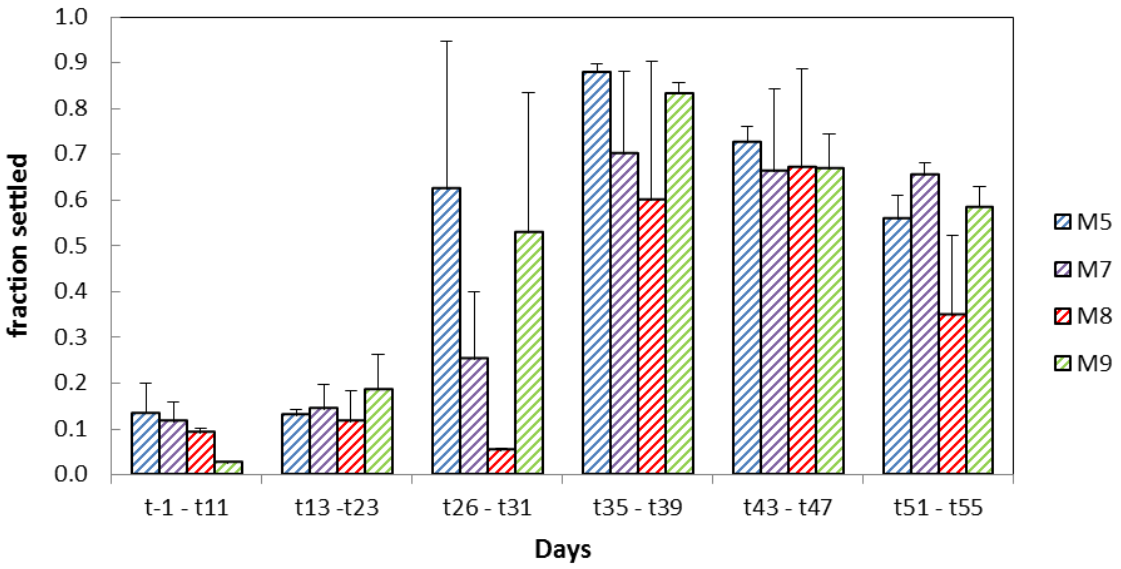


**Figure 5-8.** Fraction settled in the sediment traps derived from the water column. M defines mesocosms at different $pCO_2$ concentrations: M5 = 450 ppm, M7 = 700 ppm, M8 = 1000 ppm, M9 = control at ambient $pCO_2$. Error bars represent the standard deviation for the indicated sampling days.

## 5.4 Discussion

### 5.4.1 General observations

Most of the previous $CO_2$ enrichment mesocosm experiments have focused mainly on how phytoplankton communities responded to ocean acidification. Due to a close-to-natural behaviour of mesocosm experiments compared to laboratory conditions, mesocosms provide ideal platforms to investigate carbon dynamics as a whole whilst allowing the study of the effects of $CO_2$ on phytoplankton species and composition. The study described here was a first attempt to follow and quantify the contribution of added natural carbon to an oceanic oligotrophic system by using stable carbon isotope analysis. In perturbation experiments on enclosed systems such as the current mesocosm experiment, homogeneity of initial conditions and controlled parallel manipulations are essential in order to avoid the risk of biasing the results. Measurements of the mesocosm water salinity were used to determine the volume of each enclosure for correct manipulation (Czerny et al., 2013) and to assess the general mesocosm performances. The concurrent and consistent salinity variations among the considered mesocosms (M5, M7, M8 and M9) ensured comparable general dynamics, although on t13 a hole was spotted but quickly fixed in the ambient mesocosm M9. The hydrographic pattern observed at the beginning of the experiment was typical for post summer conditions in the Canary Islands area (González-Dávila et al., 2003) as it was characterised by high surface water temperatures (around 24 °C), overall supersaturation of surface waters with respect to $CO_2$, low inorganic nutrient concentrations and low chlorophyll *a* values (about 0.18 µg/l). The experiment had to be carried out during the autumn season for technical reasons and although not common for the area at this time of the year, a phytoplankton bloom was induced (Phase II) by addition of nutrient-rich deep waters. In this area, weakening of the thermocline and convective mixing naturally occurs from the end of February to mid-March however, in the past, occasional internal waves or passage of eddies during summer-autumn periods have increased nutrient concentrations and induced production (Arístegui et al., 2001) to levels comparable to the ones established in the present study (Figure 5-4).

### 5.4.2 $\delta^{13}C$ distribution and dynamics

The addition of $CO_2$ enriched seawater into designated mesocosms simulated ocean acidification. The $CO_2$ manipulation formed a linear DIC concentration range among the considered mesocosms which although below prefixed levels (Figure 5-3), it was still indicative of the potential $CO_2$ concentrations expected for the next decades. The $CO_2$ treatment gradient was reflected in the isotopic signature of DIC (Figure 5-5) indicating the added lighter carbon ($\delta^{13}C_{DIC}$ =

-35.6 ‰) mixed homogeneously in the mesocosm water columns. Beside the rapid changes in carbonate chemistry speciation due to the direct additions of $CO_2$, DIC and $\delta^{13}C_{DIC}$ distribution in the mesocosms were affected by both air-sea gas exchange and biological activity. The impact of air-sea gas exchange on water column dissolved inorganic carbon isotope inventories was about an order of magnitude smaller than that of biological activity during the phytoplankton bloom phase. To assess this, two assumptions were made: constant wind speed and negligible biological activity during the oligotrophic phases. Although wind speed, recorded a few nautical miles away at 10 m height, was between 0 and 15 m/s (median 5.0 m/s), the surface waters inside the mesocosms were sheltered by the enclosure bags (about 1.5 m above the water level) therefore almost no wind stress was present. However wave motion was able to transfer through the mesocosms bags promoting physical gas exchanges. Following Schulz and Riebesell (2013) a constant wind speed of 2 m/s was derived and used for isotopic air-sea exchange calculations. Based on undetectable changes in chl *a* concentrations during the first phase (Figure 5-4), the biological effect was assumed to have little importance for the observed variations in the mesocosm DIC and $\delta^{13}C_{DIC}$ distribution.

During photosynthesis, the lighter isotope of carbon, $^{12}C$, is preferentially taken up relative to the heavier isotope, $^{13}C$ (O'leary, 1981; Gruber et al., 1999). In the present experiment, the isotopic signature of the particulate organic carbon in the water column rapidly decreased following nutrient addition (deep water) confirming the incorporation of the light carbon component into phytoplankton and the consequent associated increase of $\delta^{13}C_{DIC}$ in the surrounding seawater (Figure 5-6). Within each pool, good agreement between concentrations and carbon isotope dynamics was established. A slight temporal disagreement between the peak in chl *a* and the biomass (POC) maximum was however noticed. The delay (one sampling day) was most likely due to the composition of POC itself, which includes extracellular polymeric substances and phytoplankton detritus. Assuming these particles were the main constituents of the exported material, it was possible to derive the isotopic signature of the phytoplankton ($\delta^{13}C_{phyto}$) and assess the isotopic transfer of biologically active carbon. Unlike the study by de Kluijver et al. (2010), direct phytoplankton to bacteria transfer dynamics could not be revealed as group specific labelled biomarkers were not used. However, by combining measurements of DOC concentration and $\delta^{13}C_{DOC}$, potential bacterial behaviour was indirectly defined. The rapid increase in DOC concentrations following the phytoplankton bloom, and the successive slow build up following the collapse, was partly associated with bacterial biomass dynamics. Bulk $\delta^{13}C_{DOC}$ measurements, were instead used to define phytoplankton-bacteria interactions. Bacterial production is significantly correlated with planktonic primary production as phytoplankton derived organic matter is an important food source for heterotrophic bacteria (Cole et al., 1988). The weak response observed in the $\delta^{13}C_{DOC}$ signal might indicate that bacteria relied only partly on

freshly produced material while the increase in DOC concentration could be explained by an intensive metabolism of bacterial excretions. Similar results were obtained in incubation (Norrman et al., 1995) and past mesocosm experiments (de Kluijver et al., 2010, 2013) where bacterial $\delta^{13}C$ never fully reached the signature of the labelled $^{13}C$ value of the corresponding algal POC although stronger responses were obtained. It must be noted that the uncertainties associated with $\delta^{13}C_{DOC}$ measurements in the present study were relatively high ($\sigma$ = 0.71 ‰) and this might have masked some underlying signal. Moreover, contrary to previous studies in which the isotopic signal was intensified through the use of labelled $^{13}C$, here the small difference in the isotopic signature of the two organic carbon pools and the high DOC background concentration compared to the produced POC, might have constrained further the quantification of potential isotopic incorporation into the DOC pool.

Mesocosm water columns were homogeneously mixed without formation of any thermo- or halo-cline that could separate the upper water from the deeper water. This situation facilitated particle mixing throughout the full water column and consequent deposition of settling material. Prior to the phytoplankton bloom, particulate carbon in the mesocosm sediment traps was generally low reflecting the low biological activity in the water column (Figure 5-5), however a slight anomaly in the isotopic signature between the two pools was found with settled material being isotopically lighter than the water column counterpart. The combination of isotopically light inorganic carbon additions at the beginning and a Saharan dust event which occurred between t16 and t22, may explain this discrepancy. Stable carbon isotope composition of -26.2 ‰ measured in Saharan dust derived aerosols (Eglinton et al., 2002; Mladenov et al., 2011) may have contributed to both lower the $\delta^{13}C$ values of particulate carbon in the sediment traps and increase the DOC concentrations during the pre-bloom phase. During and after the phytoplankton bloom, carbon isotopic signature in the mesocosm sediment traps mirrored the isotopic signature of POC in the water column (although shifted by one sampling day) confirming the sinking material was formed of freshly produced aggregates. However the $\delta^{13}C$ measured in the sediment traps was not affected by the magnitude of the formed material. Although POC concentrations in the post bloom phase reduced by half (51 % drop) compared to the bloom phase, the isotopic signature did not vary significantly. Similarly, the particulate carbon build up in the sediment traps did not affect the isotopic signature of the exported material. The relatively constant isotopic signatures of the particulate carbon pools in the post bloom phase may be explained by the low zooplankton activity; in shallow sediment traps zooplankton can largely contribute to the settling material either by production of sinking detrital carbon (feeding products and faecal pellets) or by active swimming into the trap (Buesseler et al., 2007). However, in the present study mesozooplankton abundance was low and showed a late development (Algueró-Muñiz, pers. comm., 2016), so that the majority of the material in the water column and consequently in the sediment traps was

derived from phytoplankton cell lyse and aggregates. In contrast, in a previous mesocosm experiment (de Kluijver et al., 2013), high mesozooplankton biomass was observed and this was estimated to have contributed for 82% to the carbon isotopic signature of the sediment material.

### 5.4.3 $CO_2$ effects on carbon isotope distribution

The increasing anthropogenic $CO_2$ emissions and the consequent oceanic uptake are altering the marine environment. One line of evidence of this $CO_2$-induced change is a reduction of the isotopic ratio of surface ocean waters known as $^{13}C$ Suess effect. In this study, the addition of isotopically light $CO_2$ in mesocosm systems simulates the oceanic $CO_2$ accumulation therefore the evaluation of any $CO_2$ derived effect could improve our understanding of future ecosystem responses. Increasing inputs of isotopically light carbon decreased the isotopic ratio of seawater DIC. Elevated $CO_2$ concentrations showed a clear negative effect on the isotopic signatures of the dissolved inorganic carbon (Table 5-2) as a consequence of proportional dilution of the added lighter carbon within the mesocosm water column. During the bloom and post bloom phases a similar $CO_2$ effect on phytoplankton and sediment material isotopic ratios was observed (Table 5-2), however no clear effect was noticed on $\delta^{13}C$ signature of the DOC pool during any phase. Higher $CO_2$ levels had no significant effect on inorganic nutrient uptake (Table 5-1) or carbon production/consumption (Table 5-2). In agreement with previous $CO_2$ enrichment mesocosm experiments where phytoplankton bloom development was based on chl *a* (Riebesell et al., 2007), POC (Schulz et al., 2008) or labelled phytoplankton stocks (de Kluijver et al., 2010), in this study phytoplankton bloom intensity and evolution was independent of $CO_2$ concentrations. However, in the post bloom phase, the highest $CO_2$ level mesocosm (M8) showed increased levels of chl *a* with generally higher POC concentrations in the water column compared to the other treatments. Interestingly, less material was found in the mesocosm M8 sediment trap and this was predominantly characterised by a lower amount of the $^{13}C$ fraction compared to low $CO_2$ level mesocosms (Figure 5-8). An explanation for the lower sediment material accumulation could be that in mesocosm M8, phytoplankton exudates were rapidly used by bacteria and the intense microbial remineralisation of freshly produced DOC contributed to the observed decreased export, to the slight isotopic enrichment of $\delta^{13}C_{DOC}$ (1.5 ‰) in the water column and to the low and stable (Figure 5-5) $\delta^{13}C_{DIC}$ values during the post bloom phase. This is in agreement with previous mesocosm experiments where significantly higher bacterial biomass was observed at higher $CO_2$ levels compared to present $pCO_2$ during the post bloom phases (Engel et al., 2004; de Kluijver et al., 2010).

In this study a $CO_2$ effect on phytoplankton fractionation was observed (Figure 5-7 C). Previous studies have shown that one of the major factors controlling the fractionation of carbon

into phytoplankton is the availability of aqueous $CO_2$ with highest fractionation values under high $CO_2$ availability (Hinga et al., 1994; Laws et al., 1995; Kukert & Riebesell, 1998; Burkhardt et al., 1999). In agreement with their work, in the current study, isotope fractionation between $CO_2$ and phytoplankton was higher in the higher $CO_2$ conditions however, the response did not follow a linear concentration gradient but it was mainly detected in the highest $CO_2$ treatment mesocosm M8 suggesting the possible presence of a threshold level. An additional explanation for the different fractionation value could be linked to a shift in phytoplankton community composition. It has been shown that isotopic fractionation by autotrophic phytoplankton can be taxon-specific (Laws et al., 1997; Pagani et al., 2002; Vuorio et al., 2006) with generally higher fractionation values ($\varepsilon$ =23.0 ± 3.7 ‰) noticed for green algae and lower values ($\varepsilon$ =15.6 ± 3.3 ‰) for diatoms (Van den Meersche et al., 2009). Based on the observed fractionation range ($9.6 < \varepsilon < 16.5$ ‰), the mesocosms must have been dominated by diatoms. Chemtax analyses confirmed a diatom dominated system as high abundance of bacillariophyta (diatoms) and almost complete absence of chlorophyte (green alga) was found (Bach, pers. comm., 2016). In organic cyst-forming dinoflagellates, stable carbon isotope fractionation relative to the isotopic composition of aqueous $CO_2$ was estimated between 9.0 and 12.7 ‰ (Hoins et al., 2015), values that are in good agreement with the ones observed in the present study. In contrast, no dinoflagellates were found in the high $CO_2$ treatment mesocosm (Bach, pers. comm., 2016) and this could have contributed to the observed higher fractionation value ($\varepsilon$ = 15.8 ‰) in mesocosm M8 compared to the other mesocosms. In addition, there has been speculation that mesocosms M8 developed the raphidophyte *Heterosigma*, a toxic microscopic alga known to benefit from elevated $CO_2$ (Fu et al., 2008) which could have further restructured the phytoplankton community structure. Whether it was the high $CO_2$ availability, the dominance of a specific planktonic group or the combination of both, in this mesocosms experiment elevated $CO_2$ concentrations positively affected the stable carbon isotope fractionation in phytoplankton.

## 5.5 Conclusion

This mesocosm study provided a baseline to assess the effects of increasing $CO_2$ concentrations in an oligotrophic oceanic system. This study was the first to employ stable isotope analysis of unlabelled natural carbon in order to examine carbon transferring mechanisms and dynamics during a phytoplankton succession. Additions of isotopically light $CO_2$ for the $CO_2$ enrichment established an isotopic gradient in the DIC pool which, subsequent to the nutrient-induced phytoplankton bloom, was reflected both in the water column and sediment trap particulate carbon pools. However no significant $CO_2$ effects were observed in the either particulate or dissolved organic carbon stocks. Interestingly, export of material to the deeper

layer during the post bloom phase decreased under high $CO_2$ conditions, however the response was not gradual but was only found for the highest $CO_2$ treatment (~1000 ppm) mesocosm. A $CO_2$ effect on phytoplankton fractionation was observed and similarly, the response was predominantly noticed in the highest $CO_2$ treatment mesocosm. High $CO_2$ concentrations affected fractionation values either directly as a consequence of the higher $CO_2$ availability or indirectly as a consequence of the phytoplankton community composition change observed in the highest $CO_2$ treatment mesocosm.

In general, results from this mesocosm experiment proved that stable isotopes analysis of each of the individual carbon system pools within the marine environment are suitable for the assessment of carbon dynamics in an oceanic system. However when stable carbon isotopes are applied to estimate future oceanic interactions that are relevant for our climate, careful evaluation of the underlying processes is needed. Stable isotopic analysis confirmed that the material accumulating in the sediment trap was similar to that in the water column, an observation statement that is widely assumed when estimations of past natural changes in oceanic and atmospheric $CO_2$ are performed. However, this study confirms that other factors (allochthonous carbon, phytoplankton fractionation and community composition) rather than $CO_2$ alone can influence the isotopic signature of the water column and the sediment material, therefore it is important to identify and understand the processes driving these changes in order to hindcast past and forecasts future oceanic $CO_2$-driven effects.

# Chapter 6: General Conclusions and Future Work

## 6.1 Conclusions

The aim of this project was to determine whether measurements of the stable carbon isotope composition of seawater samples could refine our understanding of the biogeochemical interactions among carbon compartments within the marine system. Three main objectives were set in order to assess the feasibility of using such approach:

1. The establishment of adequate protocols for the collection, preparation and analysis of seawater samples in order to determine the isotopic composition of all the individual inorganic and organic carbon forms within the marine system.
2. The determination of the isotopic distribution and abundance of carbon species within the different pools in natural and semi-enclosed (mesocosm) ecosystems, and their changes over an experimental study.
3. The determination of the effects of increasing $CO_2$ concentrations on the isotopic distribution and abundance of carbon species within the different pools of mesocosm systems.

This study presents and assesses the main processes that influence the isotopic distribution of carbon species in two different climate zones, a high latitude North Atlantic subarctic area and a subtropical oligotrophic environment. The combination of isotopic and non-isotopic carbonate system parameters together with standard oceanographic measurements and biological data (plankton community development and composition) have provided a holistic view of the mechanisms that affect carbon dynamics within the two marine systems. The use of mesocosm platforms have allowed the quantification of the isotopic variations of carbon species under different $CO_2$ conditions and provided an effective way to identify pathways for carbon in different marine environments over the study period. The present study contributes to our understanding of the effects of rising $CO_2$ on primary production and carbon export and provides a new line of interpretation for the potential response and feedback that the oceans exhibit in the global carbon cycle.

Results from this thesis can also be linked to the wider context of the paleoclimate research. In paleo-oceanography, the study of the variations of the stable carbon isotope composition from different natural archives have largely been used for reconstructing past Earth's climate and the analysis of these proxy records have given indications of past physical and chemical oceanic properties and estimations of past atmospheric $CO_2$ concentrations and paleo photosynthetic

activity during specific geological periods. However, to reconstruct the past state and evolution of oceanic and climate conditions from stable carbon isotope records it is necessary to calibrate these records against independent but correlated measurements of the current oceanic conditions. Results from this work showed that variations in the isotopic signature of the various marine system compartments are in relationship with both environmental and biological factors confirming the validity of using stable carbon isotope analysis as an effective tool for paleoclimate interpretations.

The first part of the thesis (Chapter 2 and 3) mainly focused on the methodological aspects and analytical strategies employed for the measurements of stable carbon isotope ratios in seawater samples. Chapter 2 specifically presented a state of the art of the current analytical techniques for the determination of the stable isotope composition of dissolved and particulate inorganic and organic carbon in aquatic samples with the use of Isotope Ratio Mass Spectrometry (IRMS) instruments. The review addressed methodological issues concerning data reporting and offered indications for the production of universally comparable datasets. Sources of error derived from analytical systems can be reduced by continuous monitoring of instrument performances and periodic checks on the reproducibility of the measurements. Correction procedures must be carefully applied and in order to have inter-comparable results, the algorithms applied should be reported. The accuracy of the analyses must be assessed by running international certified reference materials (CRMs) and continuous inter-laboratory comparisons. In this study, the use of standard materials provided by the International Atomic Energy Agency (IAEA, Vienna) and by the Scottish Universities Environmental Research Centre (SUERC) stable isotope laboratory, East Kilbride, for the calibration of our instruments (Appendix A and B) assured both universal and inter-laboratory accuracy of our isotopic measurements. Additionally, the use of seawater reference materials from A.G. Dickson and D. Hansell for $\delta^{13}C_{DIC}$ and $\delta^{13}C_{DOC}$ measurements, respectively, although not isotopically certified, guaranteed internal analytical consistency. In this work, the measured seawater reference materials $\delta^{13}C_{DIC}$ and $\delta^{13}C_{DOC}$ values were similar to previously reported results assuring also inter-comparability of our measurements. In particular, repeated measurements of CRMs from D. Hansell were crucial to prove the feasibility of using the herein developed TOC-IRMS system for both quantitative and isotopic measurements of DOC in seawater samples.

The new modified TOC-IRMS combination is described in Chapter 3. The need for the $\delta^{13}C_{DOC}$ measurements in mesocosm experiments was the driving factor for the development of an accurate, precise and rapid method for quantitative and isotopic analysis of marine DOC samples. Measurements of the stable isotopic composition of DOC in seawater have historically been problematic and to date analyses are still quite laborious and challenging. The isotopic signature of DOC in seawater samples is difficult to characterise due to the refractory nature of the sample

compounds, the generally low concentrations (< 80 µM) and high inorganic salt content (30-35 g/l) that contribute to negatively affect the precision of the results. With the new in-house TOC-IRMS system it was possible to perform parallel measurements of DOC concentrations and stable isotopes with a precision of ± 1 µM and ± 0.5 ‰ for DOC and $\delta^{13}C_{DOC}$, respectively, in less than 20 min per sample when a sequence was analysed. The mechanical and chemical modifications performed on the system reduced background signal variations from previously reported changes of up to 8 ‰ to a 3 ‰ difference and measurements of up to 100 consecutive samples did not appear to be affected by loss in oxidation efficiency nor sample salt content. The use of sequential trapping and cryo-focusing allowed $\delta^{13}C_{DOC}$ measurements of samples with concentrations as low as 40 µM with a precision of ± 0.4 – 0.6 ‰. The optimised TOC-IRMS system was used to perform $\delta^{13}C_{DOC}$ measurements of more than 170 samples from 3 mesocosms experiments. The general idea was to link the $\delta^{13}C$ signatures of the organic material to the inorganic pool in order to have a full picture of the carbon isotope dynamics within the marine system.

Isotopes integrate ecological processes in space and time (depending on the turnover rate of the element within a specific pool) giving an indication of the magnitude of such processes. Owing to the isotopic fractionation associated with physical and biological processes, the origin and movement of carbon within an ecosystem can be traced. The second part of this thesis (Chapter 4 and 5) focussed on the stable carbon isotope datasets from 3 long-term mesocosm experiments, two from North Atlantic fjords and one from Canary Island waters. The biogeochemical interactions among carbon compartments within the different ecosystems were assessed. The two sites represent biogeochemically different regions with the North Atlantic fjords characterised by relatively cold, nutrient rich and highly productive waters while warm and oligotrophic waters were representative of the Canary Islands area. Although a direct comparison between the two sites was not carried out, in general similar stable carbon isotope dynamics were observed for both areas.

Chapter 4 includes data from the two North Atlantic fjord mesocosm experiments. The chapter described exhaustively data processing and calibration procedures for the determination of the $\delta^{13}C$ signatures of the inorganic and organic pools within the mesocosm systems. About 700 isotopic measurements were performed with calculated precisions (1$\sigma$) of 0.09 ‰, 0.62 ‰ and 0.12 ‰ for the $\delta^{13}C_{DIC}$, $\delta^{13}C_{DOC}$ and $\delta^{13}C_{POC}$ datasets, respectively. The unavailability of supplementary biological and oceanographic data precluded an in-depth analysis of the biogeochemical processes controlling the isotopic distribution of carbon species in the mesocosm systems, however some early stage conclusions could be drawn. It was found that additions of isotopically light $CO_2$ were proportionally transferred to the organic compartments through photosynthesis and bacterial activity. Moreover, the stable carbon isotope composition of the

material found in the sediment traps was mirroring the suspended counterpart suggesting direct export of freshly produced material in all mesocosm systems.

Chapter 5 revealed the biogeochemical interactions among carbon compartments within the individual mesocosm ecosystems. Isotopic and non-isotopic carbonate system parameters were combined with a wide range of other oceanographic measurements gathered from the Gran Canaria mesocosm experiment in order to assess the transfer mechanisms and dynamics of carbon during a phytoplankton succession. It was noticed that increasing additions of isotopically light $CO_2$ showed a proportionally negative effect on the isotopic signature of the dissolved inorganic carbon. During the course of the experiment, air-sea gas exchanges affected the distribution of $\delta^{13}C_{DIC}$ in the water column, however the effects were about one order of magnitude smaller compared to the isotopic variations caused by the biological activity. During the bloom, the incorporation of carbon into phytoplankton was rapid and this was proportionally reflected in the isotopic signature of the particulate organic carbon while only a weak response was detected in the $\delta^{13}C_{DOC}$ pool. With the development of the phytoplankton bloom the isotopic signature of the settled material mirrored the overlaying water column $\delta^{13}C_{POC}$ confirming sinking of newly produced material. Besides the assessment of the isotopic distribution of carbon in the mesocosm systems, the effects of increasing $CO_2$ levels on carbon dynamics were determined. Phytoplankton bloom intensity and evolution was independent of $CO_2$ concentrations and higher $CO_2$ levels had no significant effect on inorganic nutrient uptake or carbon production/consumption. No significant $CO_2$ effects were observed in the either particulate or dissolved organic carbon stocks. A $CO_2$ effect on phytoplankton fractionation was however observed, with higher isotope fractionation values between $CO_2$ and phytoplankton found in higher $CO_2$ conditions. The effect was either a direct consequence of the higher $CO_2$ availability or an indirect consequence of a shift in phytoplankton community composition.

Overall this thesis has shown that stable carbon isotope analyses of seawater samples provide a valid and elegant way to assess the major biogeochemical interactions among individual compartments within the marine system. However, it is essential to have accurate and precise isotopic measurements of all the individual carbon species and refer to some of the main oceanographic and biological data to provide a solid interpretation of an isotopic dataset.

## 6.2 Future work

Assessment of the ratio between heavy and light carbon in natural pools makes isotopes particularly appropriate for evaluating the existence or magnitude of key processes involved with carbon cycling. Small variations from a background signal in the carbon isotope signature of a specific component within a pool can provide important information on the origin and movement

of the compound in question. The presence of various biogeochemically interconnected forms of carbon (dissolved and particular inorganic and organic) within the marine system makes stable carbon isotope analysis exceptionally stimulating. The combination of a wider interest in global carbon cycling with the rapid improvement in technology offered scientists precise and relatively easy to use commercially available instrumentation for isotopic analysis. In our laboratory, the state of the art mass spectrometer and the related connected peripherals allowed to have accurate and precise analysis of stable isotopes in seawater samples for all the carbon forms. However, the present work was delivered as an innovative but isolated instance. The connection to a network of other stable isotope laboratory facilities would be beneficial for the future running of our laboratory. Continuous cross laboratory calibration, adoption of common protocols and distribution of mutual isotopic standards would guarantee an appropriate reporting of future isotopic data. The precision of the isotopic measurements obtained in this work were good and always within the specifications of each analytical method. However, the developed TOC-IRMS system, although able to perform $\delta^{13}C_{DOC}$ measurements of seawater samples with precision and accuracy comparable to the only two other similar set-ups reported in the literature (Panetta et al., 2008; Lalonde et al., 2014), would need further improvements if isotopic variations in lower concentrations samples have to be assessed. Full automation and single but repeated measurable injections of the sample are viable options for a potential improvement of the analytical outputs. In this study, mesocosm platforms could be interpreted as individual ecosystems in which most of the natural biogeochemical interactions occurred. However, mesocosms are semi-enclosed systems and as such they were subjected to limited natural variability both in terms of physico-chemical conditions (water mass circulation and exchange) and biological structure (selective marine biota communities). Furthermore, the relatively shallow depths (about 20 m) of the mesocosm systems represented a limitation for the assessment of isotopic variations due to processes like export and remineralisation of organic matter below the euphotic zone. Future application of parallel stable carbon isotope measurements could focus on specific oceanic areas such as upwelling, deep water formation and oxygen minima zones where distinct physico-chemical and biological events occur. Stable carbon isotope measurements in these regions could provide spatial and temporal distribution profiles of carbon and help to identify and quantify specific carbon pathways, microbially mediated processes and important trophic interactions.

Nevertheless, the isotopic datasets were put in relationship with environmental parameters in order to successfully assess the processes controlling carbon dynamics under different $CO_2$ levels. Based on the outputs of the present work a further step would be the realisation of specific biogeochemical models that relay on the current isotopic datasets to identify interactions and quantify fluxes of carbon within the marine environment. Ideally, the hypothetic models should be able to reproduce the experimentally simulated natural versus future oceanic $CO_2$ conditions in

mesocosm systems with the potential of subsequently extending the quantification of carbon fluxes to an open ocean ecosystem. The eventual identification of new effective parameters could also help to improve existing numerical models designed to quantify past, present and future oceanic carbon uptake.

The interconnections among carbon species in the marine system is regulated by biogeochemical processes. Physico-chemical and biological processes also control the dynamics of other life supporting elements such as nitrogen. Dual isotope analysis ($\delta^{13}C$ and $\delta^{15}N$) offer additional constraints for assessing the contribution of significant sources and transferring mechanisms within compartments of a particular ecosystem. Future mesocosm and field experiments should therefore include the analysis of stable nitrogen isotopes and integrate the contribution of multiple sources to carbon dynamics to better understand ecosystem functioning and responses to the devastating current anthropogenic activities. The set of instruments currently present in our laboratory is suitable for the stable isotope analysis of dissolved and particulate inorganic and organic carbon and nitrogen in seawater samples, however future work, including isotopic calibration of new standard material and the preparation of a detailed protocol for the recovery of the dissolved and particulate nitrogen fraction from seawater samples, is required. The development of a successful method for dual analysis would certainly open the door to new approaches for investigation of organic and inorganic matter sources and cycling in marine ecosystems.

# Appendices

# Appendix A

## Validation method of $\delta^{13}C$ standards for isotopic analysis of dissolved inorganic carbon in seawater samples

## A.1 Introduction

This section describes the validation process and the analytical procedure for the determination of the most suitable laboratory standards for stable carbon isotope ($\delta^{13}C$) analysis of dissolved inorganic carbon (DIC) in seawater samples. The instrument used was a GasBench II preparation device connected to a Delta V Advantage isotope ratio mass spectrometer (both Thermo Fisher Scientific). Several tests were performed prior the final validation analysis took place.

## A.2 Experimental

### A.2.1 Chemicals and Materials

In total, nine carbonate samples were used including one internationally certified (primary) standard from the International Atomic Energy Agency (IAEA, Vienna) and three secondary standards from the Scottish Universities Environmental Research Centre (SUERC) stable isotope laboratory, East Kilbride. The standards are listed in the table below (Table A-1).

| ***Standards*** | ***$\delta^{13}C$ value (‰)*** | ***Source*** |
|---|---|---|
| NBS 18 - Calcite | -5.014 | IAEA |
| MAB - Marble | +2.48 | SUERC |
| NA - $NaHCO_3$ | -4.67 | SUERC |
| CA - $CaCO_3$ | -24.24 | SUERC |
| CS - $CaCO_3$ | - | Sigma Aldrich Lot 110M0081V |
| NS - $Na_2CO_3$ | - | Sigma Aldrich Lot BCB K8268V |
| NF - $Na_2CO_3$ | - | Fisher Scientific Lot 074365 |
| CM – Marble | - | Carrara (07/2004) |
| DIC CRM Batch 144 | - | A.G. Dickson laboratory (Scripps Institution of Oceanography, USA) |

**Table A-1.** Carbonate samples used for the laboratory standard determination experiment.

Additional samples were prepared by diluting sodium carbonate standards (NS and NF) in ultrapure water (Milli-Q water, resistivity 18.2 MΩ·cm) to obtain liquid state standards (NSL and NFL) of concentrations typical of marine dissolved inorganic carbon (2.0-2.4 mmol $kg^{-1}$). Carbon dioxide extraction was performed by acidification with Ortho-Phosphoric Acid ($H_3PO_4$) (ACS grade, Fisher Scientific). A 100 µl gastight syringe (Hamilton) with a stainless steel removable needle was used to inject the acid into 12 ml borosilicate Exetainer sampling vials (Labco), with round bottom. Vials were sealed with butyl rubber septum caps. Liquid samples were injected by using a 1 ml gastight PTFE Luer Lock termination syringe (Hamilton). The GasBench II is equipped with a GC oven where a 27.5 m long (0.32 mm i.d., 10 mm film thickness) fused silica Poraplot Q column (Agilent Technologies) was installed. A 100 µl sample loop was mounted to the Valco eight port switching valve. High purity helium gas (CP grade N5.0, BOC) was used as flushing and carrier gas, while pure carbon dioxide (Research grade N5.0, BOC) was used as reference gas.

### A.2.2 General instrument set-up

The instrument set-up consisted of three devices connected in a continuous flow: a CTC Analytics PAL autosampler, a Thermo Scientific GasBench II and a Thermo Scientific Delta V Advantage isotope ratio mass spectrometer. The general instrument settings are described below:

*PAL Autosampler*

- Syringe - 10 µl
- Fill stroke - 10
- Pullup delay - 62 s
- Sample tray - 12 x 8, 12 ml Exetainer vials
- Flushing method - Internal 1
- Analysis method - Internal 2

*GasBench*

- He pressure - 1 bar
- $CO_2$ reference pressure - 1.2 bar
- Flush fill flow rate - ~ 120 ml/min
- Capillary column temperature - 70 °C

*IRMS*

- Emission - 1.50 mA
- Trap - 40 V
- Electron energy - 124 eV
- High Vacuum (MS valve close) - $8.2*10^{-8}$ mbar
- High Vacuum (MS valve open) - $1.8*10^{-6}$ mbar

Isodat 3.0 software controls ion generation, mass separation, ion detection, ion source tuning, data evaluation and storage. The software operates in three modalities:

- Instrument Control - It monitors and controls IRMS parameters
- Acquisition - It is used for running a sequence
- Workspace - It allows to setup methods and sequences and it is used to review data

### A.2.3 Standards preparation

Borosilicate Exetainer vials were washed several times (at least three) with RO water and placed in an ultrapure water bath to soak overnight. Vials were then moved into an oven at 150 °C to be baked out overnight. Vial caps were soaked in a 10 % HCl solution bath, rinsed with ultrapure water and dried into an oven at a temperature of 60 °C overnight. Damaged septa were discarded and substituted with new ones.

Carbonate samples were weighed using a Precisa ES 125SM laboratory balance. Target weights were determined by calculating the weigh percent carbon for each sample type and they ranged between 0.1 and 0.3 mg. After careful weighing, the samples were placed into pre-cleaned and appropriately labelled vials. The vials were tapped on the table a few times to allow the entire sample to fall to the bottom of the vial. In case liquid samples were to be analysed (Dickson CRMs, NFL and NSL), sample vials were filled with a small amount (80 µl) of $H_3PO_4$. Sample vials were then capped and carefully placed into the sampling tray.

### A.2.4 Analytical procedure

Four analysis days were performed for this validation experiment. During the first and second day the run consisted of 62 samples while 81 samples were analysed in each of the last two runs. The analysis sequences were arranged with sample vials placed as follow: 2 empty tubes, 2 vials containing only $H_3PO_4$ (80 µl), 2 with $H_3PO_4$ (80 µl) plus ultrapure water (1 ml), 8 or 11 series of 6 vials each containing the standards and interspaced by one vial containing 1 ml of Dickson CRM water.

Each sample vial was flushed with He gas before the analysis. The flush fill needle was attached to the PAL autosampler and from Isotadat Instrument control the T-valve was opened to allow the helium line to be purged for at least 10 minutes. The FlushFill single needle sequence was launched from Isodat Acquisition mode. When the helium FlushFill sequence was completed, the T-valve was turned back to switch the helium flow off and in the PAL autosampler the flush fill needle was substituted by the sampling needle.

Helium flushed vials containing the standards were spiked with 80 µl of $H_3PO_4$ while the ones pre-loaded with $H_3PO_4$ were filled with 1 ml of liquid sample injected through the septum. Vials with samples and $H_3PO_4$ were left for 22 hours to allow equilibration.

Previous analysis of samples, water background signal and $CO_2$ reference gas intensity were closely monitored and recorded. Stability and linearity tests were performed and focus parameters were adjusted. The analysis of each sample consisted of 4 injections of the reference gas followed by 6 injections of the sample gas (carried by Helium). The method file name is *$CO_2$ in He with delay.met,* while the analysis sequence is stored in the laboratory desktop under the filename *Cal-tray.seq*.

Following analysis, the SSH (Santrock et al., 1985) algorithm was applied to the measured ratios in order to correct for ion species contribution. Finally, the samples were mathematically corrected for linearity according to the following equation:

$$\delta_{corr} = \delta_{meas} - m * A$$

where $\delta_{meas}$ is the measured value, A represents the peak area and m is the correction factor.

## A.3 Results

### A.3.1 Analytical performance of IRMS

Four hours were needed to reach a vacuum pressure of $1.8*10^{-6}$ mbar after the opening of the MS valve. Water background signal on cup 3 (m/z 18) dropped below 1.5 V after two hours of turning on the filament. From the autofocus procedure, optimal focusing parameters for the $CO_2$ reference gas were determined and the maximum signal was reached when ion source currents and lens were arranged as follow:

- Emission - 1.48 mA
- Trap - 38.80 V
- Electron energy - 114 eV
- Extraction - 80 %
- Extraction Symmetry - -1.64 %
- X-Focus - 19.61 %
- X-Focus Symmetry - 1.54 %
- X-Deflection - 47.74 %
- Y-Deflection - 25.05 %
- Y-Deflection Symmetry - -6.52 %

From the on-off test on the reference $CO_2$ gas, the standard deviation of 10 consecutive $\delta^{13}C$ values was 0.03 ‰. The regression slope derived from 10 consecutive $\delta^{13}C$ values (generated by a 0.1 bar increasing step of the reference $CO_2$ gas pressure) versus their related amplitudes was 0.02 ‰/V. Both stability and linearity analysis results were within the Delta V Advantage specification values of $\sigma < 0.06$ ‰ and slope < 0.06 ‰/V, respectively.

The reference $CO_2$ gas was adjusted to give a reference peak signal of amplitude similar to the range of the samples to be analysed (m/z 44 = 6200 mV, m/z 45 = 7200 mV, m/z 46 = 8700 mV). The average area (mass 44) of all the $CO_2$ reference injections was 87.19 ± 0.47 V*s (n = 988) while the standard deviation of the $\delta^{13}C$ values was 0.09 ‰. The correction factors m (slope) applied to the samples were always less than 0.015 ‰/V although variable sample amounts were used.

### A.3.2 Data validation

In the first two analysis runs, isotopic calibration curves were generated by a two-point normalisation procedure using MAB and NA as main standards. Results are summarised in the table below (Table A-2).

| **Date 13/06/15** | | | | **Date 17/06/15** | | | |
|---|---|---|---|---|---|---|---|
| **Regression line y = 0.9854x - 30.4** | | | | **Regression line y = 0.9787x - 30.2** | | | |
| **Standard** | **$\delta^{13}C$ average (‰)** | **Std Dev (1σ)** | **n** | **Standard** | **$\delta^{13}C$ average (‰)** | **Std dev (1σ)** | **n** |
| **MAB** | 2.48 | 0.02 | 6 | **MAB** | 2.48 | 0.02 | 6 |
| **NA** | -4.67 | 0.06 | 6 | **NA** | -4.67 | 0.04 | 6 |
| CS | -40.87 | 0.04 | 6 | CS | -40.58 | 0.08 | 6 |
| NS | -39.99 | 0.11 | 6 | NS | -39.80 | 0.06 | 6 |
| NF | -8.75 | 0.05 | 6 | NF | -8.74 | 0.17 | 6 |
| CM | 2.26 | 0.04 | 6 | CM | 2.23 | 0.05 | 6 |
| NSL | - | - | - | NSL | -39.60 | 0.03 | 6 |
| NFL | - | - | - | NFL | -8.83 | 0.03 | 6 |
| Dickson CRM | 1.06 | 0.04 | 9 | Dickson CRM | 1.06 | 0.04 | 8 |

**Table A-2.** Statistics for carbonate standards validation on day 1 and 2. Compound in bold were used as main calibration standards to determine the regression line equation.

The third analysis run was used to determine the isotopic values of the carbonate standards by creating a standard curve using a three-point normalisation. The carbonate samples used as main standards were MAB, NA and CA. The primary standard NBS-18 was run as unknown. As a final check, the last run was a back-calculation analysis in which CM, NS and the internationally

certified standard NBS-18 were used as main calibration standards. Results from the last two analysis runs are summarised in the following table (Table A-3).

| **Date 08/12/15** | | | | **Date 09/12/15** | | | |
|---|---|---|---|---|---|---|---|
| **Regression line y = 0.9709x - 36.6** | | | | **Regression line y = 0.9737x - 36.5** | | | |
| **Standard** | **$\delta^{13}C$ average (‰)** | **Std Dev (1σ)** | **n** | **Standard** | **$\delta^{13}C$ average (‰)** | **Std dev (1σ)** | **n** |
| **MAB** | 2.48 | 0.02 | 6 | MAB | 2.45 | 0.04 | 6 |
| **NA** | -4.67 | 0.06 | 6 | NA | -4.71 | 0.08 | 6 |
| **CA** | -24.24 | 0.11 | 6 | CA | -24.24 | 0.14 | 6 |
| NBS-18 | -5.01 | 0.08 | 6 | **NBS-18** | -5.01 | 0.08 | 6 |
| CS | -40.30 | 0.09 | 6 | CS | -40.26 | 0.11 | 6 |
| NS | -39.61 | 0.08 | 6 | **NS** | -39.80 | 0.13 | 6 |
| NF | -8.62 | 0.19 | 6 | NF | -8.71 | 0.11 | 6 |
| CM | 2.22 | 0.04 | 6 | **CM** | 2.24 | 0.04 | 6 |
| NSL | -39.52 | 0.06 | 6 | NSL | -39.72 | 0.05 | 6 |
| NFL | -8.68 | 0.04 | 6 | NFL | -8.80 | 0.05 | 6 |
| Dickson CRM | 1.09 | 0.07 | 14 | Dickson CRM | 1.07 | 0.05 | 12 |

**Table A-3.** Statistics for carbonate standards validation on day 3 and 4. Compounds in bold were used as main calibration standards to determine the regression line equation.

All data from the four analyses were then combined in order to calculate the final values of the carbonate compounds and to determine the most adequate laboratory standards to be used for $\delta^{13}C$ analysis of seawater DIC samples (Figure A-1).

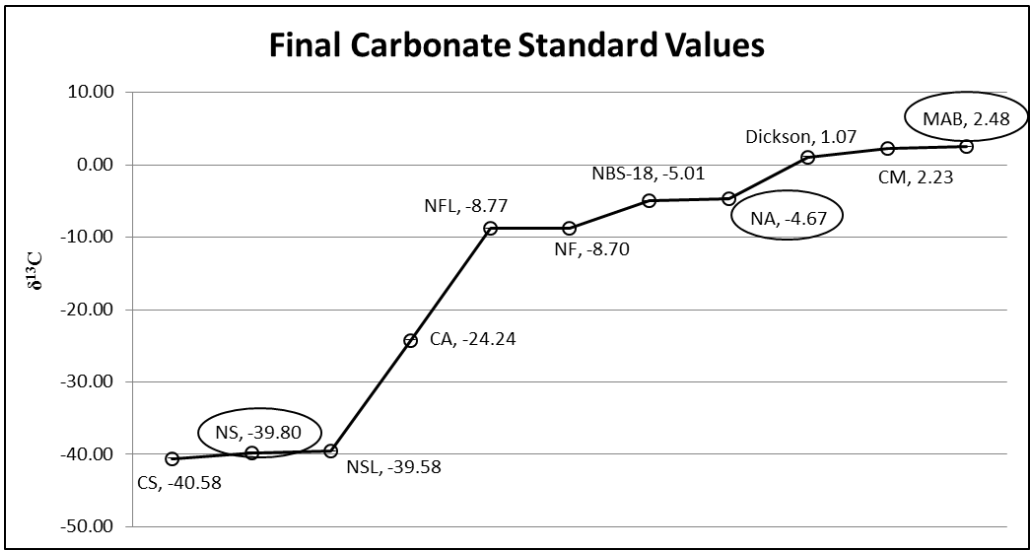


**Figure A-1.** Final carbonate standard isotopic values. The circles identify the most suitable laboratory standards for seawater $\delta^{13}C_{DIC}$ analysis.

Potentially all of the above carbonate standards can be used as laboratory standards for $\delta^{13}C_{DIC}$ analysis but MAB, NA and NS were chosen as main laboratory standards as they showed the lowest isotopic variation and covered a large isotopic range (-39.80 ‰ < $\delta^{13}C$ < +2.48 ‰).

## A.4 Comments

Two different reference $CO_2$ gas cylinders were used for this standard validation test: one for the first two analysis runs and a second one for the last two days. Isotopic signatures were $\delta^{13}C$ = -30.56 ± 0.01 ‰ and $\delta^{13}C$ = -36.59 ± 0.06 ‰ for cylinder one and two, respectively. The substitution of the reference gas cylinder did not affect the analysis output as the reference gas was not used as reference material for isotopic calibration procedures.

It must be pointed out that the last two analyses were performed about six months after the first two analysis runs were made. The waiting time was due to the initial unavailability of the internationally certified reference material (NBS-18) which was delivered in November 2015. Liquid samples NFL and NSL, although stable within runs, showed variations between the runs higher than 0.08 ‰, probably due to some minor atmospheric contamination of the ultrapure water used to prepare the standards. The isotopic signature of Dickson CRM samples showed little variation ($\delta^{13}C_{DIC}$ = 1.07 ± 0.01 ‰) however, its use as main laboratory standard is still under investigation as inter-batch variability has not been assessed yet. Nonetheless Dickson CRMs are useful as internal standards to check analysis consistency as they proved to be reasonably stable for at least six months.

## A.5 Conclusion

This series of analysis identified the most suitable laboratory standards to be used for stable carbon isotope analyses of seawater DIC samples by using a GasBench-IRMS setup. The validation also provided the $\delta^{13}C$ values for nine carbonate compounds along with statistical evaluation of the values. The results are summarised in the following table (Table A-4)

Appendix A

| *Standard* | *$\delta^{13}C$ average (‰)* | *Std Dev (1$\sigma$)* | *Acc (%)* |
|---|---|---|---|
| MAB | 2.48 | 0.02 | 99.95 |
| NA | -4.67 | 0.02 | 99.96 |
| NS | -39.80 | 0.16 | 100.01 |
| NBS-18 | -5.01 | 0.002 | 99.97 |
| CS | -40.58 | 0.28 | 100.01 |
| CA | -24.24 | 0.01 | 99.99 |
| NF | -8.70 | 0.06 | 100.03 |
| CM | 2.23 | 0.02 | 100.14 |
| Dickson CRM | 1.07 | 0.01 | 99.99 |

**Table A-4.** Isotopic values of the carbonate standards used in the validation analysis.

# Appendix B

# Validation method of $\delta^{13}C$ standards for isotopic analysis of particulate carbon in seawater samples

## B.1 Introduction

This section describes the validation process and the analytical procedure for the determination of the most suitable laboratory standards for stable carbon isotope ($\delta^{13}C$) analysis of particulate organic carbon (POC) and particulate inorganic carbon (PIC) in seawater samples. The target was to identify a series of four to five standards bracketing the full natural marine particulate carbon isotopic signature range ($-30 < \delta^{13}C < -15$). The instrument used was a Flash 2000 Elemental Analyser (EA) connected to a Delta V Advantage isotope ratio mass spectrometer through a ConFlo IV interface device (all Thermo Fisher Scientific). Several tests were performed prior the final validation analysis took place.

## B.2 Experimental

### B.2.1 Chemicals and Materials

In total 15 compounds were used for this laboratory standard validation analysis including three primary standards from the International Atomic Energy Agency (IAEA, Vienna) and 3 laboratory standards from the Scottish Universities Environmental Research Centre (SUERC) stable isotope laboratory, East Kilbride. The standards are listed in the table below (Table B-1). The EA was equipped with a prepacked (chromium oxide, high quality copper and silvered cobaltous/ic oxide) quartz reactor CHN (Thermo Fisher Scientific) for samples combustion. Following the reactor chamber an IRMS adsorption filter filled with magnesium perchlorate (ACS grade, Sigma Aldrich) was installed. The EA oven was accommodating a 3m stainless steel separation column NC/NCS (Elemental Microanalysis) for chromatographic sample gas separation. High purity helium (CP grade N5.0, BOC) was used as carrier gas, high purity oxygen gas (99.995 %, BOC) pulses were injected to each sample to allow better combustion and pure carbon dioxide (Research grade N5.0, BOC) was used as reference gas.

| *Standards* | *$\delta^{13}C$ value (‰)* | *Source* |
|---|---|---|
| IAEA-600 Caffeine | -27.77 | IAEA |
| IAEA-CH-6 Sucrose | -10.449 | IAEA |
| NBS 18 - Calcite | -5.014 | IAEA |
| MAB - Marble | +2.48 | SUERC |
| NA - $Na_2CO_3$ | -4.67 | SUERC |
| CA - $CaCO_3$ | -24.24 | SUERC |
| Urea | -39.79 | IVA Analysentechnik, Lot 33802174 |
| Urea (red label) | -40.81 | Elemental Microanalysis Batch 128785 |
| Caffeine | - | Fisher Scientific Lot 1482314 |
| Sucrose | - | Acros Organics Lot A0333146 |
| L-Glutamic Acid HCl | - | Acros Organics Lot A0324778 |
| L-Glutamic Acid | - | Acros Organics Lot A0225694 |
| Potassium hydrogen phthalate | - | Fisher Scientific Lot 1152106 |
| NS - $Na_2CO_3$ | - | Sigma Aldrich Lot BCB K8268V |
| CM – Marble | - | Carrara (07/2004) |

**Table B-1**. Compounds used for the laboratory standard determination experiment.

### B.2.2 General instrument set-up

The instrument set-up consisted of three devices (Flash 2000 EA, ConFlo IV and IRMS) connected in a continuous flow and configured as follow:

*Flash 2000 Elemental Analyser*

- MAS 200R Autosampler for solid samples - 1 drum, 32 positions
- He pressure - 180 kPa
- He Carrier flow rate - 100 ml/min
- He Reference flow rate - 100 ml/min
- $O_2$ pressure - 160 kPa
- $O_2$ Injection flow rate - 180 ml/min
- Left Furnace - 1020 °C
- Right Furnace - 400 °C
- EA Oven - 40 °C
- Cycle Run Time - 450 s
- Sampling Delay - 18 s
- Oxygen Injection End - 3 s
- TCD Detector Signal - 1000 µV

*ConFlo IV*

- He pressure - 1.0 bar
- $CO_2$ reference pressure - 0.9 bar
- Sample Pre-Split - 1:10 dilution
- Dilution Valves - All closed, 0 % dilution

*IRMS*

- Emission - 1.49 mV
- Trap - 38 V
- Electron energy - ~120 eV
- High Vacuum (MS valve open) - $1.9*10^{-6}$ mbar
- Instrument Configuration - ConFlo IV + Flash EA
- $CO_2$ reference peak intensities -m/z 44 = ~6700 mV
  - m/z 45 = ~7800 mV
  - m/z 46 = ~9600 mV
- Methods - Carbon Meas.met, FlashEA.eam

Isodat 3.0 software controls ion generation, mass separation, ion detection, ion source tuning, data evaluation and storage. The software operates in three modalities:

- Instrument Control - It monitors and controls IRMS parameters
- Acquisition - It is used to control and configure EA status and parameters. It allows to launch an analysis sequence
- Workspace - It allows to setup methods and sequences and it is used to review data

### B.2.3 Standard preparation

Carbon compounds were weighed using a Precisa ES 125SM laboratory balance. Target weights were determined by calculating the weigh percent carbon for each sample type and in order to cover the marine particulate carbon concentration range their weights were between 0.1 and 1.0 mg. The compounds were weighed into Ultra-light weight tin capsules (8 x 5 mm, Elemental Microanalysis) using a stainless steel small spatula (Thermo Fisher Scientific) and a pair of stainless steel curved points forceps (Elemental Microanalysis) to fold and tightly close the tin containers. Capsules were then carefully placed into the autosampler tray.

### B.2.4 Analytical procedure

Prior to measurements, EA reactor and GC column were conditioned. The process was undertaken in three steps by increasing the furnace temperature to 400, 750 and 1020 °C and

column temperature to 40, 75 and 120 °C. Each heating step was kept for about two hours and interspaced by a leak check. The system was then left overnight in the last stage to remove organic material and water from the column, connection lines and all the other system components. After conditioning, water, nitrogen and argon (for leak check) background were monitored from the IRMS instrument control panel.

Three analysis days were performed for this validation experiment. The first analysis consisted of 21 samples while in the second validation test 26 samples were analysed. The last analysis was a Primary to Secondary standard evaluation and consisted of 31 samples. The analysis sequences started with empty tin capsules (blank measurements) followed by duplicate or triplicate analysis of the standard compounds (Table B-2).

| ***Analysis day 1 - 08/07/2015*** | ***Analysis day 2 - 16/07/2015*** | ***Analysis day 3 - 26/11/2015*** |
|---|---|---|
| 3 x Sn Capsule | 2 x Sn Capsule | 1 x Sn Capsule |
| 2 x Empty filter | 2 x Empty filter | 3 x Urea |
| 3 x Urea | 2 x Urea (red label) | 3 x Caffeine |
| 2 x Sucrose | 2 x Urea | 3 x Sucrose |
| 3 x Caffeine | 2 x Glu-Ac | 3 x IAEA-CH-6 |
| 2 x Glu-Ac HCl | 2 x NS | 3 x IAEA-600 |
| 3 x Glu-Ac | 2 x MAB | 3 x MAB |
| 3 Urea (red label) | 2 x NA | 3 x CM |
| | 2 x CA | 3 x Glu-Ac HCl |
| | 2 x Sucrose | 3 x Phthalate |
| | 2 x Caffeine | 3 x NBS-18 |
| | 2 x Phthalate | |
| | 2 x Glu-Ac HCl | |

**Table B-2.** Run sequences of the 3 analysis for the particulate carbon laboratory standards validation

Empty and pre combusted (450 °C for at least 4 hours) glass fiber filters (25 mm, Whatman GF/F) were added to the analysis sequence in order to check both their carbon content and isotopic signal for successive blank corrections of seawater particulate carbon samples (collected on pre-combusted filters).

The analysis of each sample consisted of 3 injections of the reference $CO_2$ gas followed by the eluted sample gas (carried by Helium). One sample cycle lasted for about 15 minutes. The method file can be found in the laboratory PC subfolder Flash EA with the name *Carbon Mes.met.*

Following analysis, the SSH (Santrock et al., 1985) algorithm was applied to the measured ratios in order to correct for ion species contribution. Finally, the samples were mathematically corrected for the blank contribution according to the following equation:

$$\delta^{13}C_{sample} = \left[\frac{(\delta^{13}C_{meas} * A_{meas}) - (\delta^{13}C_{blank} * A_{blank})}{(A_{meas} - A_{blank})}\right]$$

where $\delta^{13}C_{meas}$ and $\delta^{13}C_{blank}$ are the isotopic ratios of sample and blank (empty Sn capsule), respectively and $A_{meas}$ and $A_{blank}$ are the respective peak areas (signal intensities).

## B.3 Results

### B.3.1 Analytical performance

System stability was reached after overnight conditioning. With the MS valve opened and the ion source switched on, the water background signal on cup 3 (m/z 18) dropped below 1 V, nitrogen background (m/z 28) was less than 3 V and argon signal (m/z 40) was low (<200 mV) and within specification. Leak checks were all successful with carrier and reference flow rates lower than 3 ml/min after 120 - 150 s. The average area of the reference $CO_2$ gas was 133.2 ± 4.8 V*s (n = 234) while the standard deviation of the $\delta^{13}C$ values was 0.05 ‰. The area of the blank measurements for mass 44 was on average 7.5 ± 2.8 V * s with $\delta^{13}C$ values varying from -32 to -27 ‰. The carbon content of a tin capsule was measured by the EA thermal conductivity detector (TCD) and its contribution to the samples signal was between 0.3 and 2.5 ‰ for standards with high (~350 µg) to low (~40 µg) carbon content, respectively.

### B.3.2 Data validation

Isotopic values of the standards included in the first and second analysis were determined by a back calculation from the Primary to Secondary standard analysis (third analysis). Results and statistical evaluations are summarised in the following tables (Table B-3, B-4 and B-5).

| **Date 08/07/15**<br>**Regression line y = 1.0685x + 32.635 $R^2$ = 0.9997** | | | | | |
|---|---|---|---|---|---|
| **Compound** | **Established $\delta^{13}C$ (‰)** | **Measured $\delta^{13}C$ (‰)** | **Std Dev (1σ)** | **n** | **Acc %** |
| **Urea (red label)** | -41.35 | -41.40 | 0.03 | 3 | 100.1 |
| **Glutamic Acid** | -13.72 | -13.74 | 0.10 | 3 | 100.2 |
| **Caffeine** | -33.40 | -33.33 | 0.05 | 3 | 99.8 |
| Urea | -39.84 | -39.68 | 0.16 | 3 | 99.6 |
| Sucrose | -26.45 | -26.81 | 0.15 | 2 | 101.4 |
| Glu-Ac HCl | -10.91 | -10.92 | 0.06 | 2 | 100.1 |

**Table B-3.** Statistics for carbon standard compounds analysed on day 1. Compounds in bold were used as main calibration standards to determine the regression line equation.

| **Date 16/07/15**<br>**Regression line y = 1.01965x + 31.04999 $R^2$ = 0.9999** | | | | | |
|---|---|---|---|---|---|
| **Compound** | **Established $\delta^{13}C$ (‰)** | **Measured $\delta^{13}C$ (‰)** | **Std Dev (1σ)** | **n** | **Acc %** |
| **Urea** | -39.84 | -39.91 | 0.12 | 2 | 100.2 |
| **MAB** | +2.48 | 2.42 | 0.23 | 2 | 97.8 |
| **Sucrose** | -26.45 | -26.60 | - | 1 | 100.6 |
| **Caffeine** | -33.40 | -33.44 | 0.22 | 2 | 100.1 |
| **Phthalate** | -30.49 | -30.25 | 0.10 | 2 | 99.2 |
| **Glu-Ac HCl** | -10.91 | -10.83 | 0.06 | 2 | 99.2 |
| Urea (red label) | -41.35 | -41.31 | 0.12 | 2 | 99.9 |
| Glutamic Acid | -13.72 | -13.79 | 0.15 | 2 | 100.5 |
| NS | -39.80 | -39.69 | 0.23 | 2 | 99.6 |
| NA | -4.67 | -4.99 | 0.74 | 2 | 106.9 |
| CA | -24.24 | -24.96 | 0.39 | 2 | 103.0 |

**Table B-4.** Statistics for carbon standard compounds analysed on day 2. Compounds in bold were used as main calibration standards to determine the regression line equation.

| **Date 26/11/15**<br>**Regression line y = 1.02521x + 37.79731 $R^2$ = 0.99996** | | | | |
|---|---|---|---|---|
| **Compound** | **Measured $\delta^{13}C$ (‰)** | **Std Dev (1σ)** | **n** | **Acc %** |
| **IAEA CH-6** | -10.48 | 0.08 | 3 | 100.3 |
| **IAEA-600** | -33.40 | 0.06 | 3 | 100.0 |
| **NBS-18** | -4.90 | 0.19 | 3 | 97.7 |
| **MAB** | 2.41 | 0.06 | 3 | 97.1 |
| Urea | -39.84 | 0.14 | 3 | 100.0 |
| Caffeine | -33.40 | 0.06 | 3 | 100.0 |
| Sucrose | -26.45 | 0.07 | 3 | 100.0 |
| Carrara Marble | 2.23 | 0.30 | 3 | 99.7 |
| Glu-Ac HCl | -10.91 | 0.22 | 3 | 100.0 |
| Phthalate | -30.49 | 0.12 | 3 | 100.0 |

**Table B-5.** Statistics for carbon standard compounds analysed on day 3. Primary standards (in bold) were used to determine the regression line equation.

## B.4 Comments

Only the Primary standards were used as main standards to determine the $\delta^{13}C$ values of the other standard compounds. Although a $\delta^{13}C$ value for the urea standard was reported ($\delta^{13}C$ = -39.79 ± 0.16 ‰, certificate of analysis No. 147273), the compound was not included in the calibration curve as a main standard because it had passed its expiration date (19/01/2015). The same consideration was taken for the urea-red label standard ($\delta^{13}C$ = -40.81 ± 0.18 ‰, certificate of analysis No.B2174) whose expiration date was on the 2nd of July 2013. The two standards were however recalibrated and the new given values were within the uncertainties for the first urea standard ($\delta^{13}C$ = -39.84 ± 0.14 ‰) and slightly depleted for the urea-red label standard ($\delta^{13}C$ = -41.35 ± 0.12 ‰).

Two reference $CO_2$ gas cylinders with different calculated stable carbon isotopic signatures ($\delta^{13}C$ = 30.55 ± 0.14 ‰ and $\delta^{13}C$ = -36.69 ± 0.14 ‰) were used for this standard validation test. However analysis output was not affected as the reference gas was not used as reference material for isotopic calibration procedures.

The isotopic signature of the blank showed a 5 ‰ variation among analysis. The variation was probably due to the different tin capsule batches used during the three days of analysis that

might have contained traces of distinctive carbon sources. However the day to day discrepancy was not an issue as the measurements were corrected using the blank value determined on the day of the analysis where a single tin capsule pack was used for all the standards.

## B.5 Conclusion

This series of analysis identified the most suitable laboratory standards to be used for stable carbon isotope analyses of particulate carbon samples by using an EA-IRMS setup. The $\delta^{13}C$ values of the 4 most suitable secondary standards compounds are summarised in the following table (Table B-6).

| *Standard* | *$\delta^{13}C$ average (‰)* | *Std Dev (1$\sigma$)* |
|---|---|---|
| Urea | -39.84 | 0.08 |
| Caffeine | -33.40 | 0.05 |
| Sucrose | -26.45 | 0.15 |
| L-Glutamic Acid HCl | -10.91 | 0.06 |

**Table B-6.** Summary of the values of the most suitable carbon isotope standards for particulate carbon analysis of marine samples.

# Appendix C

## Isodat PreCon analysis sequence script

## C.1 Introduction

This section describes the modifications carried out on Isodat 3.0 software in order to control the operations of a modified PreCon device (Thermo Fisher Scientific). The PreCon was used as interface between a Shimadzu 5000A Total organic carbon (TOC) analyser and a Delta V Advantage IRMS via GasBench II in order to perform stable carbon isotope analysis on dissolved organic carbon samples.

## C.2 Instrument description

The PreCon device consists of a high flow part (20-25 ml/min) which carries the sample from a sample container (100 ml glass vessel) to a 1 mm internal diameter (i.d.) stainless steel tube (trap 2) and a low flow part (1-2 ml/min) which transfers the sample from trap 2 to trap 3 (0.5 mm i.d. stainless steel tube) and subsequently to the GC column of the GasBench II prior IRMS analysis. The traps are automatically moved in and out of a liquid nitrogen (LN) bath in order to capture and release, respectively, the $CO_2$ contained in the analyte stream. The sample container was substituted by a new trapping loop (60 cm long, 1/8 inch i.d. stainless steel tube filled with Ni wire) connected through a custom made valve system in order to receive and trap the $CO_2$ gas generated by the TOC analyser and to transfer it in a continuous flow to the PreCon high flow part (Figure C-1).

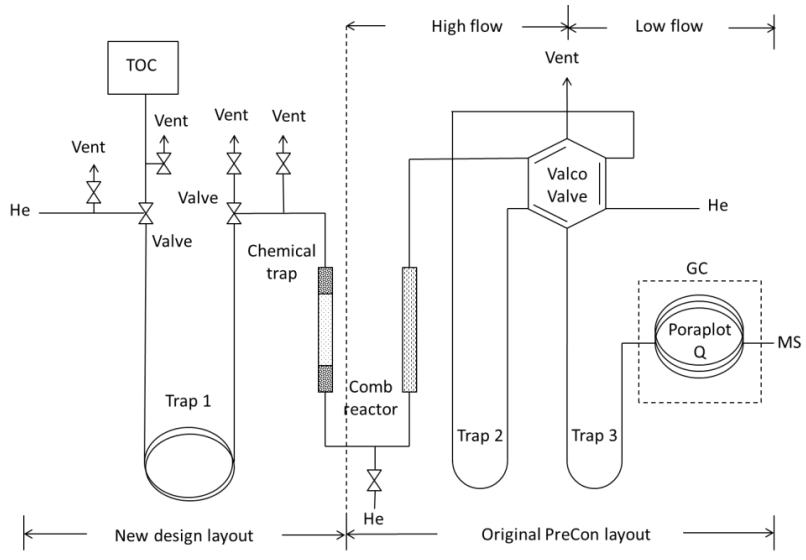


**Figure C-1.** Schematics of the modified PreCon device. Adapted from Brand (1995)

## C.3 Rationale

PreCon vents, valves and traps allow the sampling gas to be carried to the IRMS through purification, drying, oxidation and pre-concentration steps via the various components at adequate flow rates. The operation of valves and traps must be carefully controlled in order to avoid contamination by atmospheric gas intrusion and sample overlapping. Isodat 3.0 software allows configuration and control of the operation procedures. The mechanical modifications carried out on the PreCon device caused a variation in the gas flow path so that the conventional analytical sequence was not suitable for the new design. Moreover, in order to allow stable carbon isotope analysis on dissolved organic carbon samples in a continuous flow, valves switching and traps operation must match the TOC analysis timing. New Isodat PreCon software configuration settings were therefore necessary.

## C.4 PreCon Scripts

The Isodat PreCon software is installed under the same configuration as the GasBench II. The use of the GasBench II device as interface between the PreCon and the IRMS allows for reference gas ($CO_2$) pulses introduction and chromatographic separation (GasBench GC oven). The PreCon acquisition script must therefore recall the GasBench script. The script *Acquisition_for _TOC split in.isl* was created. The step by step procedure follows:

```
/=====================================================================
//ISODAT NT SCRIPT LANGUAGE (ISL): Gas Bench Acqusition Script
//=====================================================================
include "lib\stdisl.isl"
include "lib\instrument.isl"
include "lib\Continues Flow_lib.isl"
include "lib\GasBench_lib.isl"
include "lib\PreCon_lib.isl"
//---------------------------------------------------------------------------------------------------------------------
function CleanUp()
        call StopAutoDilution();
//---------------------------------------------------------------------------------------------------------------------
function InitScript()
OnBreak CleanUp;
        _Set("Gas Bench/Valco",LOAD);
        _Set("Gas Bench/Split",IN);
```

```
        _Set("Gas Bench/Reference 1",0);
        _Set("Gas Bench/Reference 2",0);
        _Set("Gas Bench/Reference 3",0);
        call PrepareHWo();

//-----------------------------------------------------------------------------------------------------------------------
main()
        call UploadSamplerMethod();
  call InitScript();
        call PeakCenter();
        call PreConPreparation();
        call ExecuteExtraScript();
        call StartAutoDilution();
        call WaitForStartSignal();
        call StartChromatogram();
        call WaitForScanEnd();

//-----------------------------------------------------------------------------------------------------------------------
```

The program starts loading the available libraries and once the GasBench_lib.isl and PreCon_lib.isl are found, all the process parameters (valves, split, reference and traps) are checked step by step and configured for initial conditions. The operation of the PreCon components is however not controlled by this script. A new script was necessary and "*PreCon_TOC_Sample.isl"* was created. The Isodat PreCon script follows:

```
/=====================================================================
//ISODAT NT SCRIPT LANGUAGE (ISL): PreCon TOC Sample Script
//=====================================================================
include "lib\Precon_lib.isl"
//-----------------------------------------------------------------------------------------------------------------------
        _UserInfo("Initialize PreCon",0,1);
        number nGCInterfaceAvail = _IsDeviceAvailable ("GC II-III Interface");
        if (nGCInterfaceAvail==1)
                        _Set("GC II-III Interface/Split",0);


                _Set("Precon/Purge",1);
        _Set("Precon/Vent 1",1);
        _Set("Precon/Vent 2",1);
```

```
        _Set("Precon/Trap 2",PC_UP);
        _Set("Precon/Trap 3",PC_UP);
        _Set("Precon/Valco",PC_LOAD);
        _Delay(2000);
        _Set("Precon/Valco",PC_INJECT);
        _Delay(2000);
        _Set("Precon/Valco",PC_LOAD);

 _MessageBox("Close Sample Loop, Open PreCon Valves",MB_OK,MB_ICONEXCLAMATION);
        _Delay(15000,1,"Depressurising sample loop...");
        _Set("Precon/Vent 1",0);
        _Delay(2000);
        _Set("Precon/Vent 2",0);
        _Delay(2000);
        _Set("Precon/Purge",0);
        _Delay(2000);
        _Set("Precon/Trap 2",PC_DOWN);
        _Delay(320000,1,"Freeze Sample to Trap 2");
        _Set("Precon/Valco",PC_INJECT);
        _Delay(20000,1,"Injecting...");

        _MessageBox("Close Precon Valves, Fill Loop with next
sample",MB_OK,MB_ICONEXCLAMATION);

 _Set("Precon/Purge",1);
        _Set("Precon/Vent 2",1);
        _Set("Precon/Vent 1",1);
        _Set("Precon/Trap 3",PC_DOWN);
        _Delay(60000,1,"Cooling Trap 3...");

        _Set("Precon/Trap 2",PC_UP);
        _Delay(260000,1,"Sample Transfer Trap 2 -->Trap 3");

        _Set("Precon/Trap 3",PC_UP);
//-----------------------------------------------------------------------------------------------------------------
```

In the Isodat language, process entries are specific for every component and they can be summarised as:

Valco Valve = LOAD/INJECT
Vents = 0(Close)/1(Open)
Traps = PC_UP/PC_DOWN
Open split = IN/OUT
The timing is defined in milliseconds.

The PreCon analysis sequence script can be divided in three main steps:

*Step 1 - Flushing of the PreCon lines*

PreCon Isodat script loads the PreCon library, checks all the available peripherals and once it founds the GasBench and the PreCon starts checking all the components and it sets them for starting conditions. With the Valco vale in the load position, Vent 1 and 2 opened and Trap 2 and 3 out of the LN Dewar, the PreCon lines are flushed with pure helium gas.

*Step 2 - Sample transfer Trap 1 -> Trap 2*

In order to avoid overpressure in the flow lines due to rapid sample gas expansion, a de-pressurisation time of 15 seconds is allowed to the sampling loop (Trap 1). Next, Vent 1 and 2 are closed and Trap 2 is lowered into the LN bath for 320 seconds allowing for complete transfer of the sample gas from Trap 1 to Trap 2.

*Step 3 - Sample transfer Trap 2 -> Trap 3*

In the last step the Valco valve is set to the inject position to allow the sample to flow through the low flow side of the PreCon. Vent 1 and 2 are opened and Trap 1 is free to receive the next sample from the TOC analyser. Prior to lifting the Trap 2 for sample release, Trap 3 is immersed into the LN Dewar for 60 second to allow for cooling of the loop before transfer in order to avoid eventual loss of sample. Trap 2 is then moved out of the LN bath and the contained sample is transferred to Trap 3 where it is frozen again. After 260 seconds Trap 3 is removed from LN container and the pre-concentrated sample can flow to the GC column and IRMS.

## C.5 Comments

The Isodat PreCon script is able to control all the process parameters that are electronically connected. Trap 1 is operated manually as no electronic mechanism is present yet. However Isodat PreCon script was configured to send the operator a message window informing about the exact time when to switch valves and operate the manual trap.

## C.6 Conclusion

A PreCon unit was modified in some of its hardware components in order to be used as an interface between a TOC analyser and an IRMS allowing for stable carbon isotope measurements of dissolved organic carbon. A direct consequence of the mechanical modifications was to update the unit software so that the modified device could be controlled by the Isodat computer program. The creation of a new Isodat PreCon script allowed for a full operation of the modified PreCon peripheral with the advantage of performing isotopic analysis in a continuous flow. The result was a constant analysis time controlled by the Isodat software able to match the TOC analysis timing allowing for good analytical reproducibility

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