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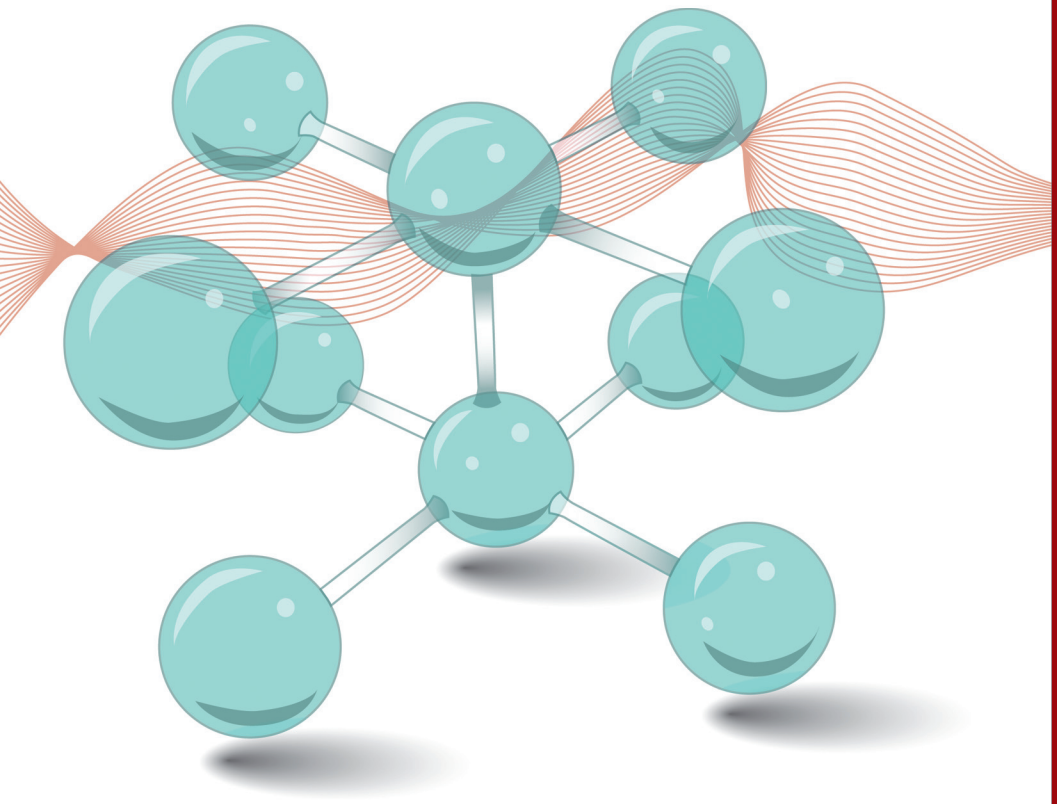

ISOTOPES: FROM THEORY TO PRACTICE

2 bis/2018

Quaderni

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ISOTOPES: FROM THEORY TO PRACTICE

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ISPRA - Istituto Superiore per la Protezione e la Ricerca Ambientale
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Daria Mazzella
ISPRA – Communications Area

Authors

Daniela BERTO, Nicoletta CALACE, Federico RAMPAZZO, Flavia SACCOMANDI

This text was drawn up with the help of

Camilla ANTONINI (ISPRA, BIO CIT)

Giuseppe ARCANGELI (Istituto Zooprofilattico Sperimentale delle Venezie)

Maddalena BONANOMI (Thermo Fisher Scientific)

Lucia BONGIORNI (CNR ISMAR Venice)

Rossella BOSCOLO BRUSA' (ISPRA, COS MLG)

Michele BOSCOLO NALE (Azienda Agricola di Chioggia)

Giuseppe BOSCOLO PALO (Consorzio di tutela del radicchio di Chioggia IGP)

Federica CACCIATORE (ISPRA, COS MLG)

Amelia DE LAZZARI (CNR ISMAR Venezia)

Tomaso FORTIBUONI (ISPRA, BIO CIT)

Guido GIAZZI (Thermo Fisher Scientific)

Claudia GION (ISPRA, BIO ACAM)

Giordano GIORGI (ISPRA, BIO ACAM)

Otello GIOVANARDI (ISPRA, BIO CIT)

Liliana KROTZ (Thermo Fisher Scientific)

Malgorzata Marcelina FORMALEWICZ (ISPRA, BIO ACAM)

Ines MARINOSCI (ISPRA, GEO DES)

Seta NOVENTA (ISPRA, BIO ACAM)

Massimo PELEGGI (ISPRA, BIO ACID)

Roberto PIRO (Istituto Zooprofilattico Sperimentale delle Venezie)

Sasa RAICEVICH (ISPRA, BIO CIT)

Francesca RONCHI (ISPRA, BIO CIT)

Luca SIMONOTTI (Thermo Fisher Scientific)

Luisa STELLATO (University of Campania "Luigi Vanvitelli")

Federica TOSI (Istituto Zooprofilattico Sperimentale delle Venezie)

Umberto TRALDI (Thermo Fisher Scientific)

Staff of the Harbormaster's Office of Chioggia

PREFACE

This Booklet intends to provide a summary of use of a versatile and innovative investigation technique which can be applied in a wide range of fields, from geology and hydrogeology to environmental studies, to identification of food adulteration, preservation of the artistic heritage and medical diagnostics.

The work, supported by a basic theoretical part, examines a series of applications for evaluating nitrate contamination in internal waters using an isotopic approach, traceability of food and agricultural products, characterization and study of the origin of organic substance, operation and dynamics of food webs and of bioaccumulation of pollutants in sea coast and transition environments, traceability of fishing chain products, national implementation of isotopes in the context of the European framework directive on marine strategy, and techniques for identifying plastic polymers.

This work, the outcome of developed and consolidated synergies between the Department for monitoring and safeguarding the environment and for preservation of biodiversity and the National Center for environmental characterization and protection of the coastline, marine climatology and operative oceanography, expressed in terms which are accessible to the wider public of non-experts, can represent a stimulus for a different and innovative technical and scientific approach.

Maurizio Ferla
Director of the National Center for environmental characterization and protection of the coastline, marine climatology and operative oceanography

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INTRODUCTION

The term isotope (from the Greek *iso-topos*, same place) means atoms of the same chemical element which have the same atomic number, but have different atomic mass numbers. Practically speaking, isotopes have the same number of protons and electrons (same chemical properties) and a different number of neutrons (different physical properties). They may be natural or artificial, stable or unstable.

Quantification of the ratio between two isotopes of the same element has considerable potential for establishing whether two chemically similar elements are of different origin, as their sources differ. The isotopic distribution characterizing sources can be affected by phenomena of various kinds which, in their turn, can cause significant variations in the final products. These variations can be detected by techniques for analyzing stable isotopes, in fact many chemical elements have isotopes which are useful for this purpose (Figure I.1). Constant technological progress has made it possible to develop tools which can measure tiny variations in the abundance of stable isotopes with a high degree of precision and accuracy (mass spectrometry). So today we can count on a wide range of isotopic measures for application in various fields of science.

The isotopic composition of a sample is equal to the ratio between the abundance of the heavy isotopic form compared to the light one (for example $^{13}C/^{12}C$) and is expressed as a deviation, in parts per thousand, from a standard benchmark material internationally identified. Isotopic abundance is expressed according to the following equation:

$$\delta(‰) = \left(\frac{R_{sample} - R_{standard}}{R_{standard}}\right) \times 1000$$

where R is the ratio between abundances of the masses of the heavy isotope and of the light one, R_{sample} is R measured in the sample and $R_{standard}$ is R measured for the standard. A positive value of δ shows that the heavy isotope has been enriched in the sample compared to the standard, while a negative value shows that in the sample the heavy isotope has been depleted (Michener and Lajtha, 2007).

The possibility of differentiating two samples on the basis of isotopic analysis is related to the phenomenon of isotopic fractionation by processes which may be chemical (e.g. nitrification, ammonification), physical (e.g. evaporation and condensation) and biological (e.g. photosynthesis,

assimilation, excretion). These may in fact alter the isotopic signature of the compounds, provoking fractionation of the distribution of the isotopes among the reactants and products and producing a variation of the isotopic delta of the products compared to the reactants (Brand et al., 2014). The terms “signature”, “marking” or “composition” therefore mean the isotopic values which arise a consequence of these processes and which characterize in specific way a certain environmental sample.

Generally speaking, two kinds of isotopic fractionation can be distinguished:

1) Equilibrium isotope fractionation (thermodynamic): due to a difference of binding energy of the isotopes in the compounds. This implies that:
   - Heavy isotopes accumulate in oxidized products;
   - The reaction is favored at low temperatures, since at high temperatures the differences between isotopes are attenuated;
   - The process is not significant in case of chemical reactions of gaseous substances and of biological reactions.

2) Kinetic: due to different speed of reaction of isotopes.

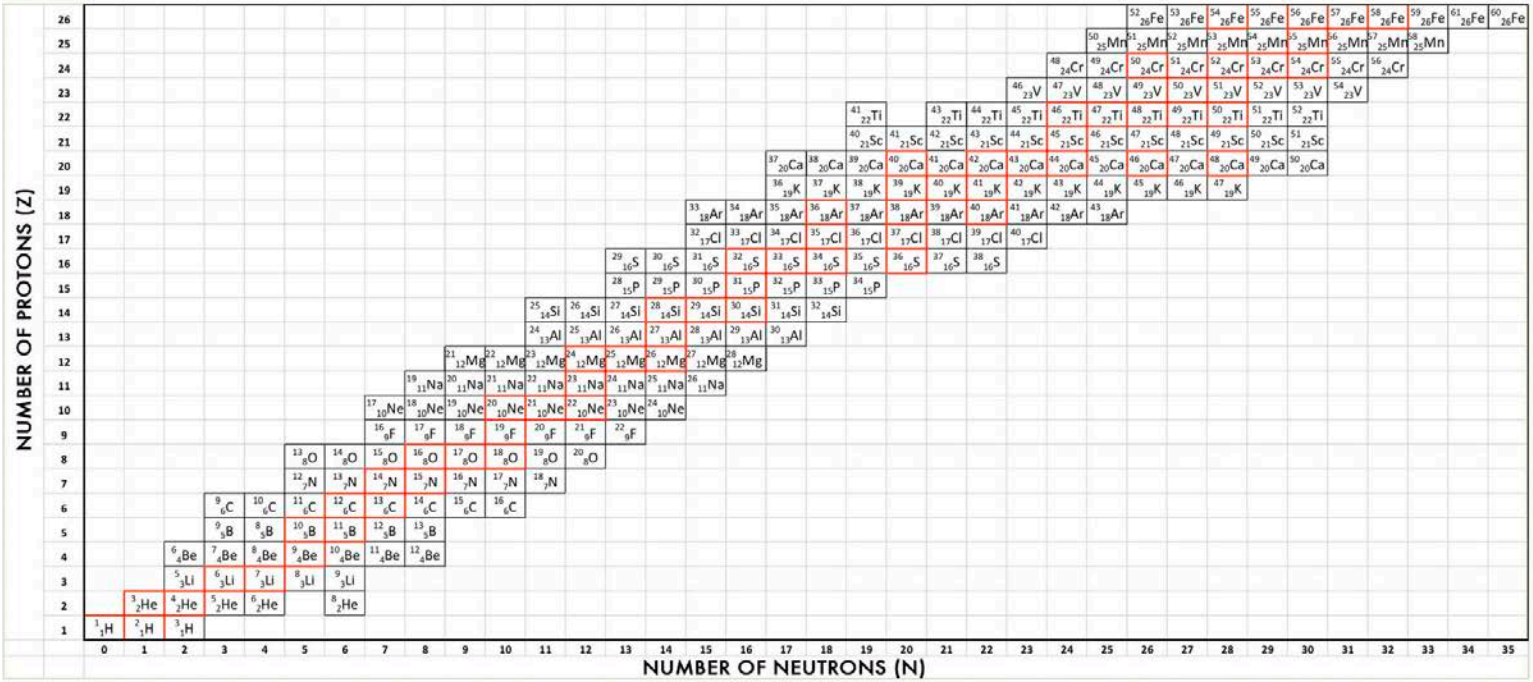

*Figure I.1: Stable isotopes generally have a proton/neutron ratio less than 1.5 (*Michener R., Lajtha K., 2007*)*

In the reactions described above, one generally observes that rapid, irreversible and unidirectional processes are favored, that is when products are easily distanced by reactants, for example in processes involving evaporation, diffusion, etc. Moreover, the breaking of bonds formed by light isotopes is favored, and the preferential distribution of light isotopes is in the products, while heavy isotopes remain in the reactants.

Given a chemical substance AB featuring the presence of a certain isotopic distribution of the element X we can calculate the fractionation factor (α_{AB}) by dividing the ratio of the number of isotopes X in the product A by the number of the isotopes X in the product B.

$$\alpha_{AB} = \frac{R_A}{R_B} = 1 + \left[\frac{(\delta_A - \delta_B)}{1000}\right]$$

where

$$R = \frac{X_p\ [atoms\ of\ the\ heavy\ isotope\ (rare)]}{X_l[\ (atoms\ of\ the\ light\ isotope\ (abundant)]}$$

The fractionation factor is however normally replaced by the factor of isotopic enrichment (ε) which is defined as (α^{-1})*1000.

Analysis of stable isotopes is a highly specialized scientific method supporting various disciplines: environmental sciences, forensic sciences, medicine, etc.

In the field of environmental sciences, there are many applications, ranging from geology to ecology, including the study of environmental chemical and physical processes.

Applications in the field of geology range from hydrogeological and stratigraphic to petrological studies. In hydrogeology, for example, stable isotopes of the water molecule (^{18}O and ^{2}H) record the average altitude of the reload area of the aquifer and any phenomena of evaporation, allowing distinction between waters coming from different hydrogeological circuits. They also allow one to study water-rock interaction. In the stratigraphic field, carbon, nitrogen, sulfur and chlorine isotopes allow one to acquire information concerning the evolution of the diagenetic process of the sediment they come from (solid carbonates). In the field of petrology, study of isotopes allows one to understand the origin of magmas and their differentiation processes. In addition, determination of the isotopic composition of oxygen and hydrogen in intrusive, effusive and metamorphic rocks allows one to study geodynamic processes.

In the field of ecology, analyses of stable isotopes find many applications. For example, the analysis of isotopic fractionation of iron, due to the bacterial activity, allows one to trace the origin of such microorganisms. Analysis of carbon and nitrogen isotopes allows one to study the trophic relations and flows of matter among the main components of an ecosystem (e.g. organic matter, primary producers, primary and secondary consumers) and may also be used to investigate chemical and biological processes taking place both on the ecosystem level and on the level of individual organisms.

Analysis of stable isotopes has been used, in some cases, to determine the causes of the water, atmospheric and soil pollution, such as the origin of nitrate contamination in surface and groundwater.

Study of stable isotopes has recently become significant in the field of quality controls on some food products (wine, oil, honey, fruit juices) and adulteration of mineral waters and sugars. It has turned out to be very important in the study of authenticity of food items: in this case, the analysis of the different isotopic ratios of specific molecules present in food items allows one to identify their origin, as they come from different raw materials or materials processed in different ways, for example by biological or industrial synthesis.

Table I.1 shows the main elements whose stable isotopes have been applied for various studies.

Table I.1: Main elements and isotopes used in various fields of science. The table shows the natural abundance of isotopes, the international benchmark standard used for the analysis of stable isotopes and their certified isotopic abundance (the isotopic distribution in standard benchmark materials is different from the natural one).

Element	Isotopes	Abundance (%)	International Standard	Absolute abundance of the standard ($R_{standard}$)
Carbon	^{12}C ^{13}C	98.892 1.108	Vienna Pee Dee Belemnite (VPDB)	^{13}C :^{12}C = 0.0112372
Nitrogen	^{14}N ^{15}N	99.635 0.365	Atmospheric Nitrogen (air)	^{15}N :^{14}N = 0.0036765
Oxygen	^{16}O ^{17}O ^{18}O	99.759 0.037 0.204	VSMOW in water, VPDB in CO_2 or Carbonated	VSMOW=0.0020052 VPDB=0.0020672 both for ^{18}O :^{16}O
Sulfur	^{32}S ^{22}S ^{34}S ^{36}S	95 0.75 4.21 0.014	Vienna Cañon Diablo meteorite troilite (VCDT)	^{34}S :^{32}S = 0.0450045
Boron	^{10}B ^{11}B	19.9 80.1	Boric Acid NBS-951	^{11}B :^{10}B = 4.0437
Chlorine	^{35}Cl ^{37}Cl	75.8 24.2	Standard Mean Ocean Chloride (SMOC)	^{37}Cl :^{35}Cl = 0.324
Iron	^{54}Fe ^{56}Fe ^{57}Fe ^{58}Fe	5.82 91.66 2.19 0.33	Mean between terrestrial and lunar rocks	^{56}Fe :^{54}Fe = 15.7028

References

Brand W. A., Coplen T.B., Vogl J., Rosner M., Prohaska T., 2014. Assessment of international reference materials for isotope-ratio analysis (IUPAC Technical Report), Pure Appl. Chem.; 86(3): 425–467

Michener R., Lajtha K., 2007. Stable Isotopes in Ecology and Environmental Science, Blackwell Publishing Ltd

1. MAIN ELEMENTS USED IN ISOTOPIC ANALYSIS

The following describes some of the chemical elements most commonly used in isotopic analysis, not only for environmental purposes.

1.1 - Carbon

The two main reserves of carbon in nature are organic carbon and carbonates. They have different isotopic footprints due to the different processes involved (Figure 1.2). Inorganic carbon (carbonates) takes part in the balance of exchange among: atmospheric carbon dioxide - dissolved bicarbonate ion - solid carbonate; these exchange reactions lead to an enrichment of the heavy isotope in solid carbonate form ($\delta^{13}C$ equals 0‰). Vice versa, kinetic reactions mainly involving organic carbon, through processes of photosynthesis, determine a concentration of the lighter isotope in the organic material produced ($\delta^{13}C$ equals about −25‰) (Michener and Lajtha, 2007).

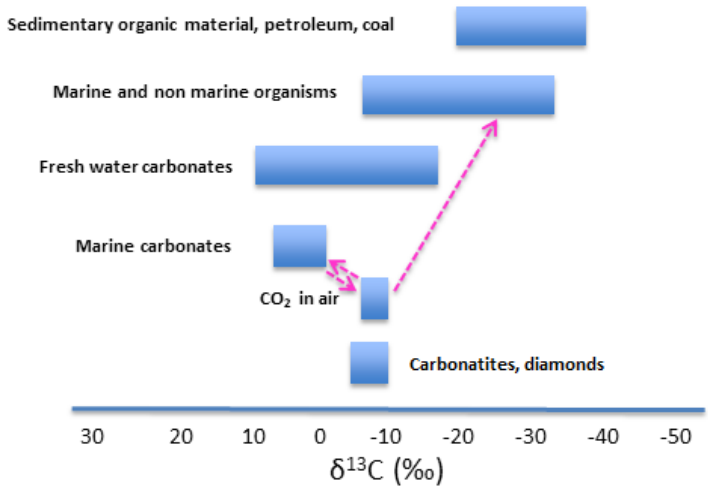

Figure 1.2: Isotopic footprint in carbon present in nature (Hoefs, 2015)

Fractionation of organic carbon is mainly associated with the kind of plant, which operates photosynthesis. Terrestrial plants, classified as C3 and C4, follow two different metabolic paths. Both generate organic substance featuring $\delta^{13}C$ values, which are more negative than that of carbon dioxide (~-7‰), since during photosynthesis, the organic substance produced accumulates the light isotope rather than the heavy isotope.
C3 plants, characteristic of temperate climates, produce a compound with three carbon atoms (Calvin cycle) with an average value of $\delta^{13}C$ equal

to -26.5‰. C4 plants generate a compound with 4 carbon atoms (Hatch-Slack cycle) characterized by a $\delta^{13}C$ value around -12.5‰.

Starting from this kind of knowledge, fractionation of carbon of organic origin has been investigated for many applications, including the study of food webs (Post et al, 2002). The chemical composition of animal tissues is related to the food resources they assimilate, so the tissues reflect the isotopic composition of the diet (De Niro & Epstain 1978, Vander & Rasmussen, 2001). Enrichment among primary producers and consumers (herbivores) has been estimated to be around +5‰, whereas the enrichment is less marked (+1‰) at higher levels of the food chain (Tykot, 2004; Lelli, personal communication). So the isotopic value found in the tissues of an organism can be used as an indicator of its position in the food chain, but since variation in value of $\delta^{13}C$ associated with steps along the food chain is relatively insignificant, these data are mainly used to track down the primary source of carbon (Layman et al. 2012).

Analyzing stable isotopes of carbon also allows one to differentiate terrestrial food webs from marine ones. "Marine" carbon in fact comes from dissolved inorganic carbon (dissolved bicarbonate) characterized by an isotopic value of about 0‰, hence higher than that of atmospheric carbon dioxide, which is around -7‰. This difference is preserved at every trophic level both in the marine and terrestrial environment. (Figure 1.3)

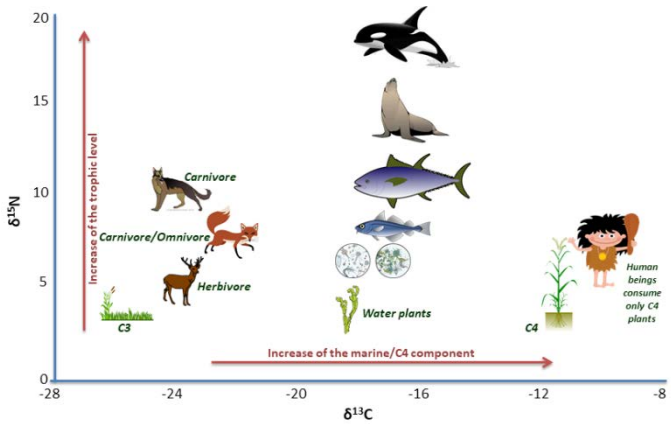

Figure 1.3: Variations of carbon and nitrogen isotopes (‰) in different organisms of the terrestrial and marine food chain

1.2 - Nitrogen

Nitrogen is the main element of the terrestrial atmosphere (about 78%), but despite this, the majority of organisms are unable to use it in its gaseous form.

Atmospheric nitrogen therefore has to be converted into forms which can be used, and this happens naturally through a series of chemical reactions mediated by microorganisms that fix nitrogen and which live both in the soil and in fresh water (*Clostridium, Azotobacter, Rhizobium leguminosarum,* actinomycetes), and produce isotopic fractionation of nitrogen differentiating the values of $\delta^{15}N$. The first phase of fixation (Figure 1.4) sees formation of ammonia nitrogen ($\delta^{15}N$ equals about 1‰) from atmospheric molecular nitrogen (Michener & Lajtha, 2007; Butterbach-Bahl et al., 2017).

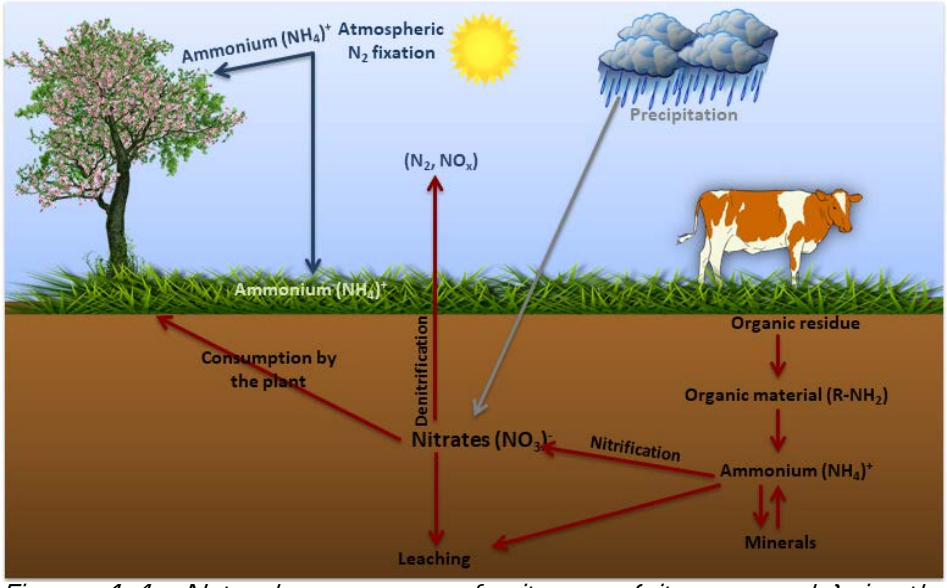

Figure 1.4: Natural processes of nitrogen (nitrogen cycle) in the terrestrial environment

Reactions, which generate further transformations of nitrogen in the soil and water, are mineralization, volatilization, nitrification and denitrification, processes also mainly mediated by microorganisms (Figure 1.4). For example, volatilization is associated with the loss of ammonia from the soil into the atmosphere. This process features a high degree of fractionation which produces ammonia depleted in ^{15}N, leaving the residual ammonium ion present in the soil enriched in the heavy isotope. The whole process of transformation of nitrogen into nitrate involves several steps of reaction, each of which produces an enrichment in the residual nitrate substrate, which can be as much as 30‰.

Denitrification (i.e. use of nitrate instead of oxygen as substrate for oxidation of organic matter) is a process mediated by bacteria, able to provoke a high degree of isotopic fractionation of residual nitrate. Also depending on environmental conditions, bacteria activity discriminates

"lighter" isotopic forms, causing - in the residual nitrate - an enrichment of the heavy isotope compared to the molecular nitrogen produced.

Figure 1.5 shows the main processes involving the nitrogen cycle in a marine environment (Ryabenko, 2013).

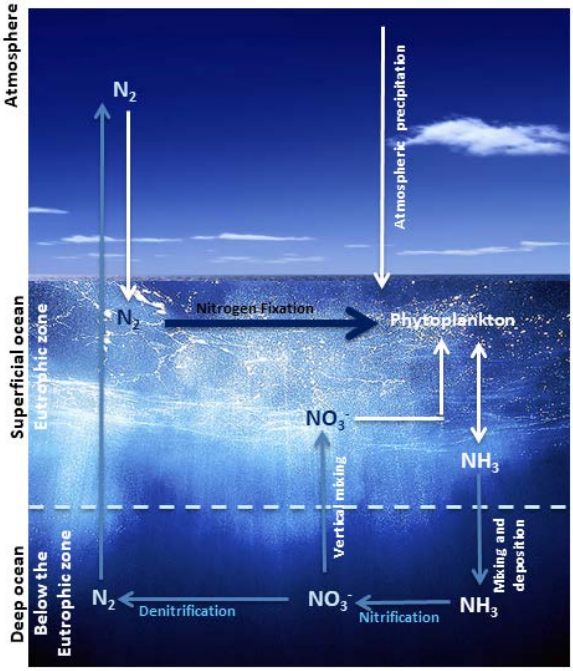

Figure 1.5: Natural processes of nitrogen (nitrogen cycle) in the marine environment

Knowledge of the whole nitrogen cycle in terms of isotopic distribution, in association with the carbon cycle, is useful both for understanding the features of the food chain and relations among some kinds of anthropic pressure on the environment and impact on the ecosystem.

1.3 - Oxygen (water)

During phenomena of evaporation and precipitation, isotopic fractionation of oxygen (and of hydrogen) takes place (Figure 1.6).

The amount of fractionation depends on the temperature and on other climatic and geographical factors, for example latitude, altitude, seasonality and continentality.

Water tends to evaporate with an equilibrium reaction regulated by temperature. The vapor phase is characterized by an enrichment in ^{16}O ($\delta^{18}O$

< 0) and in ^{1}H, since the molecules of $^{1}H^{16}O$ are lighter and hence tend to evaporate more easily. Vice versa, the liquid phase will be richer in $^{2}H^{16}O$, $^{1}H^{18}O$ and $^{2}H^{18}O$, heavier molecules ($\delta^{18}O$ and $\delta^{2}H > 0$).

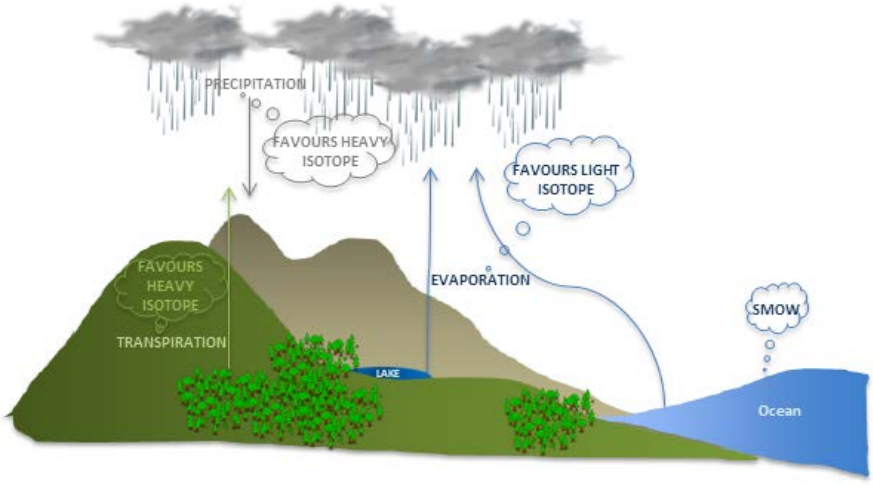

Figure 1.6: Natural processes of oxygen (water cycle)

Precipitation involves depletion of the heavy isotope as latitude and altitude increase. In the same region, precipitations in the cold months are characterized by negative isotopic compositions, while during the warm months, they are enriched with heavy isotopes (more positive δ due to seasonal effect). Finally, precipitations are more enriched with the heavy isotope as one moves inland from the coast.

Also in the vegetative cycle of plants, the processes of water adsorption and evapotranspiration determine an enrichment of heavy isotopes (^{2}H and ^{18}O), depending on the plant species and the "isotopically" different water the various plants have available for photosynthesis.

1.4 - Hydrogen

Hydrogen is ubiquitous in the environment and features the greatest difference between its stable isotopes (hydrogen and deuterium) in terms of mass. The typical isotopic distribution in some natural matrices is shown on Figure 1.7.

Hydrogen together with oxygen is used for characterization of the water cycle at different altitudes and latitudes, and in some applications involving biochemical processes.

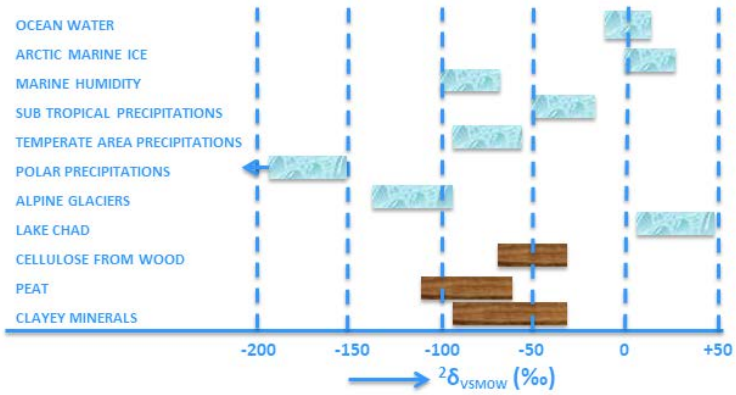

Figure 1.7: Isotopic footprint of hydrogen in some natural matrices

1.5 - Sulfur

Sulfur has four stable isotopes but generally, the isotopic ratio used refers to the abundances of ^{34}S and of ^{32}S, which are the two most abundant isotopes (4 and 95% respectively). Natural fractionation of sulfur takes place due to the effect of microbial processes, which reduce sulfate to sulfide, and chemical processes of exchange between sulfate and sulfide and among different sulfides (Figure 1.8). Knowledge of the sulfur cycle in terms of isotopic distribution is useful for studies on food diet and, in association with carbon and nitrogen, permits to understand the features of the food chain and allows the biogeochemical characterization of some environmental processes.

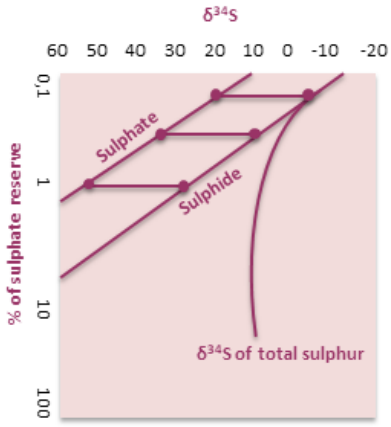

Figure 1.8: Natural fractionation of sulfur (Hoefs, 2015)

1.6 - Boron

Boron is a ubiquitous element present in dissolved form in natural waters since it is highly soluble. Its presence is natural in the earth's crust, but it can also be of anthropic origin, being introduced through fertilizers, waste, etc. Isotopic fractionation of boron can be due to a variety of chemical processes, especially those related to its speciation (Figure 1.9) (Wahab & Hussain, 2014). The light isotope is preferentially incorporated in the $B(OH)_4^-$ form, rather than $B(OH)_3$.

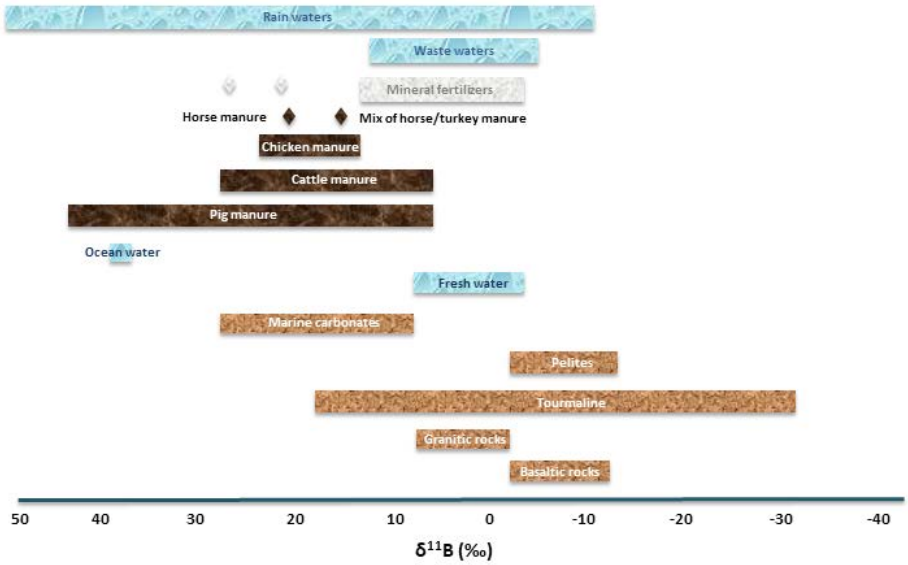

Figure 1.9: Isotopic fractionation of boron (Hoefs, 2015; Widory, 2012)

Since boron is a conservative element having the characteristic of migrating together with other contaminants such as for example nitrates and pharmaceutical substances, etc., its isotopic composition can be used together with other stable isotopes to determine the origin of the substances of interest in water.

1.7 - Chlorine

Chlorine is characterized by two stable isotopes with atomic mass 35 and 37, respectively. In the past, it was impossible to measure the minute variations of chlorine isotopic ratios, but recent mass spectrometers has made it possible to determine them with an accuracy of about 0.1-0.2‰.

Even though not many applications exist based on chlorine stable isotopes of chlorinated organic substances, they have a high potential for defining the different origins of molecules and of degradation mechanisms (Figure 1.10) (Annable et al., 2007; Hunkeler et al., 2012; Eggenkamp, 2014).

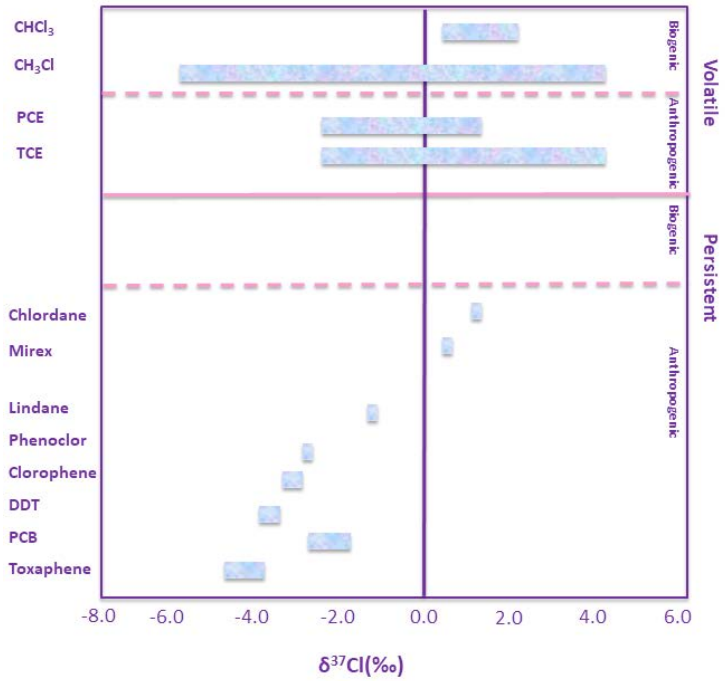

Figure 1.10: Isotopic fractionation of chlorine

1.8 - Iron

Iron isotopes are an excellent tool for understanding some natural processes, taking into account the range of stable isotopes (54, 56, 57, 58) and the importance of redox reactions involving iron in biochemical and inorganic processes. Inorganic redox reactions between Fe(II) and Fe(III) can produce deviations of the isotopic ratios which are much more marked than those generated by biochemical processes.

1.9 - Instrumentation

Mass spectrometry is a technique used to separate charged molecules, i.e. ions, according to their mass or, more properly, to their mass/charge ratio. Therefore, it is a technique, which can distinguish isotopes of the same element and calculate their isotopic ratio.

The isotopic ratios of light elements such as H, O, C, N, S are measured using the Isotope Ratio Mass Spectrometry (IRMS) technique after transformation of the samples into pure gases prior to the ionization. An example of an instrument useful for measuring isotopic ratios with excellent precision, sensitivity and linearity is the Thermo Scientific Delta V Isotope Ratio Mass Spectrometer (Figure 1.11).

This is an electron impact gas source mass spectrometer with magnetic separation and universal triple collector suitable for all standard applications, and enabling precise isotopic analysis of N_2, CO, NO, O_2, CO_2, N_2O, SO_2 but also H_2, and even CH_3Cl, CH_3Br, Ne, Ar and K by using additional collectors.

GAS	MINOR ISOTOPE	PRECISION (1σ)	LINEARITY
CO_2	^{13}C	0.06 ‰	0.02 ‰ / nA
	^{18}O	0.08 ‰	0.02 ‰ / nA
N_2	^{15}N	0.06 ‰	0.02 ‰ / nA
O_2	^{18}O	0.08 ‰	0.03 ‰ / nA
	^{17}O	0.20 ‰	0.04 ‰ / nA
CO	^{18}O	0.15 ‰	0.04 ‰ / nA
H_2	^{2}H	0.40 ‰	0.20 ‰ / nA
SO_2	^{34}S	0.10 ‰	0.03 ‰ / nA

Figure 1.11: Thermo Scientific Delta V Isotope Ratio Mass Spectrometer and Dual Inlet standard performances

The IRMS can connect in continuous flow with various peripheral devices used to produce and isolate pure gases from the samples. Some examples are: Flash Elemental Analysis (EA) to analyze stable isotopes of C, N, H, O and H after combustion or pyrolysis of the sample; GC-IsoLink-II to analyze, after gas chromatographic separation of the sample, the stable isotopes of C, N, H and O after combustion or pyrolysis of the selected molecules; LC-IsoLink for analysis of the isotope of C after separation in liquid phase. Finally, it can also interface with the GasBench II unit for headspace samples isotopic analysis, including O and H stable isotopes of O_2 and H_2 after liquid equilibration, O and C stable isotopes of CO_2 after acid attack of carbonates and O, C, H and N stable isotopes of various gases, atmospheric gases for instance (Figure 1.12).

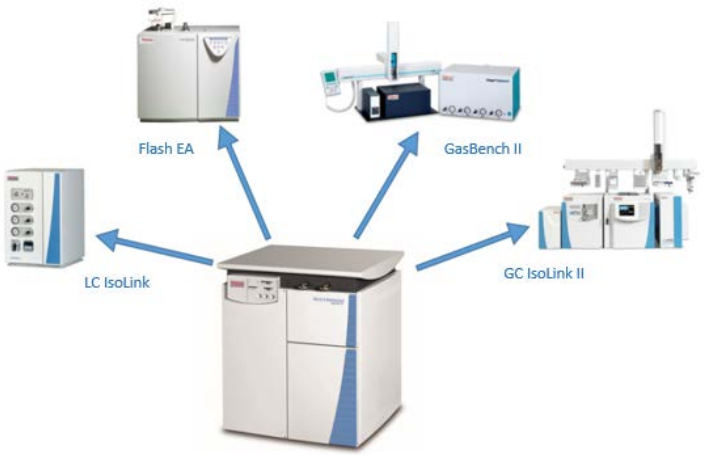

Figure 1.12: Thermo Scientific Delta V Isotope Ratio Mass Spectrometer and the various peripheral devices to be connected for different kinds of samples and analysis

The most popular of the IRMS peripherals is the Elemental Analyzer (EA). The Thermo Scientific FlashSmart Elemental Analyzer offers high performance measurements from low µg to high mg sample sizes and allows to obtain extremely accurate quantitative analyzes both at low (few ppm) and at high concentrations.

The elemental analyzer, based on a modified Dumas method, allows one to establish C, N and S by combustion or O and H by pyrolysis, and offers ample modularity in order to increase and diversify productivity and efficiency in the laboratory.

For other elements such as for example B, Fe, Sr, J, Pb, and much more, use is made of Multicollector - Inductively Coupled Plasma – Mass Spectrometry (ICP-MS) technique and Thermal Ionization Mass Spectrometry (TIMS) where the sample is transformed into atoms and ions. An example of a useful instrument for measuring isotopes of B, Fe, etc. is the Thermo Scientific ELEMENT Series ICP-MS (Figure 1.13). It is characterized by ICP-MS with a double-focusing magnetic sector for analysis stability. Together with excellent sensitivity, the Thermo Scientific ELEMENT 2 ICP-MS affords an excellent signal/noise ratio. For isotopic analyses requiring the utmost precision, the best is the Neptune Plus High Resolution Multicollector ICP-MS.

Figure 1.13: Element XR High Resolution ICP-MS for high sensitivity isotopic analyses

High precision isotopic ratios of noble gases such as He, Ne, Ar, Kr or Xe are obtained using dedicated static vacuum mass spectrometers such as the Thermo Scientific Argus VI, Helix SFT or Helix MC.

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2. MIXING MODELS

2.1 - Introduction

In recent decades, the scientific community interested in ecological studies has proposed different isotopic Mixing Models to identify contributions of different sources of food in consumers' diets.

Phillips & Koch (2002) proposed using a set of algebraic equations (Linear Mixing Models) to identify such contributions. Starting from these equations, Phillips and Gregg (2003) developed the ISOSOURCE mixing models, able to return a series of possible source partition values. The main limit of this kind of approach lies in its being "conservative": the isotopic value associated with the various sources must in fact remain constant through time, a condition, which in nature does not often hold true. Therefore, the ISOSOURCE model does not allow for incorporating uncertainties and variations that characterizes input data (uncertainty associated with the isotopic ratios of food sources and with the enrichment factors due to transfer along the food chain), that is it does not allow one to consider natural variability.

In order to overcome such a limitation, new isotopic mixing models were developed, for example MixSIR (Moore and Semmens, 2008) and SIAR (Parnell, 2008; Parnell et al., 2010). Both models, considered to be of new generation, are based on the solution of an indeterminate system of linear equations by using advanced Bayesian statistics techniques to identify proportional contributions of the food sources used by organisms. Bayesian models return the values of the proportional contributions of such sources in the form of probability distribution, expressed through descriptors of central tendency, e.g. mean, mode and confidence interval (Moore et al., 2006; Moore and Semmens, 2008; Jackson et al., 2009; Parnell et al., 2010; Fry, 2013). Besides allowing introduction of uncertainties associated with input data (in the form of means and standard deviation of sources), they also allow introduction of specific isotopic fractionation factors supplied with variability intervals and any other information which may be available beforehand, representing them in a more realistic way. In recent years, models based on Bayesian statistics have evolved considerably, providing a noteworthy contribution to the study of environmental issues, such as definition of nitrate partitioning in water (Xue et al. 2009, 2014).

One must point out that every isotopic mixing model is subject to certain limitations:

- The sources must be characterized by isotopic intervals as different as possible from each other; the more the isotopic intervals overlap each other, less powerful is the model in discriminating sources.
- In many cases, it is necessary to make prior groupings in order to limit uncertainty of probabilistic solutions (and this is possible only if one has in-depth knowledge of the system being investigated).
- Incoming data must be precise and accurate.
- Use of prior information in the models applying Bayesian statistics can lead to uncertain results, depending on the nature of the input (Moore and Semmens, 2008).

2.2. - Analysis of stable isotopes in R (SIAR)

The isotope mixing model through analysis of stable isotopes in R (SIAR) permits quantification of contributions to a mix by different sources of isotopes. This is a software, which can be installed in R (real numbers) environment (R Core Team (2014)) and is freely available on-line (https://CRAN.R-project.org/package=siar).

Generally speaking, when dealing with multiple isotopic sources, one can define a system of n equations with n-1 sources. In many environmental evaluations, however, it is not always possible to develop a defined system. Here one needs to solve an underdetermined system of equations, with n^{-2} or less variables, through statistical processing tools. SIAR is based on the solution of an underdetermined system of linear equations (n equations and n-2 variables) through use of Bayesian statistical techniques to identify the proportional contributions of the different sources in a mix.

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3. EVALUATION OF NITRATE CONTAMINATION USING AN ISOTOPIC APPROACH

3.1 - Introduction

In the context of the MIPAAF - ISPRA Convention, aimed at identifying and quantifying the origin of the nitrate content in surface and groundwater, an analytic methodology has been developed based on isotopic analyses and on mathematical processing of the data obtained.

This methodology requires knowledge of the potential sources of nitrates of domestic, agricultural or manure origin affecting a certain territory, the availability of detailed information on the use of the soil and the results of monitoring activity by Regional Agencies for Environmental Protection, as well as knowledge of the mechanisms of migration and/or chemical and physical transformation of nitrogen in different environmental matrices.

Several Italian and international studies (ISPRA Report 2015 and reference quoted there) have in fact shown that isotopic investigations can provide valid support for identifying and assessing contributions causing the presence of high nitrate values in both surface and groundwater. Recent research has proven the efficacy of these investigations in identifying and assessing contributions from multiple sources. In fact, nitrates generated by multiple pollution sources show an isotopic composition of nitrogen and/or oxygen, which is different depending on the source from which they originated.

The variation range of isotopic composition of nitrogen, according to the literature, is between -20‰ and 30‰. This range includes nitrogen fertilizers, which have an isotopic composition between -10‰ and 5‰, and sewage of manure and domestic origin with characteristic values between 3‰ and 25‰ (ISPRA Report, 2015 and reference quoted there).

Nitrates coming from use of synthetic fertilizers can thus be distinguished from nitrates originating from manure and domestic sewage. In addition, the isotopic composition of nitrogen and oxygen in the residual nitrate measured through time in a water body allows one to distinguish between denitrification and a process of dilution.

The ISONITRATE (http://isonitrate.brgm.fr/) approach was the first reference model chosen by ISPRA in the context of the abovementioned convention. This approach calls for isotopic characterization of each source in terms of $\delta^{15}N$ and/or $\delta^{18}O$ in order to discriminate their relative contribution

in surface or underground waters. To this end, in special cases, alongside isotopic composition of nitrogen and/or oxygen characteristic of the source they come from, ISONITRATE proposed the use of isotopic ratios of other trace elements (characterized by an environmental behavior similar to that of nitrates) which can provide additional information about certain kinds of sources. For example, measurement of the isotopic ratio of boron, $\delta^{11}B$ allows one to discriminate whether the source is due to domestic effluent or to other mineral and manure sources. Since during performance of the project, it was not possible to carry out isotopic analyses of boron, the SIAR isotopic mixing model was applied (Paragraph 2.2).

3.2 - Concept Model

In nature, there are two stable isotopes of nitrogen ^{14}N and ^{15}N ($^{15}N/^{14}N$ =1/272 ratio in the atmosphere) and three of oxygen ^{16}O, ^{17}O and ^{18}O (the international benchmark is the mean value of ocean water V-SMOW ($\delta^{18}O=0$)).

The main reactions, which control the dynamics of nitrogen in the various environmental sectors (soil, water and air) (Figure 3.1), are:

- Nitrogen fixation, that is when elementary nitrogen is converted into ammonia by microorganisms, both free and in symbiosis with plants;
- Organication, that is when ammonia (ammonium ion) is incorporated into the structure of the organic substance;
- Ammonization, that is when organic nitrogen is converted into ammoniacal nitrogen (ammonium ion in equilibrium with the gaseous ammoniacal form, a process also called mineralization);
- Nitrification, that is oxidation of ammoniacal nitrogen into nitrite and hence nitrate;
- Denitrification, that is reduction of nitrate and formation of N_2 e N_2O;
- Assimilative reduction of nitrate that is when nitrate is transformed into ammoniacal nitrogen, with the intermediate step of formation of nitrite.

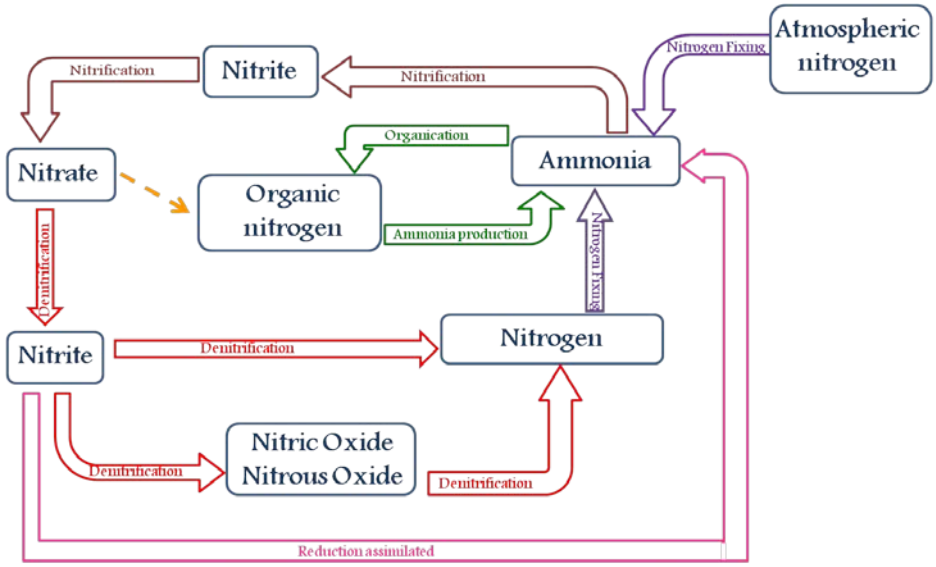

Figure 3.1: Scheme of the nitrogen cycle

Isotopic fractionation can affect every kind of reaction in which nitrogen takes part; for some, as in the case of ammonium ion formation from the organic substance, it is very small (±1‰); for others, as in the case of nitrification, fractionation can be more marked.

Actually, the formation of ammonium ion provokes almost instantly also generation of ammonia (equilibrium between ammonium ion in solution and ammonia in gaseous phase). Its volatilization into the atmosphere is characterized by a high degree of fractionation, which produces ammonia depleted in ^{15}N, leaving the residual ammonium ion on the enriched substrate. The enrichment factor (equivalent to the difference of the deltas of the substrate and product) can take on values up to 30‰.

In the case of nitrification, the whole process of transformation of nitrogen into nitrate produces an enrichment of the residual nitrogen substrate (sequential transformation reactions). Therefore, the equilibrium between ammonia in solution and ammonia in gaseous phase generates an enrichment in the solubilized fraction, as well as the equilibrium in solution between the ammonium ion and ammonia generates an enrichment in the ammonium ion.

Transformation of ammonium ion to nitrite provides a further enrichment incurred by the nitrous fraction while further oxidation of nitrogen from nitrite to nitrate is negligible in terms of isotopic fractionation.

Denitrification (i.e. use of nitrate instead of oxygen as substrate for oxidation of organic matter) is a process mediated by bacteria, also able to generate a high degree of isotopic fractionation of residual nitrate. Depending on the environmental conditions, fractionation, i.e. discrimination operated by bacteria towards "lighter" species, can vary from 1.8‰ to 40‰ (i.e. the residual nitrate results enriched in the heavier isotope respect to the produced molecular nitrogen).

However, it must be kept in mind that the speed of the processes involving nitrogen depends on many environmental factors, for example, the texture and percentage of humidity of the soils, etc.

All the knowledge about isotopic enrichment factors, which can be attributed to the nitrogen cycle, is fundamental for application of the isotopic approach to establishing the percentage contributions of nitrate sources in water.

Existence of a significant isotopic difference between potential sources is a prerequisite for using $\delta^{18}O$ and $\delta^{15}N$ to identify nitrate sources in groundwater.

Summing up, the variation range of isotopic composition of nitric nitrogen is between -20 ‰ and +30 ‰ and especially:
- values of ^{15}N around zero (-10 ‰; +5 ‰) identify nitrogen mineral fertilizers (nitric and ammonia);
- values of ^{15}N between +3 ‰ and +25 ‰ identify manure and domestic sewages; ^{14}N is preferentially excreted with urine; hence nitrogen in residual fraction of sewage results enriched in ^{15}N.

Nitrogen compounds of atmospheric origin (rain) do not contribute substantially to the increase of concentration of nitrates in farming land (Sutton et al., 2011) and have a wide range of $\delta^{15}N$ (-20‰ to +15‰) that prevents to obtain conclusive information.

Nitrates originating from synthetic fertilizers and soil nitrogen may therefore be differentiated from nitrates from animal or domestic dejections using the $\delta^{15}N$. In addition, the trend of $\delta^{18}O$ and $\delta^{15}N$ observed in residual nitrate during denitrification makes these isotopes useful tracers for differentiating the dilution (mixing) process from denitrification, a very important process for reducing nitrate concentration in the groundwater.

3.3 - Application in the Po Valley and Friuli Veneto Plain

As already said, the SIAR isotopic mixing model returns the function of probability distribution of the presence of nitrate coming from each source in water (nitrate mass which can be attributed to a specific source/total mass of nitrate) and to obtain this one must establish:

- the number and type of sources preset in the mix;
- the isotopic signature values of the sources with their uncertainties;
- correction factors to be applied to the values of the isotopic delta of N and O of the sources;
- the values of $\delta^{15}N$ and $\delta^{18}O$ of the nitrates dissolved in the waters;
- impacts insisting on the monitoring area.

In general, when nitrates present in the waters can be potentially attributed to multiple sources, a system of equations is defined:

$$\delta^{15}N_{mix} = f_1 \times \left(\delta^{15}N_1 + \varepsilon_1\right) + f_2 \times \left(\delta^{15}N_2 + \varepsilon_2\right) + f_3 \times \left(\delta^{15}N_3 + \varepsilon_3\right) + f_4 \times \left(\delta^{15}N_4 + \varepsilon_4\right)$$

$$\delta^{18}O_{mix} = f_1 \times (\delta^{18}O_1 + \gamma_1) + f_2 \times (\delta^{18}O_2 + \gamma_2) + f_3 \times (\delta^{18}O_3 + \gamma_3) + f_4 \times (\delta^{18}O_4 + \gamma_4)$$

$$1 = f_1 + f_2 + f_3 + f_4$$

where:

- the subscripts 1, 2, 3, 4 refer to the various identified sources;
- f represent the percentage contribution of each source to the water sample, expressed as a fraction;
- $\delta^{15}N$ and $\delta^{18}O$ are the isotopic ratios of the sources;
- $\delta^{15}N_{mix}$ and $\delta^{18}O_{mix}$ are the isotopic ratios of the water samples;
- ε and γ represent the correction factors (i.e. the enrichments which the isotopes may undergo following the environmental processes they are involved in).

The sources identified for application of the isotopic mixing model are:
- groundwater: mineral fertilizers, manure, untreated domestic sewage and nitrogen naturally present in the soil;
- surface waters: mineral fertilizers, manure effluent, treated domestic effluent and nitrogen naturally present in the waters.

The majority of sources were isotopically characterized; in some cases, data taken from the scientific literature has been used (Figure 3.2).

Differently from what reported in international literature, in this study untreated domestic effluent (CivING) has been discriminated from treated domestic effluent (CivUSC); for inbound domestic sewage, isotopic footprint has shown a lower $\delta^{15}N$ than that of outbound effluent. In the case of untreated domestic wastewater (CivING), the isotopic ranges of

nitrogen and oxygen of the nitrate turned out to overlap with those of mineral fertilizers and those of nitrate naturally present in the soil.
The ranges defined for treated domestic wastewater (CivUSC) turned out to overlap with the intervals define for manure. Intervals for mineral fertilizers also turned out to overlap with those of nitrogen naturally present in surface waters.

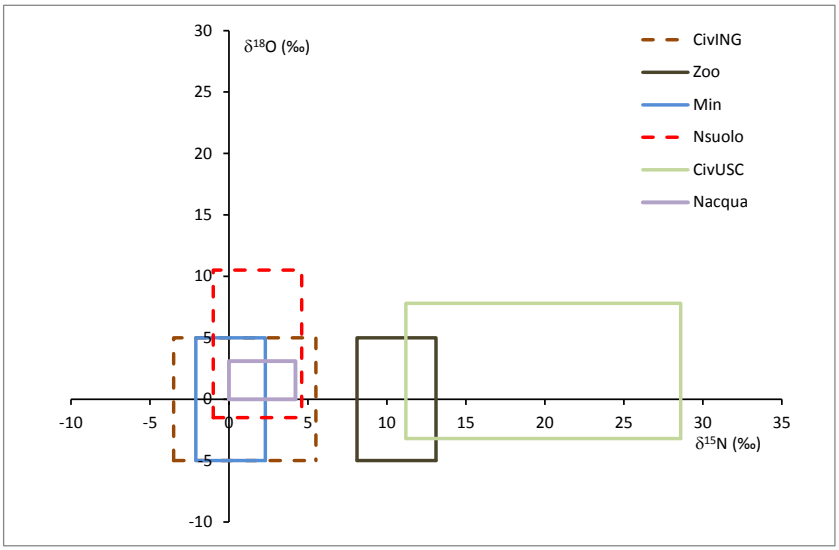

Figure 3.2: Isotopic ranges experimentally determined [ISPRA Report 217/2015] of $\delta^{15}N$ and of $\delta^{18}O$ concerning sources where:
CivING = domestic sewage coming into the purification plant ($\delta^{15}N$ has been determined experimentally on 8 samples plus two data taken from the literature (Roger, 2003) and concerning two septic tanks; $\delta^{18}O$ has been taken from the literature, (Xue, 2009 and reference mentioned there));
CivUSC = domestic sewage treated in purification plants ($\delta^{15}N$ and $\delta^{18}O$ have been determined experimentally on 8 samples);
ZOO = manure ($\delta^{15}N$ has been determined experimentally on 8 samples; $\delta^{18}O$ has been taken from the literature (Xue, 2009 and reference mentioned there)); MIN = mineral fertilizers ($\delta^{15}N$ and $\delta^{18}O$ have been taken from the literature, plus two samples determined experimentally for nitrogen);
Nsuolo=nitrogen naturally present in the soils ($\delta^{15}N$ and $\delta^{18}O$ have been taken from the literature);
Nacqua = nitrogen naturally present in the surface waters ($\delta^{15}N$ and $\delta^{18}O$ have been determined experimentally on 6 samples).

Concerning the oxygen isotope, data have been obtained experimentally both for domestic sources (going out of purification plants) and for nitrogen naturally present in the soils and in the waters. In the case of other sources characterized by a negligible content of the nitric form of nitrogen, the oxygen data have been calculated on the assumption that during the nitrification phase, two atoms of oxygen of the water (characterization isotopic interval -12‰ ÷ -8 ‰) and one of the air (23,5‰) are involved.

The correction factors (ε and γ) for mineral ($\delta^{15}N$ 5,0 ± 1,0 ‰) and manure ($\delta^{15}N$ 2,7 ± 0,2 ‰) sources to be applied for a more accurate determination of the contributions of each, have been determined on the basis of the investigations which were carried out and of results obtained on soil eluates characterized by application of the above sources. In the case of domestic sources and of nitrogen naturally present in the waters and in the soils, correction factors equal to zero have been applied. In the specific case of untreated domestic wastewater (CivING), it has been assumed that phenomena of volatilization of ammonia (the main process, which can lead to isotopic enrichment of the substrate) can be neglected because any leakage in the sewerage system rather than from the septic tanks takes place directly in the subsoil. In case of treated domestic wastewater (CivUSC), treatment processes generate further enrichment in the produced nitrate; in the case of nitrogen naturally present in surface waters and in the soils, the isotopic ratio attributed to the source already records the enrichment factors coming from the reactions of nitrate formation.

SIAR model uses correction factors to build an isotopic domain, which must contain the isotopic data of the water samples subjected to partitioning. Based on the definition of the enrichment factor, according to which nitrate is generally enriched compared to reactant nitrogen, the correction factors will be added algebraically to the isotopic ratio of the source and their uncertainties will be propagated. The revision of the corrected isotopic intervals of the sources is shown on Figure 3.3.

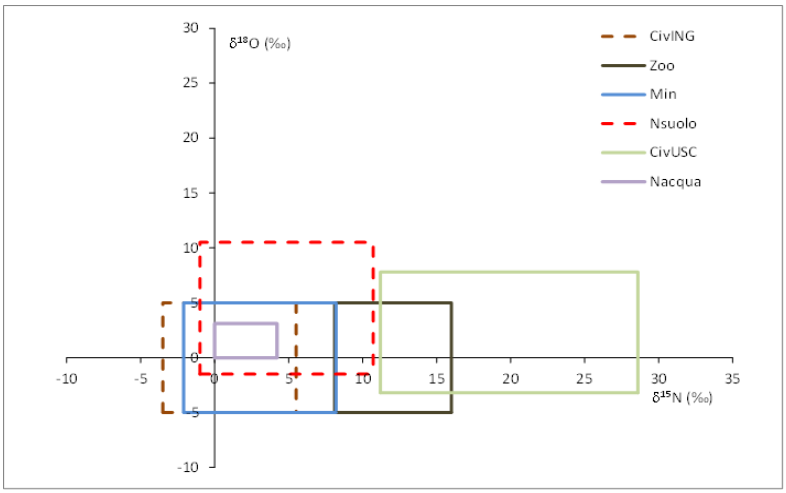

Figure 3.3: Isotopic ranges of $\delta^{15}N$ and of $\delta^{18}O$ for the sources re-determined taking into account the enrichment factors

Although in a probabilistic way and with a certain degree of uncertainty, the isotopic method has proved to be effective in estimating the proportional contribution of the relevant potential sources, which lead to the presence of nitrates in surface and underground waters, through the monitoring networks (essentially those supporting the WFD) in the regions of the Po Basin, of the Veneto Plain and of Friuli Venezia Giulia.

Results obtained in investigated areas have shown the presence of a background value of nitrates which can be attributed to the process of mineralization of the organic substance naturally present in the soils. In certain cases, they have also confirmed that a contribution of domestic origin is not to be neglected. It has also been possible to discriminate between the contribution of nitrates coming from the use of mineral fertilizers and those of manure origin. Finally, it has been possible to highlight the presence of many areas characterized by denitrification phenomena.

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4. TRACEABILITY OF FOOD AND AGRICULTURE PRODUCTS: THE CASE STUDY OF PGI (PROTECTED GEOGRAPHICAL INDICATION) RADICCHIO OF CHIOGGIA AND EXTRA-VIRGIN OLIVE OIL

4.1 - Introduction

Study of stable isotopes has recently become significant in the field of quality controls on some food and agriculture products (such as for example wine, oil, honey, fruit juices) and to monitor adulteration of mineral waters and sugars (Camin et al., 2016; Drivelos & Georgiou; Kelly et al., 2005). This analytical technique, being based on different isotopic ratios, allows one to recognize atoms or molecules, present in foodstuffs, having the same chemical structure but originating from different raw materials or raw materials which have been processed differently, for example by biological or industrial synthesis (Table 4.1).

Table 4.1: Some examples of practical application of isotopic analysis in the food and agriculture field

• *Recognition of natural flavoring agents (e.g. vanillin) from synthetic ones;*
• *Identification of the acetic acid coming from natural fermentation and of the industrially obtained one;*
• *Distinction between wild and farmed salmon;*
• *Identification of the addition of sugar other than that naturally present in various foodstuffs (honey, wine);*
• *Recognition of watered down milk;*
• *Evaluation of the geographical origin and type of food (denomination of controlled origin or DOC, protected designation of origin or PDO, protected geographical indication or PGI) and organic certifications.*

The ease of isotopic fractionation, due to the significant difference of mass among the investigated isotopes, is at the same time the strength and the limit of isotopic analysis applied to traceability of foodstuffs. In fact, the isotopic technique greatly facilitates recognition for anti-adulteration controls.

To obtain the exact identification of a product, with reference to its geographical origin, the collection of extensive data is needed. These data must take into account every possible natural variation (season, climate,

etc.) and artificial variations such as the addition of compounds characterized by different isotopic distribution (for example, fertilizing compounds and preservatives). These compounds, when introduced into the production cycle, can determine data variability that makes it difficult to precise geographical location of the product.

For all these reasons, isotopic analysis, aimed at establishing the origin of most food and agriculture products, is still in the experimental phase, although in other fields it has become a standard technique in production chains control procedures.

4.2 - Study of PGI (Protected Geographical Indication) radicchio of Chioggia

The ISPRA laboratory performed a preliminary investigation to identify the geographical origin of various *cultivars* of radicchio salad grown in different areas of Veneto. The samples, taken from Azienda Agricola di Chioggia, were provided by the Consortium for Protection of the PGI radicchio of Chioggia.

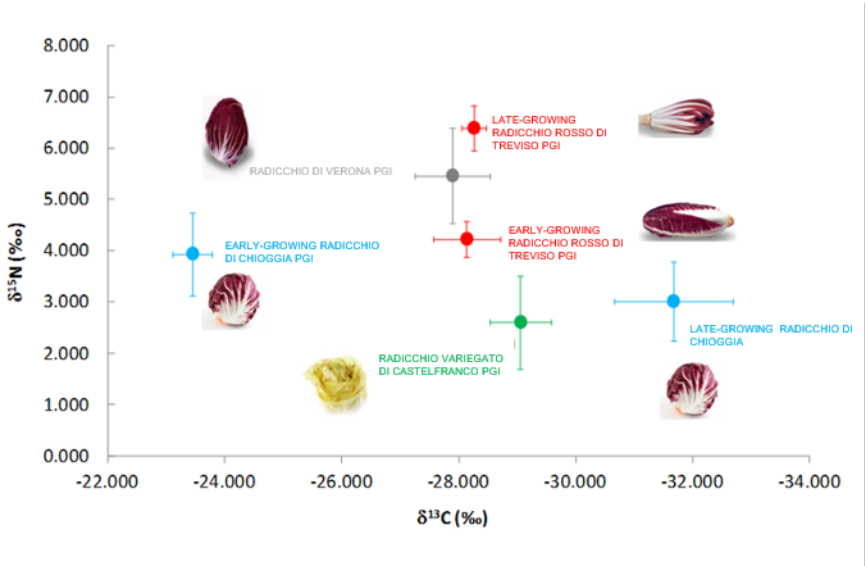

Figure 4.1: Isotopic values of carbon, on the x-axis ($\delta^{13}C$ (‰) and of nitrogen, on the y-axis ($\delta^{15}N$ (‰) of different cultivars of Veneto radicchio being analyzed (Radicchio di Chioggia PGI, Radicchio rosso di Treviso PGI, Radicchio di Verona PGI, Radicchio variegato di Castelfranco PGI)

As can be seen from Figure 4.1 the different *cultivars* of radicchio have different values for nitrogen and carbon isotopes, both for different areas of production and for different periods of harvesting.

From these preliminary results, one may suppose that isotopic analysis can be a valid tool for confirming the origin of analyzed samples, giving the right recognition to protection of the products, for example PGI radicchio of Chioggia, thus preventing food fraud, especially frequent in packaged products.

4.3 - Study on Extra Virgin Olive Oil

The ISPRA laboratory also carried out a preliminary isotopic investigation on extra virgin olive oil to evaluate its possible traceability. Figure 4.2 provides the first analytical results, showing the values of the stable isotopes of carbon associated with different *cultivars* of extra virgin olive oil. The data obtained show how some varieties can be distinguished geographically on the basis of significantly different isotopic values. However, other varieties present isotopic ratio of carbon similar to each other, thus making discrimination difficult, especially when different *cultivars* are mixed together to obtain the final product. Use of other stable isotopes (for example hydrogen, sulfur) could be decisive for improving interpretation and hence traceability.

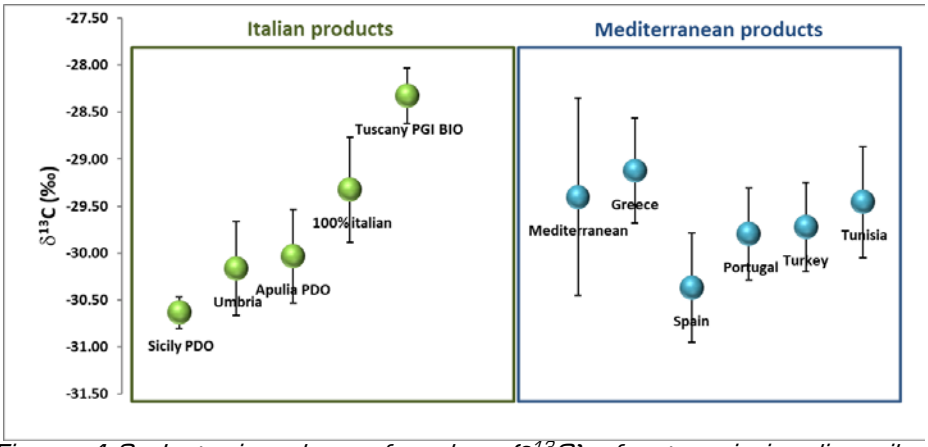

Figure 4.2: Isotopic values of carbon ($\delta^{13}C$) of extra virgin olive oil of different geographical origin.

Further statistical studies and investigations to extend the dataset may be needed to evaluate possible differences due to the location or season, or due to the substrate of harvesting (fertilizers employed, whether organic or inorganic) which might play an important role in modulating isotopic ratios.

This technique of analysis plays a strategic role in this special food and agriculture field as a tool for validating the regulation.

4.4 - References

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5. APPLICATION OF STABLE ISOTOPES TO CHARACTERIZATION AND STUDY OF THE ORIGIN OF ORGANIC MATTER, OPERATION AND DYNAMICS OF FOOD WEBS AND BIOACCUMULATION OF POLLUTANTS IN MARINE COASTAL AND TRANSITIONAL ENVIRONMENTS.

5.1 - Introduction

Different allochthonous and autochthonous sources of organic substance can play a key role in determining the biogeochemical cycle of carbon and, to a lesser extent, of nitrogen in aquatic environment. Stable isotopes ratios of carbon and nitrogen ($\delta^{13}C$ and $\delta^{15}N$) are widely used to determine the origin (autochthonous - allochthonous) and the destiny of organic matter, both in the water column and in the sediments (Fry and Sherr, 1984). Isotopic mixing models, applied to the values of $\delta^{13}C$ and to the atomic ratio between organic carbon and total nitrogen (C_{org}/N_{tot}) of the suspended particulate matter and of the sediments, have proved to be highly performing tools for qualitative and quantitative assessment of the contribution of different organic sources to the composition of organic substance present in coastal environments involved in freshwater discharge (Matson and Brinson, 1990; Thornton and McManus, 1994).

Analysis of stable isotopes is also widely applied in the field of ecology to describe flows of matter and food web relations inside a specific ecosystem. Food web relations (prey-predator) are considered to be among the main regulators of ecosystem dynamics and play an important role in mediating the responses of ecosystems to natural and anthropic disturbance such as fishing and degradation of habitats. Description of the interactions among different sections of a food web is fundamental for obtaining a picture of the state of the ecosystem, of effects due to anthropic impact and the properties of resistance and resilience of a certain ecosystem.

5.2 - Analysis of particulate and sedimentary organic matter in the area involved of the Po river delta

Different studies have shown that sedimentary organic material deposited in the coastal area in front of the Po delta is the result of a mixing of organic carbon of recent origin and humified (Tesi et al., 2007, Giani et al., 2009, Giani et al.,2010).

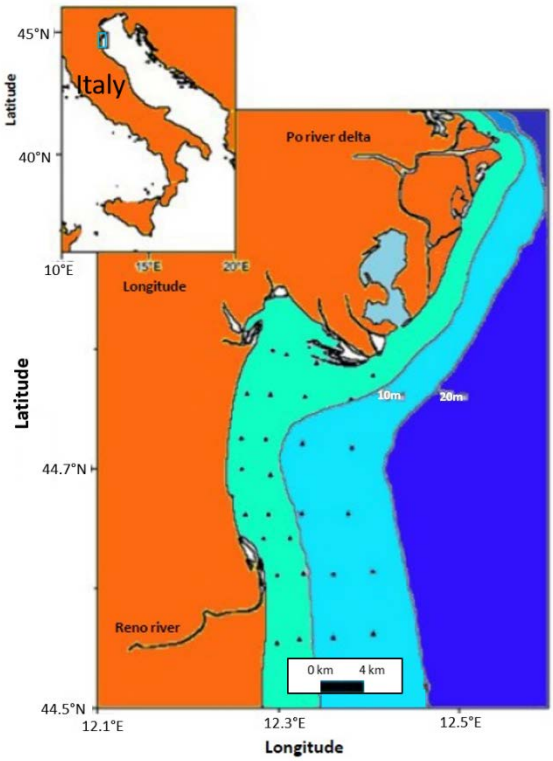


Figure 5.1: Sampling site

Giani et al. (2009) have shown how the fluvial particulate organic substance is featured by C_{org}/N_{tot} ratios very similar to those of Redfield, typical of phytoplankton, indicating an important contribution of primary fluvial production, especially in the period of low flow in the main tributaries of the area (Reno and Po). The organic substance of the marine sediments in front of the delta however show less negative values of $\delta^{13}C$ and higher molar ratios of C_{org}/N_{tot} compared to suspended particulate, suggesting either the presence of processes of degradation during sedimentation of particulate or deposit of an important component of organic substance coming from soil erosion (Figure 5.2).

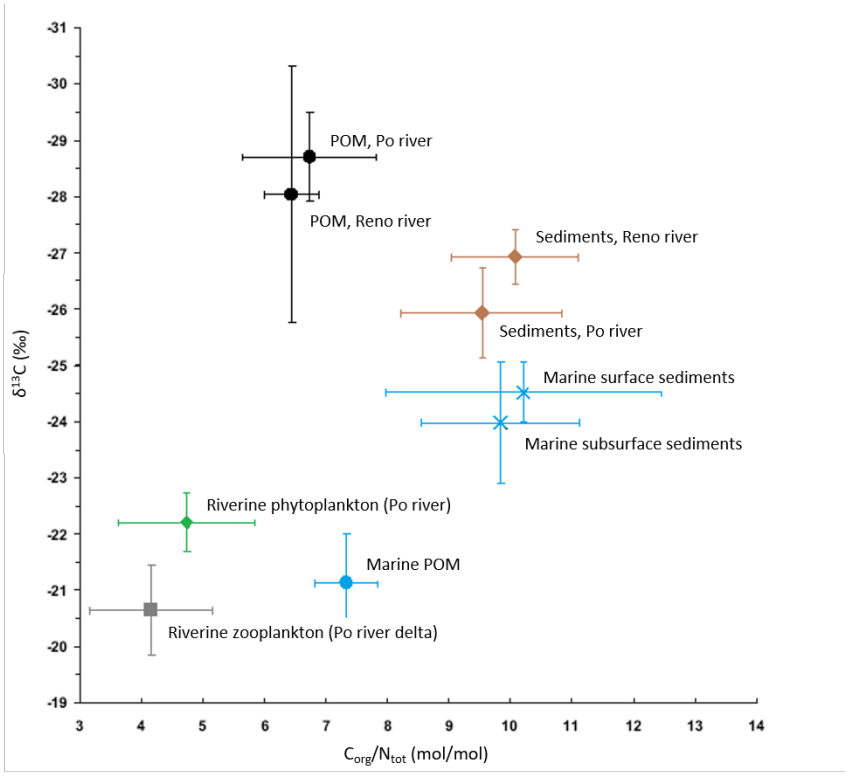


Figure 5.2: $\delta^{13}C$ vs molar ratio of C_{org}/N_{tot} for samples of surface and subsurface sediment, as well as for various matrices analyzed during the study

The isotopic mixing model applied to the surface sediments using the values of $\delta^{13}C$ and the C_{org}/N_{tot} ratio, have shown a high contribution of the organic matter of terrestrial origin compared to that of fluvial and marine origin. The greatest contribution of organic substance of terrestrial origin was observed parallel to the coastline, in an area with a bathymetry between 10 and 15 meters. On the contrary, the highest amount of marine fraction was found in offshore sediments and near Sacca di Goro lagoon, where primary production is notoriously higher. Finally, the greatest contribution of fluvial organic substance was determined for stations located near river mouths, involved in the discharge of freshwater (Figure 5.3).

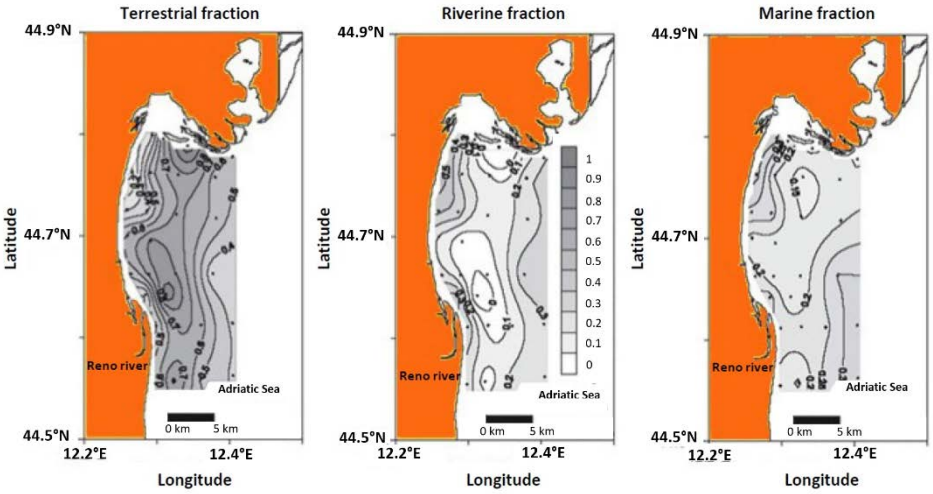


Figure 5.3: Percentage contribution of terrestrial, fluvial and marine fraction to the organic matter in surface sediments using an isotopic mixing model. Values are expressed as a fraction of the total organic substance, where the sum of three fractions is equal to 1.

The chemical and physical conditions typical of coastal environments involved in considerable fluvial discharges can influence and favor accumulation, degradation and transformation of organic substance through numerous chemical, physical and biological processes. Concerning this, a fundamental role is associated with humic substances, in particular humic and fulvic acids, which in these environments can represent as much as 80% of the *pool* of organic carbon present in marine sediments (Rashid, 1985; Calace et al., 2006). Some studies have shown how the concentrations of humic and fulvic acids[1] are inversely correlated to the distance from the coast, confirming the hypothesis that fluvial discharges may represent a source of both humified organic substances and of allochthonous organic substances of diverse origin.

Contribution of humic substances to the total organic carbon *pool* in the sediments of the north Adriatic involved in discharges of the Po river and the origin of the humic substances present have been studied in depth using isotopic and spectroscopic techniques and elementary analysis of the humic extracts (Giani et al., 2010). This has made it possible to provide a quantitative estimate of the contribution of humic acids of autochthonous and allochthonous origin. Humic carbon extracted from surface and subsurface sediments of the study area constitutes a significant percentage (about 17%) of the sedimentary organic matter. The chemical characterization of humic substances extracted has

[1] Humic and fulvic acids are natural substances which develop following recondensation of organic residues deriving from microbial biodegradation of fresh organic matter (plant or animal).

confirmed the hypothesis of a mixed origin: fluvial-terrestrial, probably associated with compounds derived from lignin, and marine, from degradation of plankton (Figure 5.4).

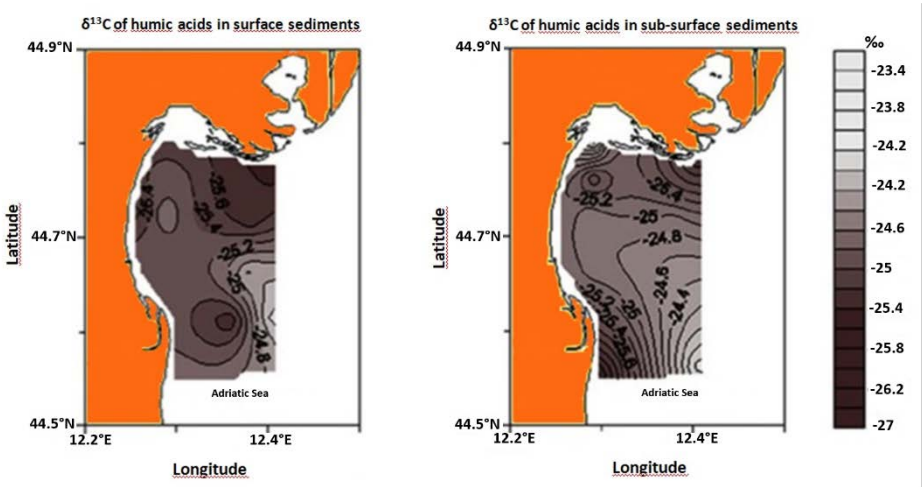


Figure 5.4: Map of the distribution of the carbon isotopic ratio ($\delta^{13}C$) values in humic acids extracted from surface and subsurface sediments

The positive correlation between the $\delta^{13}C$ of the humic acid and the percentage of marine organic matter in surface and subsurface sediments, suggests that a relevant part of the humic acid (approximately 50 %, according to the conservative mixing model; Giani et al, 2010) may derive from organic substances of marine origin.

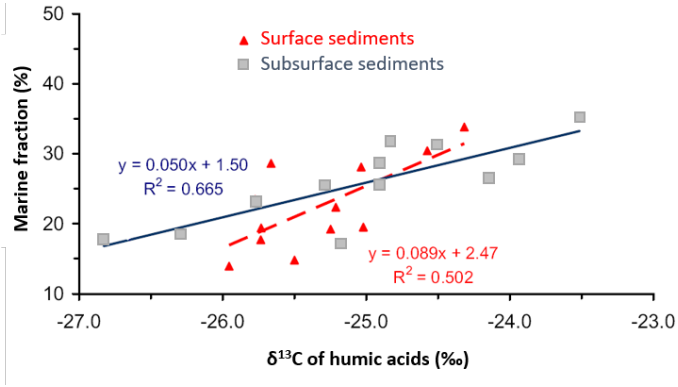


Figure 5.5: Correlation between the $\delta^{13}C$ of humic acid and the marine fraction (%) of the organic substance in surface and subsurface sediments

5.3 - Influence of the organic discharges of the Po delta as food sources for coastal macrobenthic communities

The discharge of organic matter and nutrients of terrestrial origin toward coastal marine areas can constitute a potential food reserve able to influence the diversity and operation of receiving ecosystems (the "outwelling" hypothesis). This supply can significantly influence productivity of coastal areas, for example increasing the biomass of fish resources (Dernaude et al. 2004) and hence support some important ecosystem services. Although different studies have confirmed the existence of food chain connections between fluvial/terrestrial and estuarine environments, showing the importance of terrestrial resources for estuarine communities, the extent of this phenomenon is still little studied in coastal areas influenced by rivers and especially in such complex systems as deltas. Analysis of stable isotopes of carbon and nitrogen is a useful tool for identifying food sources, their contribution to the diet of consumers and trophic levels of organisms (Fry 2006, Layman et al. 2007).

When the isotopic characteristics of potential organic sources (terrestrial plants, marine detritus, phytoplankton, etc.) are clearly distinguished, their relative contribution to the diet of consumers can be calculated using isotopic mixing models (e.g. SIAR; Parnell et al., 2010). Moreover, the isotopic data, if integrated with the biomass values and information on the structure of organisms' community, provide an opportunity to study the main flows of matter and assess the effect of multiple impacts (Quillien et al. 2016, Bongiorni et al. 2016).

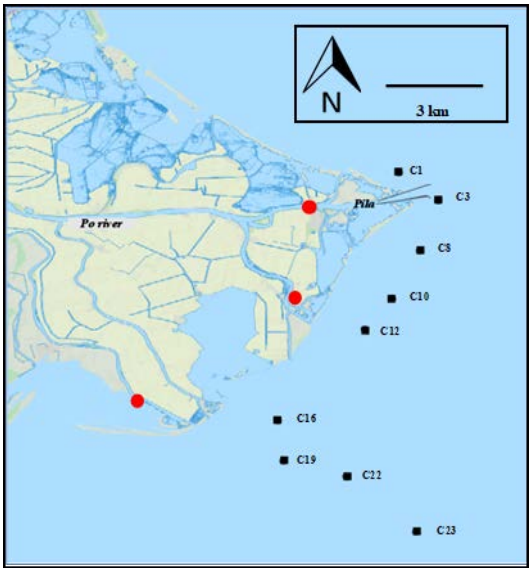


Figure 5.6: Map of the sampling stations in the prodelta area (black symbols) and inside the Po delta (red symbols).

In the context of collaboration between ISPRA and ISMAR-CNR of Venice, the hypothesis of *"outwelling"*, in relation to the fall and winter floods, was evaluated in the Po river delta-prodelta system through analysis of stable isotopes. Samples of macrofauna and potential food sources were collected in an area of the prodelta at increasing distances from the main mouth of the Po river along the *plume* (Figure 5.6). Organic sources of allochthonous

origin (terrigenous/fluvial) were also collected in areas inside the delta and the contribution of various sources (of both allochthonous and autochthonous origin) to the diet of primary consumers was estimated using isotopic mixing models.

Taking into account the trophic groups (suspensivores/filter-feeders, surface deposivores, subsurface deposivores), the values of $\delta^{13}C$ of suspensivore/filter-feeder organisms turned out to be more negative in northern stations near the Po di Pila (black circles, Figure 5.7) than in the stations in the central area (gray circles).

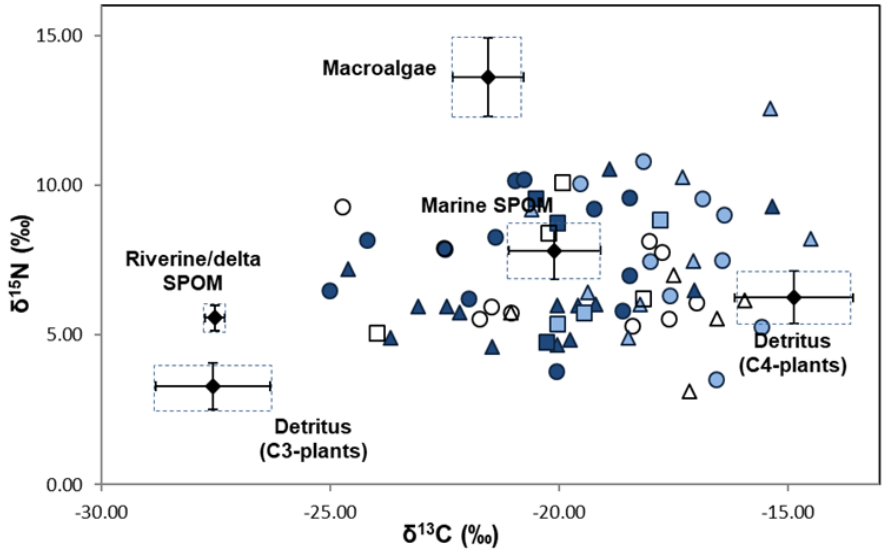


Figure 5.7: Bi-Plot of $\delta^{13}C$ and $\delta^{15}N$ (‰) values of primary organic sources corrected for isotopic fractionation taking place during food digestion and assimilation processes, and mean isotopic values in trophic groups of primary consumers: suspensivores/filter-feeders (circles), surface deposivores (triangles), subsurface deposivores (squares). The different colors indicate the station groups identified in the prodelta based on the analysis of the community structure and environmental variables: north (stations C1, C3, C8, dark blue), center (C10, C12, C16, light blue), south (C19, C22, C23, white)

A similar, though less evident, trend was found for surface deposivores, suggesting an important influence of fluvial and terrigenous discharge in the area in front of the Po di Pila. Isotopic mixing models applied to data of primary organic sources corrected for isotopic fractionation, which takes place during food digestion and assimilation processes, suggest that the main contribution to the diet of the various trophic groups of primary consumers is due to C4 type riparian plants. Furthermore, in accordance

with the observed values of $\delta^{13}C$, the contribution of the fluvial particulate and of C3 type vegetation to the diet of both suspensivores/filter-feeders and of surface deposivores, appears to diminish as the distance increases from the Po di Pila, while an increasing trend can be observed more southwards, towards the offshore stations. The results presented suggest that - especially during fall and autumn seasons with limited production, and in periods influenced by floods - the detritus originated by the vegetation of the salt marshes and of the levees, transported by the outflow of the Po river, could constitute an important food reserve for benthonic macrofauna, contributing to secondary production in the coastal areas. The data seem to suggest a close trophic connection between delta and coastal environments, further reinforcing the idea of strategies for safeguarding and integrated management of neighboring areas. The important terrigenous and fluvial contribution in terms of trophic resources is shown by the high biomasses found in the areas in front of the main mouth of the Po, as well as by the changes in communities composition of macrofauna observed along the *plume* (data not shown). This study also stresses the important relationship between granulometry, macrofauna structure and trophic flows in such highly hydrodynamic and variable environments as delta systems.

5.4 - Qualitative and quantitative characterization of anthropic sources of organic matter in the Venice lagoon.

The Venice lagoon is subject to many pressures, for example the industrial activities of Porto Marghera, the commercial and port activities in the lagoon area and the presence of the historic centers of Venice, Chioggia and minor towns. Over the years, these have given rise to several environmental problems, such as chemical pollution of water, sediments and biological matrices, resuspension of sediments and phenomena of dystrophy. In fact, the Venice lagoon is characterized by discharges of various nature, of both autochthonous and allochthonous origin (mainly fluvial, agricultural, industrial, urban, marine and atmospheric). Anthropic pressure sources for the Venice lagoon can be subdivided between punctual and diffuse sources.

The main punctual sources of pollution are the mouths of the main tributaries of the draining basin which collect industrial and domestic wastewater, as well as water washed away from the land and urban centers of the lagoon flats; from the discharge of the Campalto purification plant (the Fusina purification plant has recently been fitted with an outlet to the sea); from direct discharges by the companies of Porto Marghera, from the sewage discharges of the historic center of the cities of Venice and Chioggia and the lagoon islands.

Diffuse pollution sources are direct atmospheric deposits on the lagoon, pollution generated by navigation traffic, by surface runoff of the lagoon's historic centers and of the farmlands of the lagoon flats, by surface runoff of contaminated waters of the areas which have not yet been isolated of the Site of National Interest (SIN) of Porto Marghera and by direct erosion into the lagoon of contaminated materials which make up the embankments of the same SIN of Porto Marghera. In this context, analysis of the stable isotopes of carbon and nitrogen represent a promising investigation tool that allows to identify and characterize - also quantitatively - the different anthropic sources (urban, farming-fluvial, industrial) affecting the Venice Lagoon.

In the context of the MATTM (Ministry for the Environment)-ISPRA/2009 convention, between 2011 and 2015, ISPRA carried out several samplings aimed at isotopic characterization of the particulate and sedimentary organic matter in various lagoon and fluvial sites of the main tributaries of the flats of the Venice lagoon.

The samplings involved 28 of the ecological monitoring network stations, pursuant to the Water Framework Directive (WFD) (Directive 2000/60/EC), distributed throughout the lagoon area (Figure 5.8).

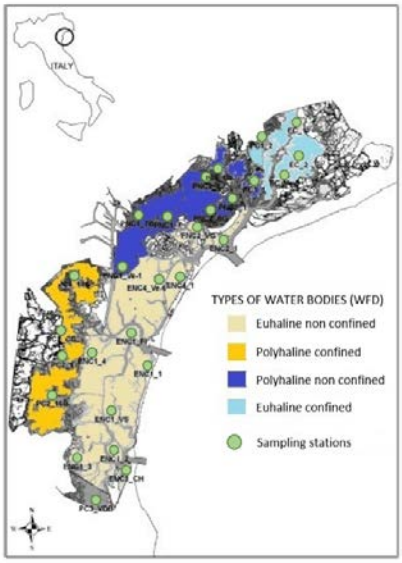
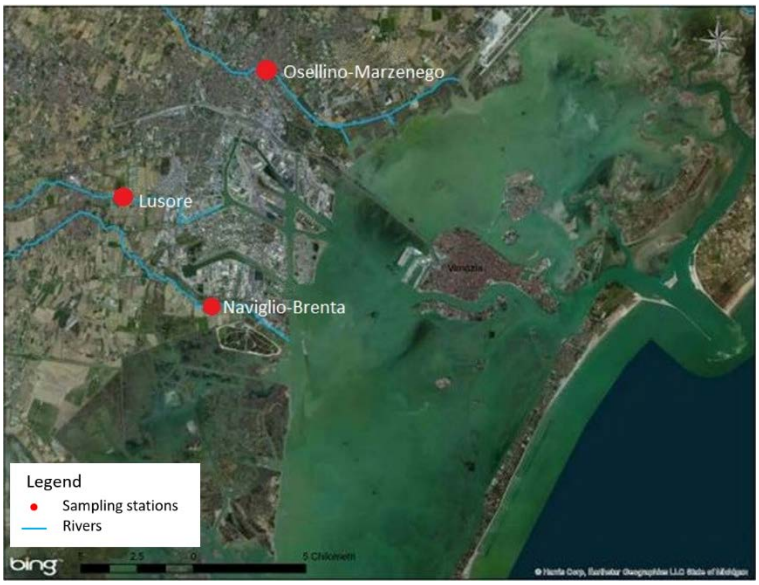


Figure 5.8: Location of the 28 lagoon sampling stations for ecological monitoring of chemical and physical parameters of water pursuant to the Water Framework Directive (WFD) (Directive 2000/60/EC)

A further 9 fluvial sites were sampled near the lagoon flats area in order to evaluate the isotopic signature of the suspended particulate coming from discharges of freshwater in the various areas of the lagoon. (Figure 5.9).

a)



b)

c)

Figure 5.9: Location of the 9 fluvial sampling stations.

Finally, a further 8 lagoon sites were sampled, in particular three stations in the industrial canals of Porto Marghera near industries of various manufacturing sectors (stations PM1, PM2, PM3), two stations in canals inside the city of Venice with free urban discharges (Celestia, Murano) and two stations in the shallows of the central lagoon chosen for being the farthest from the different possible sources (stations VS, FI). The last site was located near the outlet of a purification plant, which handles the wastewater of the city of Chioggia (Figure 5.10).

Figure 5.10: Location of the lagoon sampling stations with detail of the stations situated near the industrial canals and in the city center of Venice.

The surveys included sampling of water and sediments for the determination of $\delta^{13}C$ and $\delta^{15}N$ isotopic ratios and of a series of supporting parameters (concentration of nutrients and of dissolved and particulate organic matter) to provide better interpretation of the isotopic data.

This study together with the results reported in Berto *et al.* (2013) made it possible to implement the dataset of isotopic values of carbon and nitrogen of lagoon particulate, to identify the reference values of the main sources of particulate organic matter entering the Venice lagoon, and finally to understand their temporal variability. The information thus obtained was processed in an IsoSource mixing model (Phillips and Gregg, 2003). Table 5.1 reports the reference values of the two analytes considered for each source chosen as *end-member source* in order to calculate the mixing.

Table 5.1: Values of $\delta^{13}C$ and of POC/TPN used as benchmark for the sources used in calculating the mixing by IsoSource

Source	*$\delta^{13}C$*	*POC/TPN*	*Reference*
Untreated wastewater	*-21.9*	*1.2*	*Berto et al., 2013*
Treated wastewater	*-24.9*	*4.5*	*Berto et al., 2013*
Fluvial POM	*-26.3*	*12.1*	*River samplings*
Suspended Organic Material (SOM)	*-23.5*	*10.2*	*Samplings of stations VS and FI*
Marine POM	*-20.2*	*7.4*	*Diga Station Samplings*

It is important to note that macrophytes, though representing an important contribution of organic matter in the lagoon environment, were not used as *end-members* to calculate the mixing. The hypothesis underlying this choice consists in the belief that the macrophyte component contributes significantly to organic particulate only when it accumulates in the sediment at the end of its life cycle and is subject to sedimentary resuspension in particular conditions of re-movement due to either anthropic or natural phenomena. Concerning this, the station FI and the station VS often presented thick microalgae coating during the sampling campaigns and were therefore chosen as *end-member* stations for sedimentary resuspension, to which also the biological component excluded from the calculation is associated. The distribution of sources and values of the lagoon stations sampled is shown in Figure 5.11 as the plot $\delta^{13}C$ *vs* C_{org}/N_{tot}.

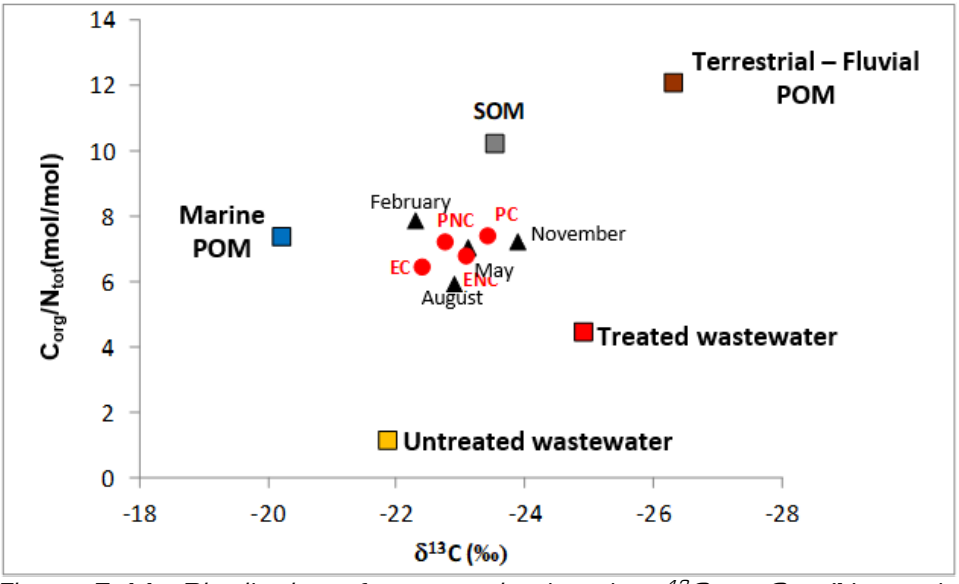


Figure 5.11: Distribution of sources in the plot $\delta^{13}C$ vs C_{org}/N_{tot} and positioning of the lagoon data obtained during monitoring performed from May 2011 to February 2012 by month and by type of water body

The chart clearly shows how the values obtained in the lagoon stations are positioned inside the space drawn by the various sources, confirming the fact that, while each station presents the prevalence of a specific source (the reason behind the initial choice), the particulate is the result of the mixing of different sources.

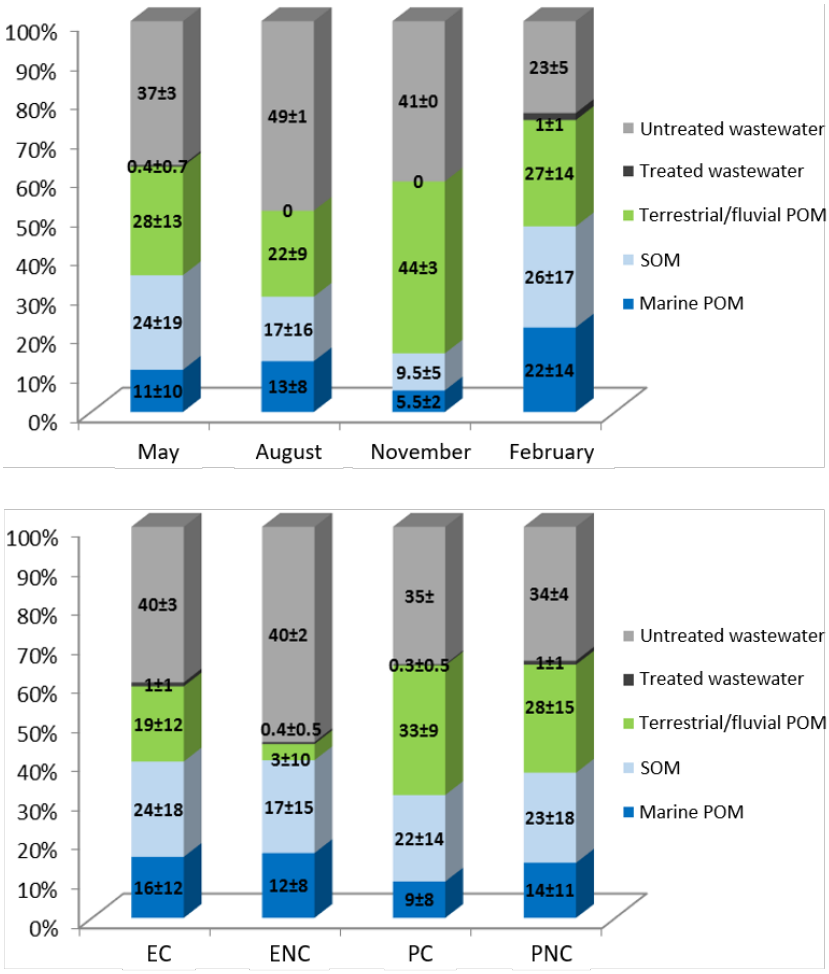


Figure 5.12: Results of the mixing model obtained during monitoring carried out from May 2011 to February 2012 mediated by month and by type of water body

The results obtained (Figure 5.12) show the prevalence of the contribution of untreated wastewater in all kinds of water body, especially in euhaline water (EC and ENC). This is probably due to the turnover times of water masses, which, especially in the northern part of the lagoon, are higher and cause less dilution of discharges and greater traceability of the sources, in accordance with what reported in Berto et al. (2013). As expected, the contribution of the fluvial-terrestrial source presents the highest percentage in polyhaline water bodies, while the marine contribution is higher in euhalines. Conversely, the resuspended fraction results on average similar in all 4 types.

The results obtained in this work, considering the complexity of the topic, call for further study in order to better represent and understand the distribution and nature of the organic matter in the marine and transition ecosystem, which turns out to be connected to both the variability of the sources and to the chemical and biological processes which affect the investigation area.

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6. TRACEABILITY OF FISHING PRODUCTS BY MEANS OF STABLE ISOTOPES

6.1 - Introduction

Fishing and aquaculture represent an important source of food with high value in terms of nutrition, income and employment. Since earliest times, fish have played a primary role in human nutrition. Awareness of the importance of fish products in the diet has increased considerably also thanks to the scientific mediation of physicians and nutritionists. Currently, the contribution of fish products to the diet in developed nations, but also in developing countries, is remarkably high. All fish products, besides providing proteins with a high biological value, balanced in their essential amino acid composition, feature a particular composition of fats, which makes them peculiar respect to the meat of terrestrial animals.

Each fish species has its own chemical and nutritional composition, which undergoes more or less marked changes during the year, depending on the fishing place, the season, but especially the reproduction period, which can change the nutrient composition. To take a few examples: sardines and mackerel have a higher variation in fat during the year than anchovy, hake or other more commonly consumed fish species. For an aquaculture product, different farming strategies - extensive, intensive in cages in the sea or in a tank, semi-intensive - offer opportunities for controlling the quality of the fish produced. Besides genetic factors, several environmental factors (temperature, salinity, pH, oxygenation, etc.), different kinds of nutrition (composition and manner), density of fish in tanks or cages, slaughtering mode and later handling and preservation treatment can influence, on various levels, the specific organoleptic features (color, flavor, etc.) of the different species and, within certain limits, the composition of the fish body, especially the lipid fraction, thus conditioning their nutritional value. In addition, the level of contamination of the aquatic environment and the use of veterinary products affects the safety of use of the product.

The increasing demand for information on the chemical and nutritional composition and origin of fish species does not come only from consumers, but also from producers: fishing and aquaculture operators, manufacturers who transform products for nutritional labeling and mass distribution demand increasing information about their product. In this context, with the creation of the Single European Market, quality, food safety and consumer information are referred to as fundamental themes of the European community farming policy in the White Paper on Food Safety of the European Community Commission (2000). In the same way, for fish products, the Code of Conduct for Responsible Fisheries and Aquaculture (FAO, 1995) laid down international principles and rules of

conduct to guarantee responsible fishing and aquaculture practices, ensuring product quality through safeguarding the environment and human health.

To do so, it was necessary to control degradation and preservability processes, considering risks related to consumption of fish products contaminated either biologically (bacteria, virus, algal toxins, parasites, etc.) or chemically (heavy metals, mercury, lead, cadmium, polychlorinated biphenyl, dioxins, etc.). Improper handling and storage of the product, from fishing to retail selling and home preservation, can negatively affect the quality and safety of use of the product.

The concept of quality referred only to the product, evolved to the concept of total quality applied to the whole production chain. Optimizing and hence controlling production procedures is the only way to guarantee consumers safety and total quality of the product, which becomes a logical consequence of the validity of the process.

Globalization of markets has led to the need for certainty concerning the origin of fish products, their traceability and hygienic quality, but also for having increasing information concerning their total quality (Håstein et al., 2001). Here, isotopic techniques can provide valid analytical support for guaranteeing the quality of the fish product in terms of distinguishing wild and farmed product, of geographical origin, of processing and storage control (EC Reg. 2065/2001) and finally of the potential bioaccumulation of contaminants (for example mercury, dioxins and organotin compounds) (Camin et al., 2016). However, the ease of fractionation, due to the significant difference of mass between isotopes, is both the strength and the limit of isotopic analysis applied to food traceability. In terms of anti-adulteration controls, this enormously facilitates the recognition method, but in terms of geographical origin, there obviously exists a natural variability of data, which makes it difficult to have precise references.

For this reason, the creation of databases that make it possible to obtain robust reference values is fundamental, also considering all the sources of variability, which could affect and determine the shift of isotopic values.

6.2 - Controlling freezing/defrosting of common cuttlefish (*Sepia officinalis*)

The degenerative processes interesting the meat of fish products after death, like those of any other animal, are mainly due to the action of aerobic and anaerobic microorganisms, and only minimally to tissue enzymes. Application of low temperatures acts on these two factors, slowing down their action and at times blocking them. It should be specified that cold does not have a sanitizing effect: it does not kill the

microorganisms, which stay alive and lively and begin to multiply again as soon as the temperature rises. The freezing process must therefore be immediate, and the cold chain must be kept constantly. Frozen fish is well preserved for more than eighteen months. Correctly frozen fish products keep their nutritional and organoleptic characteristics unaltered, like fresh products. Indeed, when fish products are frozen on board of factory ships, their nutritional features are fixed in a "very fresh" state, and their quality is superior to that of others sold fresh. Application of the so-called cold chain in fish products, at every phase from production to distribution and preservation until sale, is fundamental to ensure a fresh and healthy product. Currently, the rules to be observed for keeping the cold chain are set out in Legislative Decree No. 110 of January 27, 1992, to which distributors and sellers of refrigerated and frozen food must comply. It is fundamental to make sure that transfer of refrigerated or frozen products is undertaken without any break. Storage time of the fish and the content of CO_2 in the packaging are the two main factors, which condition the effect of the temperature on the formation of histamine, the main source of temperature dependent nutritional alarm regarding fish. In this case study, samples of *Sepia officinalis,* of Atlantic origin, both fresh and frozen, defrosted or left at a temperature of 4°C for one week ("degraded product") were analyzed and values of $\delta^{13}C$ *vs* $\delta^{15}N$ compared. The samples were taken from some wholesale companies distributing fish products, also with the help of the Harbormaster's Office of Chioggia. The results showed a variation of the stable isotopes values more evident for carbon (values of $\delta^{13}C$ more negative for the frozen product, (Fig. 6.1).

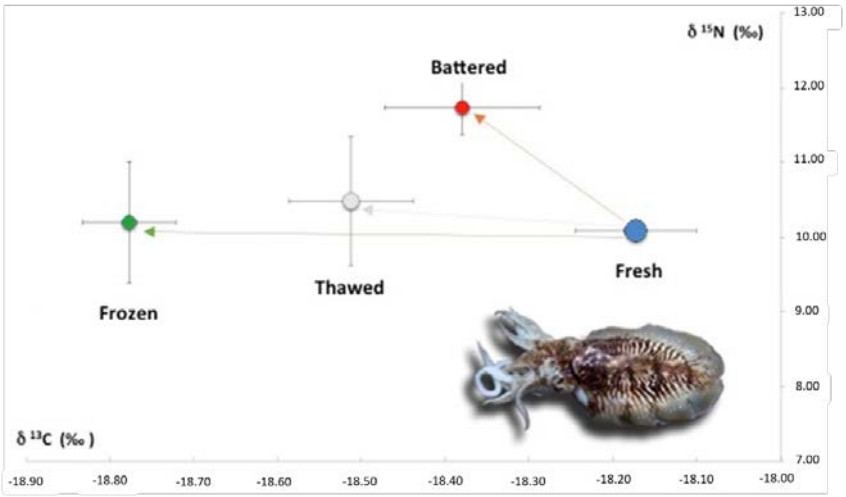


Figure 6.1: Isotopic values of $\delta^{13}C$ vs $\delta^{15}N$ in samples of Sepia officinalis fresh, frozen, defrosted and left at a temperature of 4°C for one week ("degraded product")

This result agrees with previous studies, which compared frozen samples against fresh samples (Jesus et al. 2015 and authors mentioned there). This evidence can be justified either by cells rupture with consequent release of cytoplasm during the freezing process, or by a possible microbial degradation in case of non-frozen products. Probably the isotopic variation between the frozen and the defrosted product (with a shift toward less negative values) could be associated with processes of physical/thermodynamic fractionation, which would require further study. What is certainly interesting is the variation, which appears in both stable isotopes between the fresh product and the one defined as "degraded". This difference, probably due to isotopic fractionation following microbial degradation (and the beginning of enzymatic metabolic processes) opens up interesting possibilities concerning the use of stable isotopes to control possible fraud in the fish production chain.

6.3 - Spatial variability and traceability of fishing products

Globalization of fish products has considerably increased the demand for effective and specific analytic traceability, in order to guarantee quality and safety of food as well as of consumers. The following sheets show some examples of applications useful for tracing both species and geographical origin by the use of stable isotopes of carbon and nitrogen. Combination with other analytical techniques (DNA barcoding and fatty acids analysis) is also of great interest.

Analysis of stable isotopes of carbon and nitrogen in samples of *Sepia officinalis, Sepia berthelot and Sepia pharaonis* of different origin revealed how the values of $\delta^{13}C$ provide excellent screening for tracing different species and capture areas. A significant difference can be found especially for samples of *S. officinalis* of Atlantic origin (Morocco) compared to those of Adriatic origin (Figure 6.2).

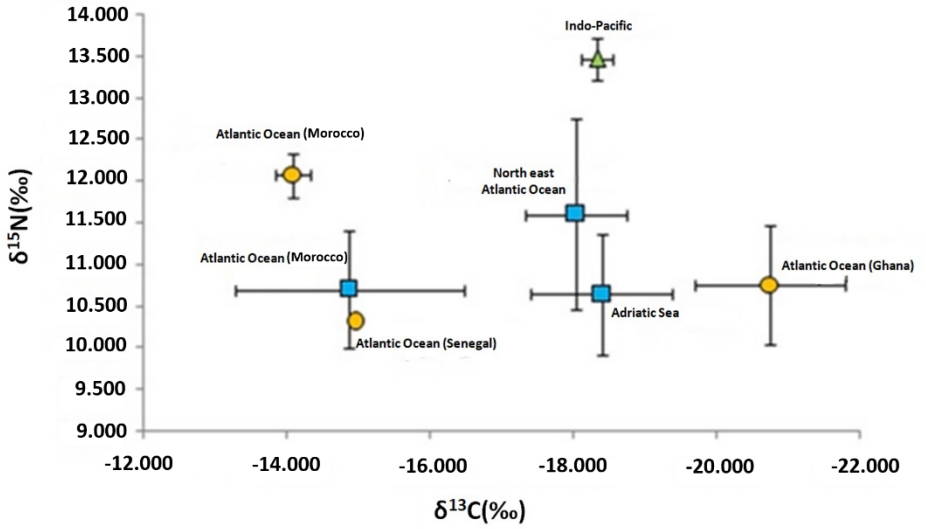


Figure 6.2: Isotopic values of $\delta^{13}C$ vs $\delta^{15}N$ in samples of Sepia officinalis, Sepia berthelot and Sepia pharaonis of different origin

The different latitude may have contributed to a variation of $\delta^{13}C$ values mainly as a function of the temperature of the water, which regulates solubility and hence concentration of dissolved CO_2 and the relevant isotopic fractionation. Besides this, the rate of phytoplankton growth, and hence that of the assimilated carbon source, differs with latitude (Takay et al., 2002 and authors mentioned there).

A further potential of stable isotopes involves the possibility of significantly discriminate between farmed products (F) and those deriving from natural stocks fishing (W). The data represented in Figure 6.3, concerning samples of fish of commercial interest (*Sparus aurata, Dicentrarchus labrax, Anguilla anguilla*) whether farmed or fished, show a differentiation of the isotopic ratio $\delta^{13}C$, a sign of different nutrition of the animals being analyzed. Farmed samples are characterized by more negative values of $\delta^{13}C$, reflecting the isotopic signal of the flour components contained in the feed.

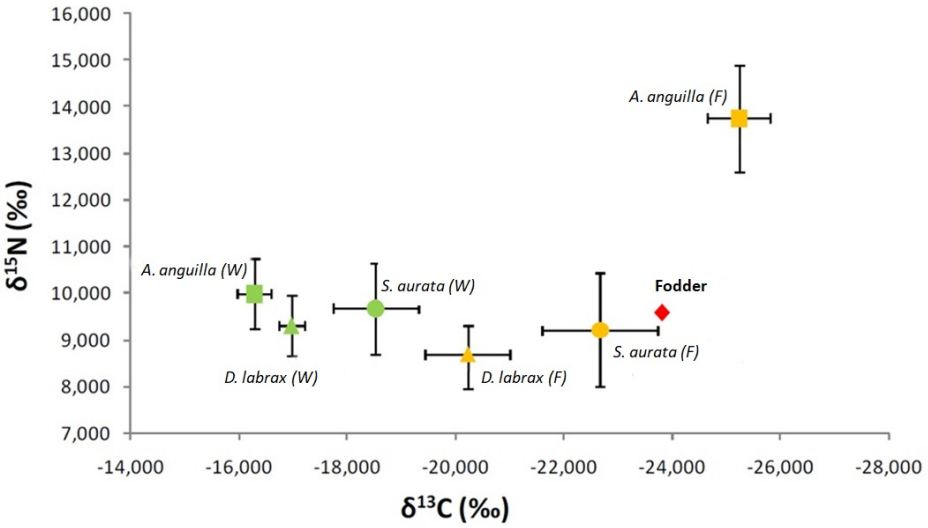


Figure 6.3: Isotopic values $\delta^{13}C$ vs $\delta^{15}N$ in samples of fish of commercial interest (Sparus aurata, Dicentrarchus labrax, Anguilla anguilla). Farmed product (F) and natural stock fishing product (W)

This approach proved to be of interest also when applied to bivalve mollusks, *Ruditapes philippinarum,* collected in various lagoons of the Po delta (Figure 6.4) in a preliminary study that involved ISPRA together with Istituto Zooprofilattico delle Venezie, Adria.

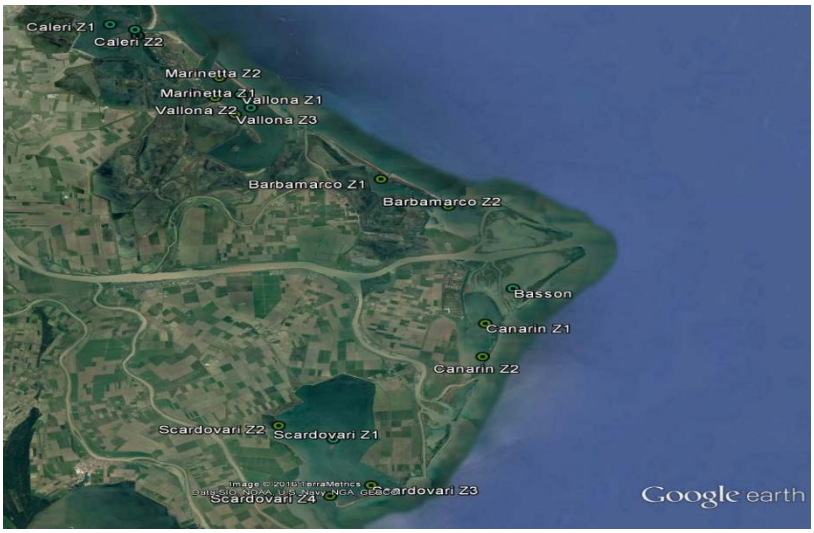


Figure 6.4: Map of sampling of bivalve mollusks, Ruditapes philippinarum, collected in various lagoons of the Po delta

Values of $\delta^{13}C$ determined in the analyzed clams were present in the range between -24.62 ‰ and -19.24 ‰ (-22.33 ± 1.32 ‰; mean ± std. dev.), these values were comparable to those reported for lagoon water particulate matter by Berto et al. (2013). $\delta^{15}N$, presenting a wide range of values between 5.33 ‰ and 11.25 ‰ (8.13 ± 1.23 ‰; mean ± std. dev.), is strongly influenced by biological processes associated with the different inputs both of nutrients and anthropic discharges which affect the area.

Figure 6.5 a, b shows the results of the isotopic analyses ($\delta^{13}C$, $\delta^{15}N$) in the samples from the different lagoons of the Po delta.

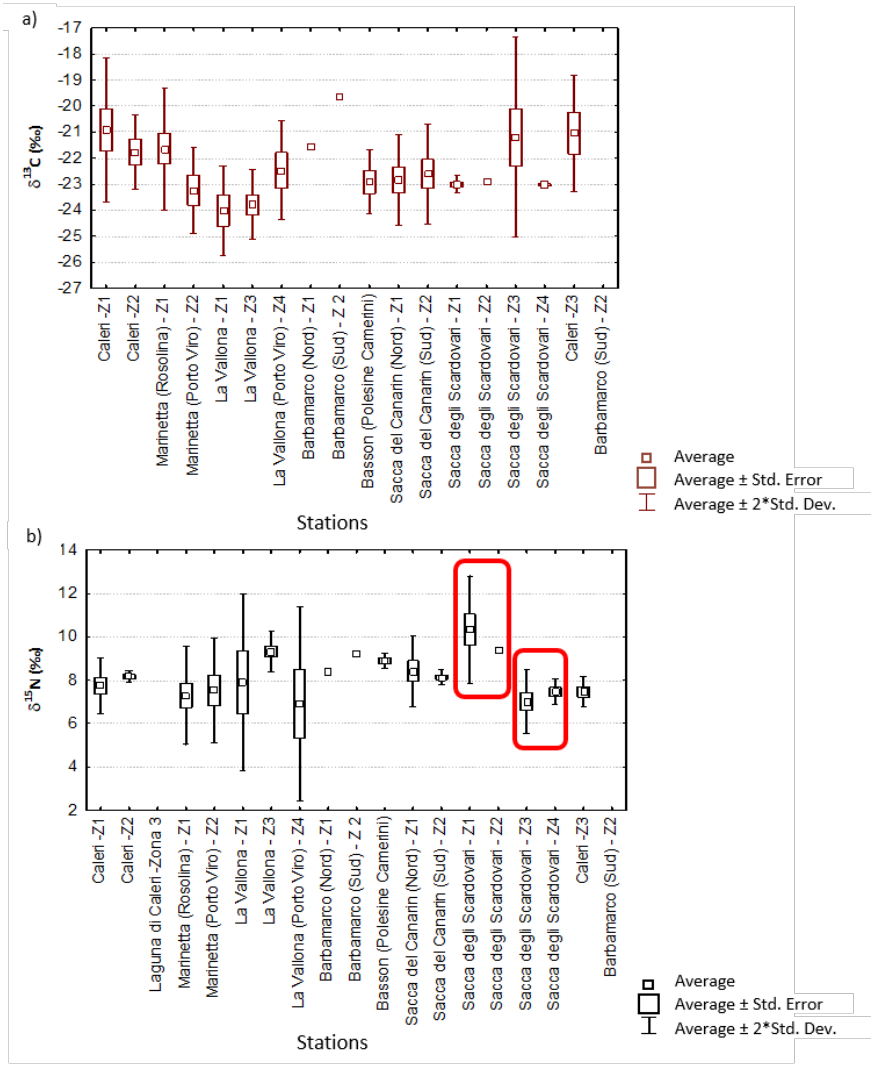
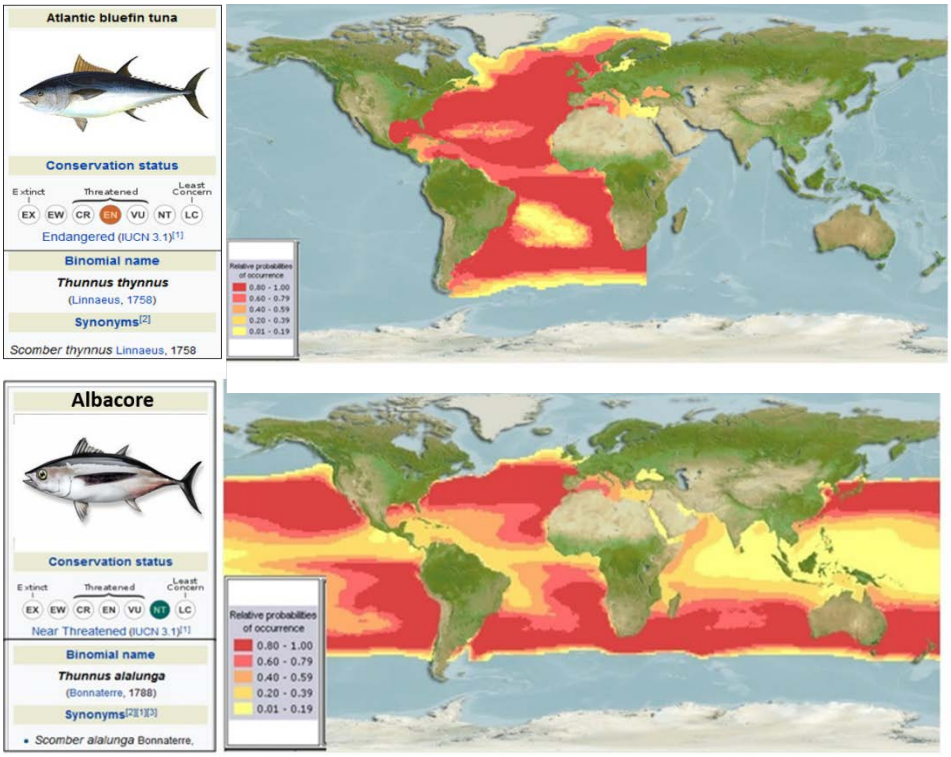


Figure 6.5: Box plots of isotopic values of carbon and nitrogen in samples of various lagoon stations of the Po delta.

The value of $\delta^{13}C$ is able to discriminate among the various lagoon areas, while $\delta^{15}N$ presents similar values in all investigated areas. A significant difference of values of $\delta^{15}N$ can be seen in the Sacca degli Scardovari, between zones 1 and 2, compared to zones 3 and 4, probably associated with the different origin of the nitrogen discharge in the area.

A further approach to traceability of fish products consists in integrating three different analytical techniques (DNA barcoding, stable isotopes of carbon and nitrogen and analysis of fatty acids). This application has been tested on epipelagic marine fish of the Scombridae family (*Thunnus alalunga, Thunnus thynnus, Auxis rochei, and Scomber scombrus).* The frames of Figure *6.6* show the species and maps of their native distribution *(http://www.aquamaps.org/)*



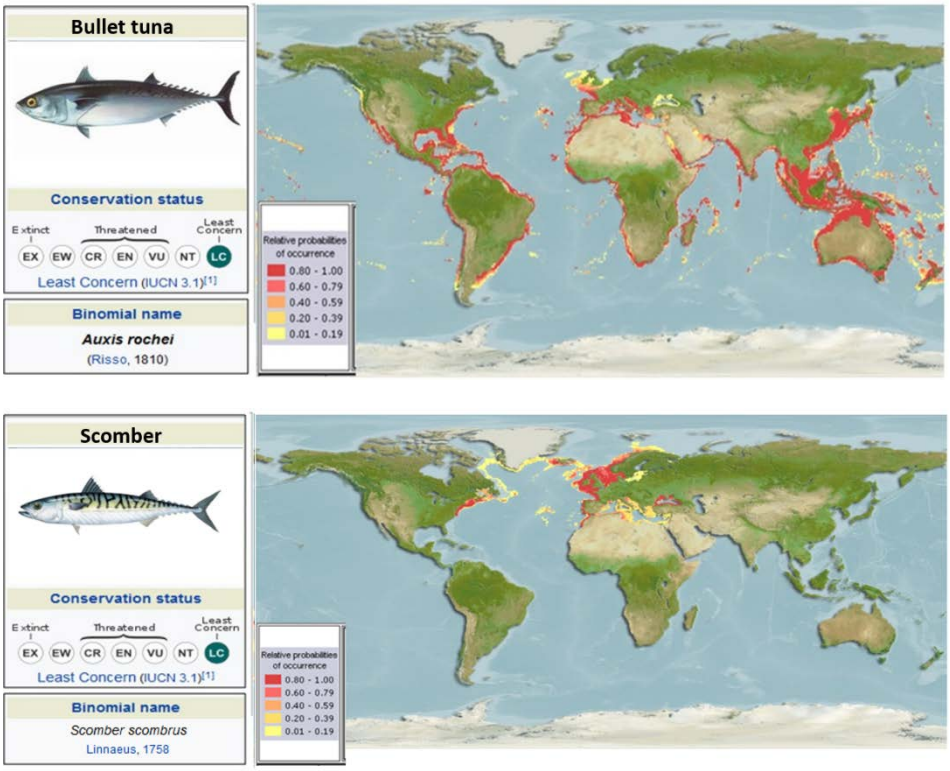


Figure 6.6: Analyzed species (Thunnus alalunga, Thunnus thynnus, Auxis rochei, Scomber scombrus) and maps of their native distribution (http://www.aquamaps.org/)

The study involved ISPRA, Istituto Zooprofilattico delle Venezie, Adria and the University of Ferrara.

Genetic analysis of DNA demonstrated some limits in discrimination between *T. alalunga* and *T. thynnus* since it only provides confirmation at genus level. This may be especially problematic concerning traceability, since fishing of bluefin tuna (*T. thynnus*) is regulated by a quota system according to EC regulation No. 302/2009 and later amendments thereto. However, the integrated analytical approach, which uses isotopes together with analysis of fatty acids tested in this study, has shown good discrimination of species (Figure 6.7 a, b) and a geographical and spatial separation between the two species of Thunnus (Figure 6.8). These results were obtained by Principal Component Analysis (PCA).

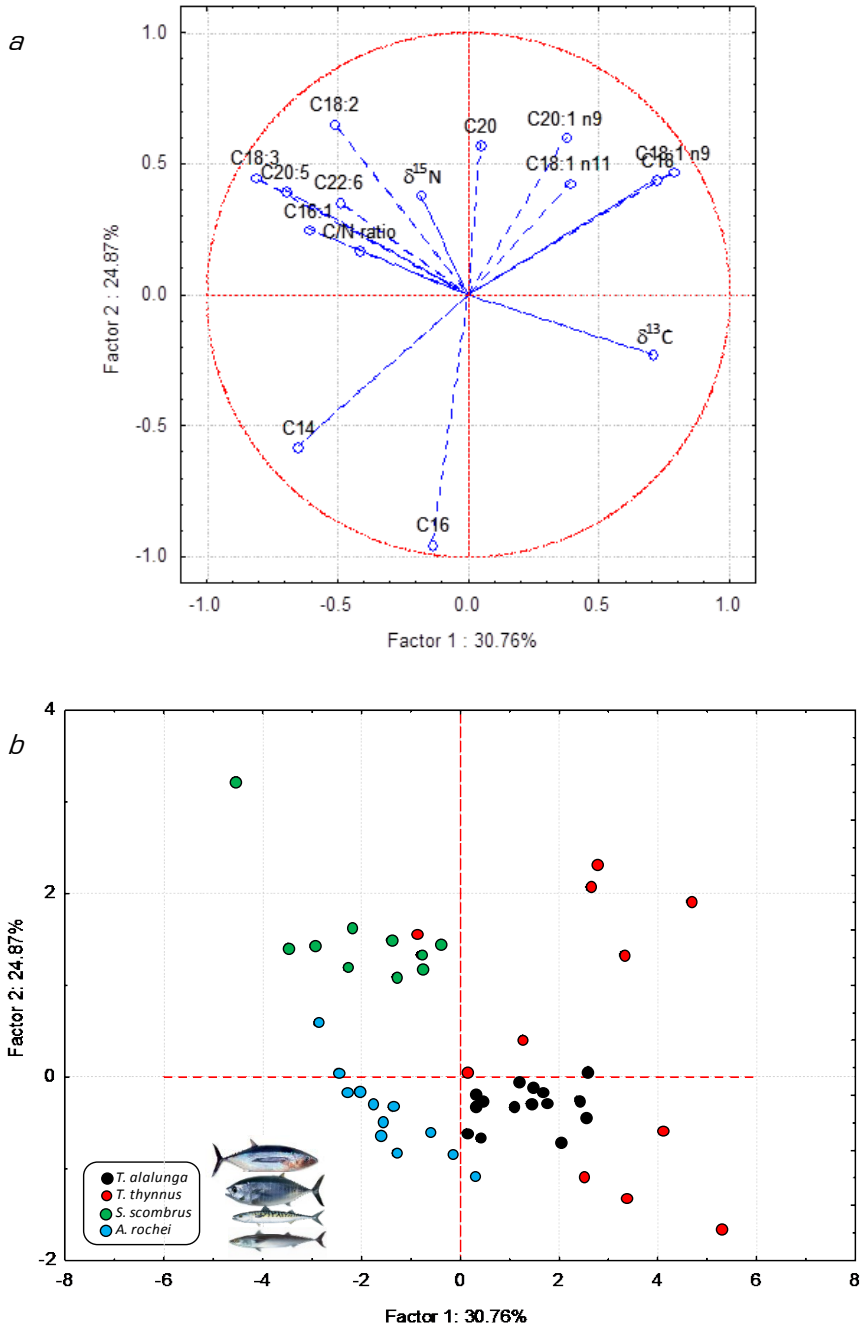


Figure 6.7: Results of the Principal Component Analysis (PCA): a) Loading plot shows the distribution of the characteristic variables compared to Factors 1 and 2; b) Score plot shows the distribution of the species analyzed in the two-dimensional plot Factor 1 vs Factor 2

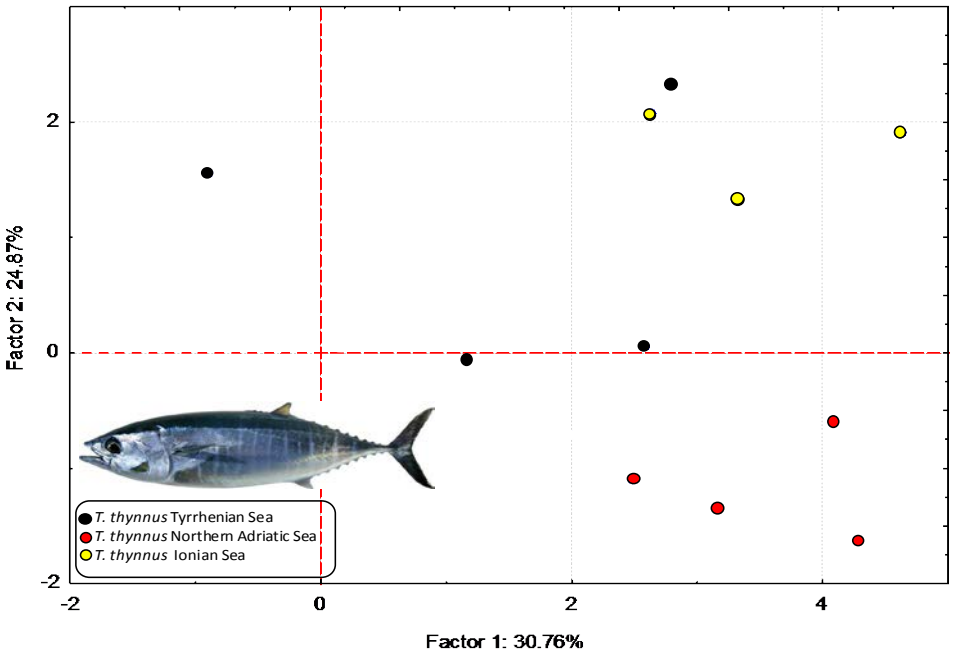


Figure 6.8: Results of the Principal Component Analysis (PCA) applied to samples of Thunnus tynnus coming from different geographical areas

6.4 - Study of bioaccumulation of contaminants in the north Adriatic food chain through analysis of stable isotopes

It has been proven that the ratio between nitrogen isotopes ($^{15}N/^{14}N$) in the tissues of a predator differs from that present in its prey, with a progressive enrichment estimated to be around 3.4 ± 1 ‰ between each trophic level (Vander Zanden & Rasmussen, 1999). Stable carbon isotopes may also be used to evaluate the trophic level in a food web (with an enrichment of about 1 ‰ between two consecutive trophic levels), however the isotopic signature of carbon is preferentially used to investigate the origin (or source) of carbon in the consumers diet(Darnaude, 2005) (Figure 6.9). In fact, it has been shown that primary terrestrial producers are characterized by more negative values of $\delta^{13}C$ compared to marine ones, with a progressive increase with the distance from the coast (Darnuade, 2005).

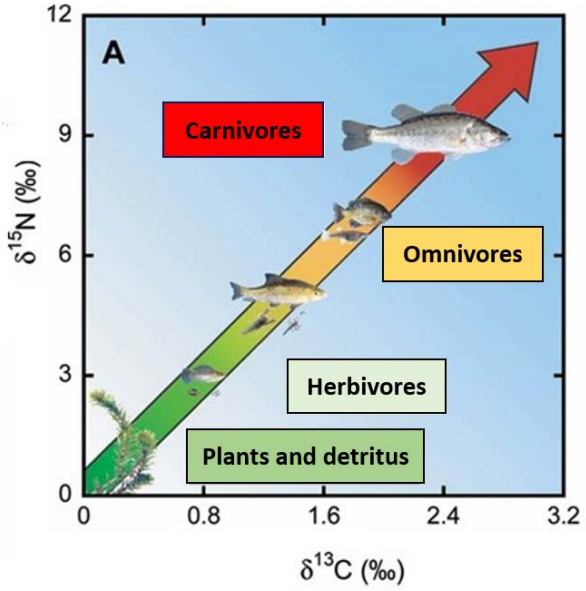


Figure 6.9: Isotopic values of $\delta^{13}C$ vs $\delta^{15}N$ in the marine food chain

Summing up, the regulatory of isotopic enrichments in ^{15}N and ^{13}C allows the use of $\delta^{15}N$ and $\delta^{13}C$ values as indicators respectively of the food chain level an organism belongs to and of the main source of primary carbon (Vander Zanden & Rasmussen, 1999).

Determination of the trophic level (TL)by measuring $\delta^{15}N$ is based on isotopic fractionation and on the relation which exists between isotopic ratios in the food source and in its consumer (Minagawa & Wada, 1984). The trophic level of an organism can be calculated according to the following equation (Vander Zanden et al., 1997):

$$LT\ consumers = 2 + (\delta^{15}N_{\ consumer/fish} - \delta^{15}N_{\ organism\ at\ the\ bottom\ of\ the\ food\ web}) / \Delta N$$

Where ΔN is equal to the mean rate of enrichment per trophic level in the food web taken into account (3.4 ‰) while $\delta^{15}N_{organism\ at\ the\ bottom\ of\ the\ food\ web}$ refers to organisms at the bottom of the food web as primary (phytoplankton) or secondary producers (zooplankton, bivalve mollusks).

Therefore, using information on the trophic level together with data on the concentration of chemical contaminants, it is possible to study bioaccumulation and biomagnification (Minagawa & Wada, 1984; France, 1995; Fuortibuoni et al., 2013) that is transfer of contaminants along the food chains through predation, with a consequent increase in concentration at the highest levels of the food chain.

Generally speaking, for substances subjected to biomagnification, critical concentrations have been reached for values of TL equal to 3/4 for freshwater fish and TL equal to 5 for marine ecosystems, with some exceptions for metabolized substances (polycyclic aromatic hydrocarbons), and for bioaccumulable ones (hexachlorobenzene, hexachlorobutadiene, mercury, dioxins and dioxin-like PCB, etc).

Following the increase in concentrations of contaminants (especially bioaccumulable ones) in aquatic ecosystems, Directive 2013/39/EU, amending Directive 2008/105/EC, introduced environmental quality standards (EQS) for biota for some classes of contaminants defined as priority substances (fluoranthene, polycyclic aromatic hydrocarbons and brominated diphenyl ethers, dicofol, perfluorooctanesulfonic acid and its derivatives, hexabromocyclododecane and heptachlore/heptachlore epoxide). For this purpose, the use of TL allows to define a specific $SQA_{biota,x}$ according to the trophic level and to the taxa through the following equation, which can be used for biota of both freshwater and marine pelagic water:

$$SQA_{biota,x} = SQA_{biota} \; / \; TMF^{(4 - TL(x))}$$

The TMF represents the trophic biomagnification factor of the investigated contaminants along the food chain and is calculated using the slope (b) of linear regression between the concentration of the contaminant (C_x) and TL (Fortibuoni et al., 2013):

$$C_x = a10^{bTL}$$

$$Log\,(C_x) = Log_{10}a + bTL$$

$$TMF = 10b$$

A first application of this approach was effected in the context of the ORGALT project (*Accumulation of organotin compounds in the food chain and in commercial fish species in the northern Adriatic 2008-2010*), with the main goal of evaluating the biomagnification of butyltin compounds (TBT, DBT and MBT) along the northern Adriatic food web. In particular, the study was referred to species of the greatest commercial interest exploited by fishing, considering a wide range of trophic levels, from primary producers to top predators. Organotin compounds (OTC), in particular tributyltin, have been widely used since the mid 1960s as biocides in anti-fouling paints. Due to their widespread use in several applications (industrial, agricultural, etc) and their specific chemical and physical characteristics, such pollutants are detected in every aquatic ecosystem, with higher concentrations in marine/coastal and lagoon environments (Berto & Boscolo Brusà, 2016). Currently, considering the dangerousness of these compounds for a wide

range of marine organisms, but also for human beings, they have been banned as biocides in anti-fouling paints (EC Regulation No. 782/2003) and included in the list of hazardous priority substances (Directive 2008/105/EC). Parallel to measurement of concentrations of OTC in tissues, the ratios of $\delta^{15}N$ and $\delta^{13}C$ have been determined, allowing evaluation of both the trophic level of the species and of the food origin, and consequently, of the contamination (Fortibuoni et al, 2013). In this work, 26 species have been analyzed, representative of different trophic levels, from plankton to top Adriatic predators. Isotopic analysis has been carried out on the samples of muscle and liver, after lipid extraction (Buchheister & Latour, 2010). The trophic level of the species has been estimated on the basis of $\delta^{15}N$ using zooplankton instead of phytoplankton as the level 2 primary consumer.

The main results of the work showed the following:

- Clear predominance of TBT compared to DBT and MBT
- A significant positive relation between concentrations of OTC and TL indicating a biomagnification factor of such compounds, with a factor of TMF>1 (falling between 3.88 and 4.62 in the liver and 2.45 in the muscle) (Figure 6.10)
- A negative–, not significant, correlation was found between $\delta^{13}C$ and the concentration of OTC. This suggests that, even though there may be a decrease in food exposure to BT with the increasing distance from the sources of coastal pollution, trophodynamic mechanisms are probably the most important factors in distribution of BT in the food web of the northern Adriatic.

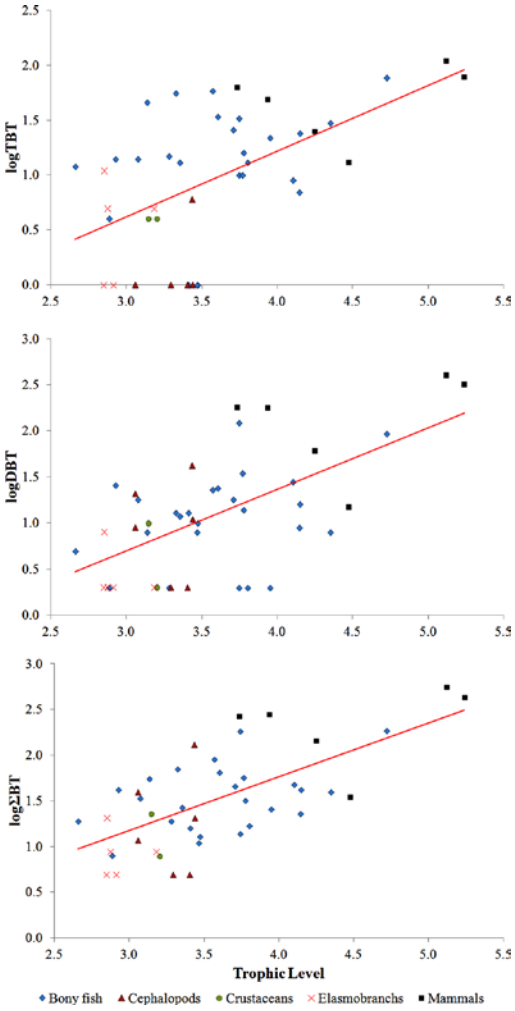


Figure 6.10: Correlations between concentrations of organotin compounds (sum of butyltin compounds - ∑BT, tributyltin- TBT and dibutyl tin- DBT) and the trophic level (TL), (from Fuortibuoni et al., 2013).

In order to explore to the best the potential offered by this technique, it is fundamental to extend as far as possible the isotopic data set by analyzing as many species as possible, of different origin, also considering any other factors which may potentially generate isotopic fractionation and hence variability of the species specific reference values (dimension classes, feeding habits, fishing methods, preservation methods).

Estimated future output of this study would be to determine the reference values for species and fishing zone that would allow the traceability of the origin of the fishing product. Further investigation is being planned to verify traceability of fishing strategies as well as of preservation methods. For

this purpose, it will be necessary to analyze fish samples of ascertained origin in order to expand the data set.

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7. USE OF STABLE ISOTOPES OF NITROGEN AND CARBON IN THE CONTEXT OF NATIONAL IMPLEMENTATION OF THE MARINE STRATEGY FRAMEWORK DIRECTIVE (MSFD)

7.1 - Introduction

Analysis of stable isotopes of carbon and nitrogen can find practical use also in the context of implementation of policies for protection and conservation of the marine environment. This is the case of the *Marine Strategy Framework Directive* (MSFD, 2008/56/CE). The purpose of this Directive is to achieve so-called "Good Environmental Status" for all the territorial waters of the European Union by 2020. Good Environmental Status means an environmental status where "marine waters preserve ecological diversity and vitality of the sea and where oceans are clean, healthy and productive" (MSFD, art. 3(5)).

Moreover, Good Environmental Status means that the conditions listed below have been achieved and maintained:

1) Use of the marine environment is sustainable, thus safeguarding the potential for use and activities of the present and future generations;
2) The ecosystems are fully functional and preserve their resilience to environmental change due to human activity;
3) Decline of biodiversity due to human activity is prevented and biodiversity is safeguarded;
4) Human activities, which introduce substances and energy into the marine environment, do not cause polluting effects.

The implementation path of the Directive called for: an initial assessment of the status of marine waters in 2012; determination of the good environmental status of marine waters and associated indicators; setting of environmental targets and monitoring programs; identification of measure programs (for a summary of the overall approach applied in Italy and of the legislative transposition, see Relazione sullo Stato dell'Ambiente 2016; MATTM, 2016).

Implementation of MSFD is carried out on the basis of 11 quality Descriptors associated with a series of criteria and indicators which have been defined Europe-wide in the context of the Decision of the Commission on criteria and methodological standards (2010/477/COM), recently integrated and reviewed on the basis of experiences gained during the first implementation phase of the Directive (2017/848/COM).

The 11 Quality Descriptors refer to a series of emerging components and properties of ecosystems or their features in terms of status or anthropic pressures, for example biodiversity (D1), non-indigenous species (D2), selective fishing of marine organisms (D3), food webs (D4), contaminants present in seafood (D9). Various criteria and indicators are associated with these descriptors the data are needed for assessment of Good Environmental Status, setting threshold values which make it possible to evaluate whether the target has been reached or not.

In particular, in the context of Descriptors regarding marine biodiversity and food webs, there are trend indicators concerning abundance and biomass of main (or most significant) functional groups, for example high productivity species or species at the top of the food web.

Especially for Descriptor 4 concerning marine food webs, the use of stable isotopes can find practical application, in order to define ecosystem indicators. In fact, this tool can provide considerable support for uniquely defining functional groups (characterizing the trophic level of the species and grouping them into uniform trophic groups) and assessing potential diet change determined by ontogenetic factors (growth).

7.2 - Monitoring plans and isotopic analyses

ISPRA, in the context of its functions of technical and scientific support to MATTM for implementing monitoring plans associated with MSFD, has developed a proposal for monitoring plans, which include the development of ecosystem indicators by application of stable isotopes of nitrogen and carbon. This proposal was later received, integrated and developed by the Work Groups set up by the MATTM, leading to definition of monitoring plans, which were later set out explicitly in the context of attachment II to the Ministry Decree of February 11, 2015 (Ministry Decree 2015).

According to the Monitoring Plan for definition of functional groups (sub-program 3.8), use of stable isotopes of nitrogen and carbon will be set out in three phases:

- a first phase of knowledge acquisition “aimed at defining the functional groups on the basis of the trophic level of the species (…) defining the organisms dimensions on which to carry out isotopic analyses for some key species and/or to fill knowledge gaps on trophic levels (…)”;
- a second phase calls for “actual monitoring activity with collection of tissues in order to analyze stable isotopes of carbon and nitrogen”;
- a third phase “should call for characterizing benthonic species which play a key role in biocoenosis present in trawlable seabeds

and of the meso-macro zooplankton acting with a top-down or bottom-up control (…) on demersal and pelagic communities".

The results of these analyses, besides filling cognitive gaps concerning trophic aspects of some significant species of the Italian seas, will also contribute to the activities of the monitoring sub-program 3.6 (Defining, testing and applying ecosystem indicators). This aims at "developing models/tools which contribute to overcoming current gaps in analysis/processing, especially for definition of Good Environmental Status (GES) and of GES targets and that contribute to making ecosystem approaches operative".

The set of monitoring and research activities mentioned above, based on application of stable isotopes of nitrogen and carbon, should therefore contribute to the overall development of nation-wide ecosystem indicators. This will allow Italy to provide itself with suitable tools in the context of the Good Environmental Status of Italian seas for marine food webs: for this Descriptor, a community-wide need has emerged for suitable technical and scientific studies in order to enable proper implementation.

7.3 - References

Ministry Decree 2015. Ministry Decree of February 11, 2015 of the Ministry for the Environment and Safeguarding of the Territory and the Sea. Determination of the indicators associated with environmental targets and monitoring programs, drawn up pursuant to Articles 10(1) and 11(1) of the Legislative Decree No. 190/2010. (15A01446) (GU Serie Generale No.50 of 02-03-2015)

EU-COM (2008). Directive 2008/56/EC of the European Parliament and of the Council of 17 June 2008 establishing a framework for community action in the field of marine environmental policy (Marine Strategy Framework Directive.

EU-COM (2010). Commission Decision of 1 September 2010 on criteria and methodological standards on good environmental status of marine waters (2010/477/EU).

EU-COM (2017). Commission Decision (EU) 2017/848 of 17 May 2017 laying down criteria and methodological standards on good environmental status of marine waters and specifications and standardized methods for monitoring and assessment, and repealing Decision 2010/477/EU.

MATTM (2016). Relazione sullo stato dell'Ambiente anno 2016. La Strategia Marina, pp. 461-488. Available at the website: www.minambiente.it/sites/default/files/archivio_immagini/Galletti/rsa_2016_170601_web_0.pdf

8. IDENTIFICATION AND CHARACTERIZATION OF PLASTIC POLYMERS

8.1 - Introduction

Plastic is an organic polymer obtained from processing of raw materials of various origin present in nature, primarily petroleum. Through a process called *"cracking"*, chains of hydrocarbon molecules are broken down, to create the basic elements of plastic, monomers. In the polymerization process, monomers are merged together again to form long chains, each of which has different features, depending on monomers used. Plastic materials offer many advantages: great rigidity or great flexibility; waterproofness, resistance to atmospheric agents, mold and fungus; resistance to corrosion; a high degree of acoustic, thermal, electrical insulation and insulation against vibrations, and finally low costs.
300 million tons of plastic are produced every year, making it one of the main risk factors for our planet and seas.

The main sources of release of plastic waste into the marine ecosystem ("Marine Litter") and their fate are shown in Figure 8.1.

Figure 8.1: The main sources of release of plastic waste into the sea and their fate in marine and coastal environments (Illustration by Cinzia Piazza)

Only in the Mediterranean, there are about 500 tons of plastic waste, constituting about 80 % of the waste present in the sea (Figures 8.2, 8.3, 8.4, 8.5), representing a serious problem for ecosystems and for marine species, as well as causing enormous social and economic damage to the coastal communities.

Figure 8.2: Plastic waste on the beach of Boccasette (province of Rovigo) (photo by Tomaso Fortibuoni)

Figure 8.3: Ghost nets at Torre Cerrano (PE) (photo by Francesca Ronchi)

Figure 8.4: Plastic pollution in transition environment (photo by Berto Daniela)

Figure 8.5: Plastic pollution (photo by Bo Eide)

The impact of this contamination has also a fallout on human health, in particular when plastic waste is degraded into microscopic fractions, so-called "microplastics", which are able to enter into organisms and therefore bioaccumulate in the food chain. Microplastics are the main problem related to contamination by synthetic polymers present in the sea. Microplastics are micro-particles measuring less than 5 mm, which arise as the product of chemical, physical and microbial degradation of plastic and as a byproduct of plastic processing and many human activities. Problems associated with these micro-particles are due to their massive presence in the oceans and to the fact that - besides containing various chemical processing additives - they can adsorb and concentrate contaminants present in water (Figure 8.6). In particular, hydrophobic contaminants are adsorbed, such as polychlorinated biphenyl, polycyclic aromatic hydrocarbons and organochlorinated pesticides - they are highly toxic for organisms and for human beings and can potentially cause chronic effects such as genetic mutation, endocrine dysfunctions and possibly cancerogenesis (Berto et al. 2017 and authors therein).

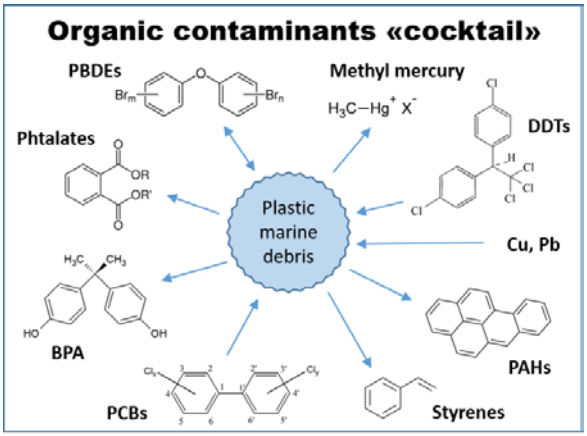
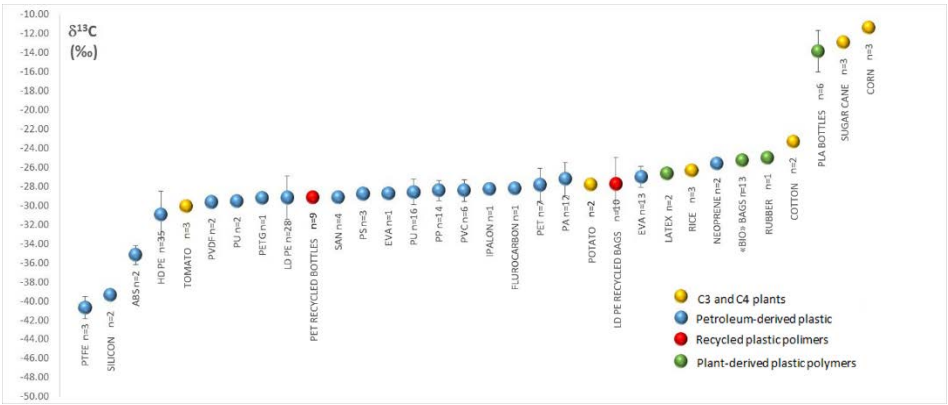


Figure 8.6: Contaminants, which can adsorb on micro-nano plastics

Recent years have seen a multiplication of efforts to replace petroleum-derived plastic with materials of plant origin (corn, rice, tomato, cotton, potato) and hence to produce a completely biodegradable "plastic". Biodegradable plastic materials are materials decomposing thanks to microorganisms (bacteria or fungi) and to chemical and physical factors (light, heat), giving as products water, carbon dioxide (CO_2) and some biomaterials.

8.2 - The study

The potential for using the analysis of carbon stable isotopes to discriminate the plastics deriving from petroleum or from plants, also considering their potential use in packaging materials as shopping bags or bottles for mineral water, has been studied by ISPRA within the DEFISHGEAR (Derelict Fishing Gear Management System in the Adriatic Region) project. Figure 8.7 shows isotopic characterization ($\delta^{13}C$) of a large number of plastic polymers of petroleum and biological origin, used for various kinds of packaging, and of possible natural precursors such as



plants with C3 and C4 photosynthetic pathway.

Figure 8.7 Isotopic characterization ($\delta^{13}C$) of a large number of plastic polymers of petroleum and biological origin, and of possible natural precursors such as plants with C3 and C4 photosynthetic pathway

This research has presented a preliminary screening of the carbon isotopic values of different kinds of plastic polymers. The synthesis mechanisms of different plastics does not seem to contribute to isotopic fractionation considering the small differences of $\delta^{13}C$ values among the main groups of petrol-derived polymers, which were analyzed. The results obtained show how analysis of carbon stable isotopes is a valid tool for

discriminating plastics derived from petrol from those derived from natural matrices (Berto et al. 2017).

Considering the lack of information about analytical methodologies able to evaluate the ratio of degradation of different kinds of plastic in marine environment, this study tested the variation of $\delta^{13}C$ in two kinds of polymers ("BIO" bags and high density polyethylene bags HD PE) (Figure 8.8) subjected to marine environmental conditions for a period of 60 days.

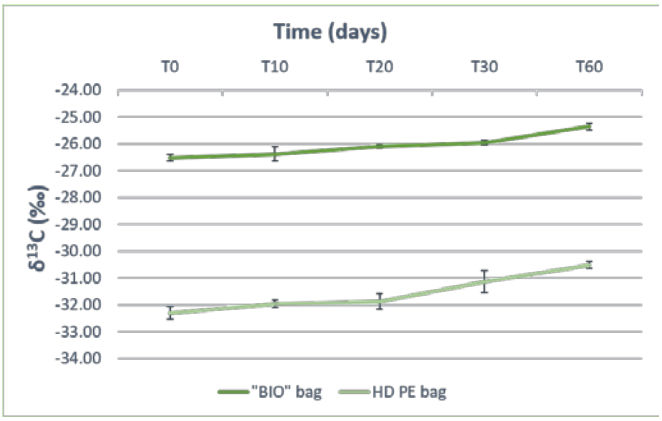


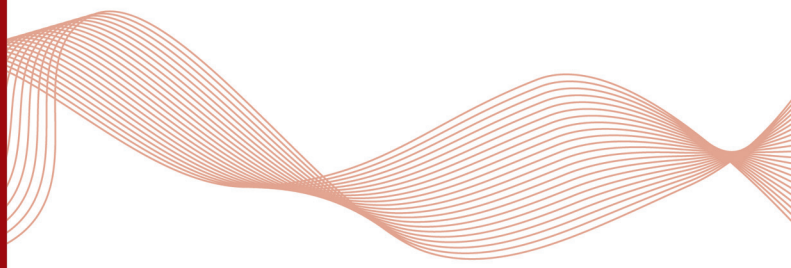
Figure 8.8: Variation over time of the carbon stable isotopes ($\delta^{13}C$) values in two kinds of polymers ("BIO" bags and high density polyethylene bags HD PE)

The shift toward less negative values of $\delta^{13}C$, observed at different times of exposure in both matrices, suggest that the isotopic analysis of carbon may be used as a valid tracer of degradability of plastic in a natural environment, though at present it is not easy to estimate the rate of degradation and the chemical, physical and biological processes involved in such isotopic fractionation.

The advantages offered by isotopic analysis, compared to other analytical techniques used for characterization and recognition of plastic compounds (UV-VIS, optical microscope, electronic microscope, NIR, RAMAN spectroscope, FT-IR), are high sensitivity, rapidity of analysis, no limitation associated with the color of the sample (a limiting factor for example in spectroscopic analysis) and, finally, the small quantity of material needed for the analysis. Concerning this, there are interesting opportunities for future applications of this analytical technique to characterize microplastics extracted from stomach contents in marine organisms.

8.3 - References

Berto, D., Rampazzo, F., Gion, C., Noventa, S., Ronchi, F., Traldi, U., Giorgi, G., Cicero, A. M., Giovanardi, O., 2017. Preliminary study to characterize plastic polymers using elemental analyser/isotope ratio mass spectrometry (EA/IRMS). Chemosphere147, 47–56



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