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“New hints on nutrients dynamics and their impact on carbon sequestration in the Mediterranean Sea”

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ABSTRACT

In the World Ocean nitrogen and phosphorus cycles are deeply linked to the carbon cycle. As nitrate and phosphate play an important role in controlling biological production, the intensity of atmospheric carbon sequestration is strongly influenced by nutrient bioavailability. This thesis aims to contribute to the understanding of the ability of the Mediterranean Sea in absorbing C and N from the atmosphere.

The Mediterranean Sea presents several biogeochemical anomalies compared to the global ocean. The high nitrate/phosphate ratios in subsurface waters and the low $^{15}\text{N}/^{14}\text{N}$ ratios in particulate and dissolved nitrogen have suggested a significant occurrence of nitrogen fixation (N_2 fixation) or an important impact of nitrogen atmospheric deposition.

This study presents, for the first time, a basin-wide overview of direct measurements of N_2 fixation, with values in the North Atlantic for comparison. Very low N_2 fixation rates ($0.052 \pm 0.031 \text{ nmols N l}^{-1}\text{d}^{-1}$) were observed in all sub-regions of the Mediterranean, in contrast to the higher values measured in the North Atlantic ($0.300 \pm 0.115 \text{ nmols N l}^{-1}\text{d}^{-1}$). Higher phosphorus (inorganic or organic) concentrations were not associated with higher N_2 fixation rates.

An isotopic evaluation of the nitrogen sources based on isotopic signature of deep nitrate, N atmospheric deposition and Atlantic inflow nitrate, provides a rough estimate of the amount of N atmospheric deposition. The range varies between 22 and 60% of the total nitrate entering the Mediterranean Sea.

Large amount of N deposition from the atmosphere can explain both P-limitation and anomalous N/P ratio in deep layers. The external inputs of N, determining the excess of N in the marine system could ultimately fuel new production and enhance Mediterranean's ability in carbon sequestration.

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1. INTRODUCTION

Marine nitrogen and phosphorus cycles are deeply linked to the carbon cycle in the ocean. As nitrate and phosphate play an important role in controlling biological production, the intensity of atmospheric carbon sequestration is strongly influenced by nutrient bioavailability.

This thesis will explore some key terms of the biogeochemical cycles of nitrogen and phosphorus in the Mediterranean Sea.

1.1 N and P cycle in the ocean

The global inventory of N is divided in geosphere, atmosphere, hydrosphere and biosphere. The bulk of the N exists in the geosphere (about 97.7%), and most of the remainder is found in the atmosphere (2.3%). Hydrosphere (0.014%) and biosphere (0.0003%), in comparison, contain relatively little N, but in the biosphere N is highly reactive and cycles rapidly.

The marine N cycle is intimately connected to the atmosphere and geosphere. On the surface, oceans receive nitrogen through N_2 through dissolution, dry and wet atmospheric deposition of organic and inorganic compounds and river inputs. Diazotrophic bacteria and phytoplankton transform the inorganic inputs in dissolved and particulate organic forms. Particulate organic nitrogen (PON) is exported in the deep layers through the sinking, while dissolved organic nitrogen (DON) is transported by physical advection and mixing. Heterotrophic bacteria, throughout the water column, provide to remineralize the nitrogen fixed forms in inorganic compounds. Assimilation of surface recycled ammonium and of upwelled deep nitrate trigger primary production in the euphotic zone. N is lost from the ocean in three different ways: denitrification, Anaerobic Ammonium Oxidation (anammox) and burial in sediments. Denitrification is a dissimilatory process

producing N_2 . It occurs in anaerobic environments, such as oxygen minimum zones and continental margins and is carried out by bacteria that use nitrate as final electrons acceptor. Anammox occurs in anaerobic conditions too, but it is performed by chemoautotrophic bacteria that oxidize ammonium to N_2 . Burial of N in sea sediments takes place generally in eutrophic areas where particulate organic matter reaches the abyssal plain or the costal margins.

In the ocean, N is present in many chemical forms, both organic and inorganic. It is present in the gas, liquid (dissolved in water), and solid phases (particulate). The most important inorganic species include N_2 , nitrate (NO_3^-), nitrite (NO_2^-), nitrous oxide (N_2O) and ammonium (NH_4^+). In organic matter, N is an essential constituent for proteins, peptides, enzymes, and genetic material (RNA and DNA). Different oxidation states characterize N compounds that range from -3 (in NH_4^+) to +5 (in NO_3^-).

At the pH and redox state of seawater, the nitrate is the most thermodynamically stable form of N, besides N_2 which is the other very abundant species derived from both dissolution from atmosphere and denitrification of organic matter. Nevertheless, the high concentration of N_2 does not avoid that N is often a limiting nutrient for primary production. This occurs because most of phytoplankton takes up N in two forms: ammonium and nitrate. Assimilation of N_2 is highly energy consuming due to its triple bond among nitrogen atoms, it is based on iron requiring molecules and the fixing 'machine' is highly vulnerable to oxygen. As a consequence, only few bacteria and archaea (diazotrophs) are able to fix molecular nitrogen in the present oxygen rich ocean.

On a global scale, the N inventory in the ocean is regulated by the balance between nitrogen fixation and denitrification [*Falkowski, 1997*]. Direct measurements of these two processes are characterized by great uncertainties due to the spatial and temporal variability, so that estimates of global balance are affected by large errors. Budgets based

on indirect geochemical parameters show estimates of N₂ fixation much lower than denitrification. This scenario let us hypothesize that nowadays oceans are loosing N. On the other hand, *Deutsch et al.* [2007] suggested a mutual feedback between these two processes, so that high denitrification rates trigger a N₂-fixation increase, stabilizing the oceanic inventory of fixed nitrogen over time.

Marine phosphorus (P) cycle is less complex than N. P contained in the continental weathering of crustal materials enters the oceans predominantly through rivers. Atmospheric deposition, the other P source, accounts less than 10% of the total inputs [*Benitez-Nelson*, 2000]. The global P budget is balanced by the sedimentary burial, the only removal mechanism of the P, as no stable gaseous forms exist for this element. As the turnover time of P is much longer (30,000 to 50,000 yr, *Delaney* 1998) than N (3000 yr, *Gruber*, 2004), P is considered the ultimate limiting factor for primary production.

In the ocean, P exists in three forms: particulate (POP), dissolved organic (DOP) and dissolved inorganic (PO₄³⁻). It is an essential element for living organisms as it takes part to the cell building in the membranes phospholipids, in the energy transporting molecules (ATP), and in genetic materials (DNA, RNA).

1.2 N/P ratio

1.2.1 Phytoplankton stoichiometry

Redfield [1934] and *Fleming* [1929] studied the stoichiometry of the phytoplankton and found that essential elements, as C, N and P were present in constant proportions of 106:16:1 in the global average population. In addition, *Redfield* [1934] observed, that the ratio of dissolved inorganic nutrients, NO₃⁻:PO₄³⁻ (N:P), in deep ocean were approximately constant, and very close to the algae stoichiometry. He argued that this overlapping was

not a coincidence, as the phytoplankton modulated the inorganic nutrient concentration according to its assimilation needs. As underlined by *Falkowski and Davis* [2004], the N:P ratio in deep ocean summarizes “the interaction of multiple processes, including the acquisition of the elements by plankton, the formation of new biomass and the remineralization of the biomass by bacteria in the ocean interior, as well as losses of nutrients from the ocean because of burial in the sediments (for example, phosphorus in apatite) or outgassing to the atmosphere (for example, production and loss of N_2 , due to denitrification)”.

On a global scale, the distribution of NO_3^- versus PO_4^{3-} can be synthesized by a line with a slope of about 16. This slope is believed to describe the N:P demand and composition of marine phytoplankton, suggesting that variability of NO_3^- and PO_4^{3-} is controlled by photosynthesis, respiration, and remineralization [*Gruber*, 2004]. It follows that the impact of the biology on the chemistry is crucial, so that an ocean without life would display completely different nutrients content and elemental proportions. When Redfield theory came out, very few investigations on inorganic nutrients in seawater had been conducted. Following studies on a global scale have confirmed this ocean stoichiometry in deep layers, even though with some variability due to concurrent processes which selectively remove one of the terms, thus validating the link between global average phytoplankton and nutrient inventory. On the other hand, since the 1930's, a great number of studies on specific phytoplankton stoichiometry demonstrated that internal N:P can diverge significantly from 16. The “anomaly” can be related to growth rate [*Goldman et al.*, 1979], light conditions [*Falkowski et al.*, 1985; *Finkel et al.*, 2006; *Goldman*, 1986], nutrient availability [*Geider and La Roche*, 2002; *Rhee*, 1978] and species composition [*Karl et al.*, 1992; *Quigg et al.*, 2003; *Klausmeier et al.*, 2004; *Bertilsson et al.*, 2003; *Ho et al.*, 2003]. In the 1970's, laboratory experiments carried out

by *Goldman et al.* [1979], highlighted that Redfield proportions are confirmed only for marine phytoplankton growing at high rates when nutrient limitation does not occur. Under nutrient limitation, on the contrary, population grows at lower rates and does display elemental ratios significantly deviating from 16. In order to reconcile these results to Redfield paradigm, they suggested that natural phytoplankton populations grow with rates very close to the maximum values and are not limited by nutrient availability. In the same years, contrasting results were found in culture experiments performed by *Rhee* [1978]. He observed that the nutrient supply ratio was reflected in the internal stoichiometry of *Scenedesmus*, for different N:P ratios, ranging from 5 to 80. In a review study, *Geider and La Roche* [2002] reached results that partially confirmed Rhee's conclusions. They summarized data from many observations and culture experiments, and concluded that the elemental stoichiometry of both algae and cyanobacteria can diverge enormously under nutrient limiting conditions. Minimum values ($N:P < 5$) are reached when nitrogen is the limiting element, with the phosphate exceeding the inorganic N, while, vice versa, maximum cellular values ($N:P > 100$) are obtained in phosphate limiting conditions. Under optimal nutrient-replete growth conditions, the cellular N:P is somewhat more constrained, ranging from 5 to 19, with most observations below the value of 16 [*Geider and La Roche* 2002]. Despite the observation that the overall average N:P composition of marine particulate matter closely approximates 16, there are significant local variations in a range from 5 to 34 . *Geider and La Roche* [2002]. Furthermore, not every phytoplankton species displays this homeostatic behaviour. For example, *Prochlorococcus* (a typical ultraoligotrophic species) shows a N:P value always higher than 16, regardless of the environmental conditions [*Bertilsson et al.*, 2003; *Heldal et al.*, 2003].

The discovery of these divergences from the classical Redfield ratio stimulated even more the debate on the mechanisms linking the phytoplankton with the nutrient distributions. Starting from experimental evidences observed by *Geider and La Roche*

[2002], *Klausmeier et al.* [2004] proposed an interesting theory to solve this problem, elaborating a model of phytoplankton physiology and resource competition. The basic idea was to obtain the optimal phytoplankton stoichiometry under diverse ecological situations. They found that, depending on the environmental conditions, phytoplankton adopts different strategies in allocating resources, and so in nutrient assimilation. For example, during a bloom, when resources are abundant, the exponential growth of phytoplankton drives the cells to produce more ribosomal RNA with respect to enzymes, proteins and pigments. The consequent internal N:P is approximately 8, lower than the canonical value of 16. On the contrary, when nutrients become scarce, phytoplankton grows slowly and the assimilation strategy changes towards a higher enzymes and proteins production. The N:P resulting from this scenario ranges between 36 and 45, depending on the limiting resource. Therefore, the Redfield ratio of 16 is an intermediate value between these two opposite extreme situations. The common conclusion resulted both from observations [*Geider and La Roche, 2002*] and the above model [*Klausmeier et al., 2004*] is that the Redfield ratio of 16 is not a universal optimum value, but an average stoichiometry of the standing phytoplankton in the ocean, where different species grow under different environmental conditions and adopt different assimilation strategies. Consequently, the deep ocean N:P represents an average of the overall phytoplankton stoichiometry. This connection between the internal algal stoichiometry and the external nutrients level, suggests that if variation of environmental conditions favoured other phytoplankton assemblage, the nutrient concentrations and their ratios in the ocean interior could change significantly, however, this would require quite a long time.

1.2.2 Primary production limiting conditions

The debate on the physiological mechanisms behind N:P has been re-flourishing in the last decade. Previously the N:P ratio of dissolved nutrients in surface waters had been

proposed as a simple index for detecting which nutrient was limiting primary production (hereafter PP) [Codispoti, 1989]. If the ratio displays values under 16, the assimilation by phytoplankton, following the Redfield stoichiometry, will lead to a depletion of inorganic N with respect to PO_4^{3-} . In this case, N will be the nutrient stopping the phytoplankton growth. Vice versa, if the water presents a N:P ratio higher than 16, PO_4^{3-} will be consumed first, becoming the limiting nutrient.

The concept of primary production limitation is borrowed from monocultures in agriculture, i.e. based on the Liebig's law, that states that growth is controlled not by the total of available resources, but by the scarcest one (limiting factor). Only by increasing the amount of the limiting nutrient, the growth of phytoplankton, and the crop totally, can be increased [Von Liebig et al., 1847]. So, when one of the essential nutrients is below the level of which organisms can assimilate it, phytoplankton growth would stop.

However, many methodological and theoretical critiques / criticisms have been raised against the use of this PP limiting index. Firstly, numerous phytoplankton species and communities have such high affinities for N and P that nutrient limitation, if it occurs, happens at concentrations not analytically detectable [Brown and Button, 1979; Goldman and Glibert, 1983], where nutrients determination are affected by large error (around 100%), lowering the reliability of this index. Secondly, the use of N:P ratio is based on the idea that phytoplankton growth depends on the external nutrient availability, as suggested by Monod [1958]. This paradigm, accepted for a long time by the scientific community, has been questioned by Droop [1973], who demonstrated that the phytoplankton growth rate is related, on the contrary, to its intracellular limiting nutrient. According to Droop [1973], the weakness of Monod model is that it does not take into account luxury nutrient uptake and storage for later growth which leads to a temporal uncoupling between growth rate and the external nutrient concentrations. Moreover, Droop's equation can be applied even to an unsteady state, while Monod model assumes the steady state as a basic condition.

Thirdly, Liebig limitation provides information about the maximum biomass that the water body could sustain, and which nutrient will first become limiting as the population increases. This does not take into account both a possible change in nutrient ratio assimilation and/or a shift in the dominant phytoplankton population. Fourthly, recent studies [Mills *et al.*, 2004; Seppala *et al.*, 1999; Shaked *et al.*, 2006; Thingstad *et al.*, 2005], have pointed out that multiple resources can limit simultaneously phytoplankton community, so that the addition of the scarcest nutrient does not necessarily enhances primary production. Three different kinds of co-limitation have been described in the literature: i) multi-nutrient co-limitation, when both nutrients are at levels too low for the uptake requirements; ii) biochemical co-limitation, when one nutrient promotes the uptake or assimilation of the other; iii) community co-limitation, when different members of the community are limited by different nutrient [Arrigo, 2005].

1.2.3 Non Redfieldian processes

Why Redfield ratio is so important? If it is assumed that all biological processes are based on Redfield's stoichiometry, it follows that, apart from the exporting of POM sinking and storage in the sediments, biology of the ocean is almost irrelevant in the global carbon sequestration [Michaels *et al.*, 2000]. Indeed, during mineralization processes nutrients and C are released exactly at the same proportions requested by phytoplankton assimilation. It follows that when deep mixing or upwelling occur, deep waters enriched in inorganic nutrient would reach the upper layer carrying up also dissolved inorganic carbon in Redfield proportions. During phytoplankton synthesis, thus, all nutrients and carbon are available, so no extra C is requested and the ocean would not demand CO₂ from the atmosphere. Assuming that most of the ocean biogeochemistry is regulated by mineralization and assimilation processes which follow the Redfieldian law (as it is obvious if we look at the main nutrients bulks proportions), we can identify two important

mechanisms effectively impacting the carbon storage in the ocean. These are: i) incomplete nutrient utilization in surface waters, ii) changes in total nitrate stock.

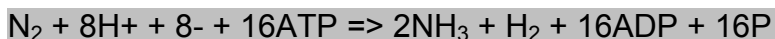
The incomplete nutrient utilization in surface waters generally occurs in the High Nutrient Low Chlorophyll (HNLC) areas, where, due to absence of peculiar micronutrients (Fe, Mg) primary production is low, and the release of C and nutrients by mineralization is not compensated by a corresponding biological assimilation. The resulting excess of dissolved inorganic carbon in the surface layer brings to a release of C to the atmosphere.

Changes in total nitrate stock can occur when external sources of N enters into the ocean or when internal sources are released to the atmosphere or geosphere. Increase of nitrate stock occurs, for example, when N_2 fixation takes place. Indeed, in most oceans, primary production is limited by nitrogen, so in surface waters, it is common to find residual phosphorus in absence of nitrate. In these conditions, diazotrophs are strongly favoured and primary production is fuelled on new sources of N and C coming from the atmosphere. Once entered into the biosphere, through a N_2 fixation process, N is transformed in organic matter and then remineralized and made available in the ocean in form of nitrate. This mechanism increases the ability of the ocean to keep in store a bigger amount of C.

Another process changing the total stock of nitrate in the ocean is denitrification. Indeed, when nitrate is used as electron acceptor during organic matter oxidation, the N_2 eventually produced is lost from the ocean to the atmosphere, decreasing the total stock of nitrate and reducing the ability of the ocean to sequester C.

1.3 Nitrogen fixation

Nitrogen fixation is a biological process performed by a limited number of prokaryotes, that reduces the atmospheric N_2 to ammonia. The reaction, catalyzed by the nitrogenase complex is presented here:



where ATP, ADP and P are adenosine-triphosphate, adenosine-diphosphate and inorganic phosphorus, respectively. The large amount of ATP required is needed to break down the triple bond of molecular nitrogen. The nitrogenase complex contains a ferredoxin which acts as an electron transfer to provide the electrons necessary for the reaction [LaRoche and Breitbarth, 2005].

In marine environment, the most known diazotroph species are non-heterocystous colonial cyanobacteria, *Trichodesmium* spp. [Capone *et al.*, 1997; Carpenter and Romans, 1991] and heterocystous-cyanobacteria (*Richelia*) symbiont of diatoms [Villareal and Carpenter, 1988]. Nevertheless, in the last decades, improvements in DNA sequence analysis have pointed out the importance of unicellular cyanobacteria [Montoya *et al.*, 2004] and other N₂ fixers evidenced with the nitrogenase genes amplification technique, but not yet isolated [Zehr *et al.*, 2001]. Diazotroph growth occurs more often in nutrient depleted surface open ocean, such as tropical and subtropical areas where surface water temperature ranges between 25 and 30 C [Langlois *et al.*, 2008]. The negative correlation between temperature and NO₃⁻ concentration in the world oceans has raised the question whether temperature too controls N₂ fixation [Capone *et al.*, 1997], or whether low nitrate concentration is a primary condition to activate the diazotrophs growth. Breitbarth *et al.* [2007] and Holl and Montoya [2005] conducted experiments separating the effect of temperature from other factors (i.e. nutrient concentration, light intensity, stratification), and demonstrated that the nitrogenase enzyme of *Trichodesmium* is inhibited in cold waters (T<20 C).

On a basin and global scale, the chemical factors limiting N₂ fixation are still an open issue. Iron (Fe) and P have been hypothesized to play a relevant controlling role. As

mentioned above, nitrogenase has a strict Fe requirement, and the iron utilization efficiency is among the lowest of any Fe-containing enzyme known [Raven, 1988]. Fe is not only important for nitrogen fixation. It controls also phytoplankton growth because of its presence in the photosynthetic machinery, and its role has been demonstrated both by the iron addition bioassay [Martin and Fitzwater, 1988], and by large scale fertilization experiments [Boyd et al., 2000] conducted in high nutrients and low chlorophyll (HNLC) regions. The iron limitation of N₂ fixation is inferred both from the iron diazotrophs physiological demand and from the positive correlation between the aeolian fluxes of Fe and the spatial and temporal distributions of cyanobacteria (*Trichodesmium*) [Duce and Tindale, 1991; Falkowski et al., 1998; Morel and Price, 2003]. On the other hand P, as well, has been considered important in limiting N₂ fixation. Sanudo-Wilhelmy et al. [2001] reported that, in the Central Atlantic ocean, diazotrophs growth appeared independent from iron levels both in sea water and within the cells of *Trichodesmium* colonies. On the other hand, they found that N₂ fixation was highly correlated to the P content of the colonies. However, it is important to remind that N₂ fixers are very efficient in P uptake. They can assimilate P both in inorganic and organic forms. For example, in depleted phosphate conditions, *Trichodesmium* produces the enzyme alkaline phosphatase that allows the extraction of phosphate from the dissolved organic phosphorus (DOP) component. Moreover, a recent study has demonstrated that four *Trichodesmium* spp. are able to break down the phosphonates compounds besides the monophosphate esters [Dyhrman et al., 2006].

Other experimental evidences on the limiting factors have been provided by Mills et al. [2004]. Their results assessed that in the Central North Atlantic N₂ fixation was co-limited by Fe and P, and that Saharan dust addition stimulated diazotroph growth.

On a global scale, the main evidences of N₂ fixation come both from direct measurements, based on the methods of acetylene reduction [Hardy et al., 1968; Stewart

et al., 1967] and ^{15}N tracer [Montoya *et al.*, 1996], and from indirect geochemical estimates of the tracer N^* [Gruber and Sarmiento, 1997] and the $^{15}\text{N}/^{14}\text{N}$ stable isotopic composition of nitrogen [Mariotti *et al.*, 1981]. The N^* is a quasi-conservative tracer defined as:

$$\text{N}^* = [\text{NO}_3^-] - 16[\text{PO}_4^{3-}] + 2.7 \mu\text{mol kg}^{-1}$$

N^* estimates the nitrate excess or deficit with respect to the canonical Redfield value of 16. Many processes, such as luxury uptake of PO_4^{3-} , preferential remineralization, atmospheric deposition, and river input, PO_4^{3-} scavenging, can explain the addition or the removal of NO_3^- with a N:P stoichiometry different than 16:1. However, the strongest signals come from N_2 fixation and denitrification, so that the first produces positive patterns of N^* , while the second, negative ones. Based on this approach, Gruber *e. Sarmiento* [1997] estimated a new global fixation rate of 28 Tg N yr^{-1} , which was about 3 times larger than the previous values [Carpenter and Capone, 1992]. Their results are confirmed by many recent studies indicating that pelagic nitrogen fixation may be seriously underestimated [Capone *et al.*, 2005; Montoya *et al.*, 2004].

The $^{15}\text{N}/^{14}\text{N}$ stable isotopic composition of nitrogen is a further indirect approach used to detect evidences of N_2 fixation, and in general to identify the N paths [Mariotti *et al.*, 1981]. Nitrogen exists in two stable isotopes, the most common ^{14}N (99.634% by atoms) and the relatively scarce ^{15}N (0.366% by atoms).

Different sources, such as atmospheric inputs, deep nitrate, atmospheric N_2 , and excreted NH_4^+ , display different isotopic ratios (signature) in response to different production processes [Hastings *et al.*, 2003; Knapp *et al.*, 2005; Liu and Kaplan, 1989; Sigman *et al.*, 2005]. When marine microorganisms uptake N from the sources they perform a fractionation, assimilating preferentially ^{14}N and ultimately the ^{15}N [Mariotti *et al.*, 1981; Montoya and McCarthy, 1995]. If the organisms uptake all the substrate available,

all the signature of the substrate (source) is transferred into the product (particles). This condition often occurs in oligotrophic environments, where N is often limiting [Filippelli, 2002]. On the contrary, when the substrate is partially consumed, the fractionation is active, and the product will display a lower signal with respect to the source (Rayleigh law). Generally, this situation occurs at the beginning of the bloom. However, the isotopic signature of atmospheric N₂ (0‰) is lower than nitrate from the deep ocean (5‰) [Liu and Kaplan, 1989]. Therefore, since no fractionation occurs during uptake, nitrogen fixation gives rise to an isotopically depleted signal of -2‰ to 0‰ in the signature of suspended particles ($\delta^{15}\text{N-PON}_{\text{susp}}$). It follows that primary products based on N₂ fixation will be depleted in ¹⁵N respect to ¹⁴N.

1.4 Atmospheric deposition and terrestrial inputs

Atmosphere is an important pathway for the transportation of many natural and pollutant materials from the continental areas to the ocean [Prospero, 1996]. The impact of the atmospheric inputs on different ocean regions depends on the local characteristics, such as the proximity of deserts or industrialized regions, and on the strength of the resting winds. However, over the last decades, atmospheric anthropogenic fixed nitrogen entering the open ocean has rapidly increased and it is expected to continue to grow in next years [Duce et al., 2008]. It has been estimated that N inputs could account for up to about a third of the ocean's external nitrogen supply and up to ~3% of the annual new marine biological production [Duce et al., 2008].

The role of N inputs has been largely discussed by Fanning [1989]. He observed that P-limited ocean areas, such as the North Atlantic and the North Pacific, are downwind of the most populated and urbanized regions of eastern Asia and North America. He attributed this coincidence to a mechanism whereby airborne pollutant N would be used by

phytoplankton, determining a P depletion of surface waters that would lead to a P limitation. However, due to the difficulties in sampling on the spatial scale of the ocean, only few data are available for most marine regions. Furthermore, seasonal variability is often poorly described because most of the data derived from short time-scale studies. So, most of the atmospheric flux estimates are affected by large uncertainty [Prospero, 1996].

The isotopic signals have been recently used in order to evaluate the relative weight of atmospheric inputs on the overall N supply to the ocean. A clear negative signature characterizes NO_3^- and NH_4^+ in the rain, ranging from -3 ‰ to +2 ‰, and from -12 ‰ to 2.2 ‰, respectively [Baker et al., 2007; Yeatman et al., 2001]. When this N enters the marine biological cycle, the low signature can be transferred in seawater. If the importance of the atmospheric fluxes was relevant with respect to the other sources, dissolved and particulate nitrogen would show a clear light isotopic signature [Baker et al., 2007].

Unlike N, P does not present any gaseous forms. So, the atmospheric bulk is not significantly impacted by P compounds as the dissolved and particulate forms involved in the marine cycle are supplied mainly from the rivers [Avila et al., 1998]. As a consequence of their different concentrations, the N/P ratio of atmospheric inputs is extremely high. For examples, values reported for the Atlantic Ocean are always over 1000 [Baker et al., 2007]. The N:P ratio higher than 16 in deep waters of peculiar regions has been attributed to the atmospheric deposition inputs [Villareal and Carpenter, 1988].

The mechanisms transferring these signature from surface to deep layers are still debated. The physical dynamics and the biological functioning of the impacted area seem to play an important role. In deep convection or dense water formation regions, physical pump can drive to the abyssal a large portion of surface water with its peculiar chemical and physical properties [Villareal and Carpenter, 1988]. In turn, surface high N:P ratio can fuel a non Redfieldian biological pump [Geider and La Roche, 2002] that, depending on the intensity of particles export, could impact the deep layers.

1.5 Study area: the Mediterranean Sea

The Mediterranean Sea is a semi-enclosed basin, surrounded by lands and connected to the Atlantic Ocean through the Strait of Gibraltar. Other adjacent seas are the Black sea and Red sea. The thermoaline circulation is driven by the excess of evaporation with respect to precipitation and river inputs, so that the Mediterranean Sea can be defined a salinification basin with an anti-estuarine circulation. Depending on the scale, three thermoaline cells can be described: one principal open cell on basin scale, and two secondary closed cells on a smaller scale [Lascaratós *et al.*, 1999]. The principal cell involves only the surface and intermediate layers. The Atlantic Water (AW) enters at surface through the Gibraltar Strait and, during its eastward path, its physical properties rapidly change, as the high evaporation rate elicits an increase of salinity, producing the Modified Atlantic Water (MAW). In the Levantine basin a further increase of evaporation and continental dry winds make the MAW even saltier and denser, so that it sinks down through subduction to the intermediate layer, forming the Levantine Intermediate Water (LIW) [LIWEX group, 2003]. The main circulation path is concluded by the outflow of the LIW at the Gibraltar Strait after crossing the entire Mediterranean [Ovchinnikov, 1984].

The secondary thermoaline cells involve deep water formation. The open ocean convection, driven by strong evaporation and cooling of surface waters, takes place both in the Eastern and Western Mediterranean, in the Adriatic and the Gulf of Lyon, respectively [Pollak, 1951; Ward, 1996].

The anti-estuarine circulation at the Gibraltar Strait, where nutrient-poor subtropical Atlantic water enters the basin, does not contribute to a significant fertilization of the Mediterranean surface layer. Because of this relatively low concentrations in the intermediate layers and because of the limited external inputs (rivers contribute do not impact significantly open ocean areas, Ribera d'Alcalà *et al.* 2003) the basin presents a

pronounced oligotrophy, with a clear decreasing gradient in nutrient concentrations from West to East. As opposed to the global ocean, the Mediterranean sea displays intriguing anomalous nutrient ratios in intermediate and deep layers, where nitrate/phosphate ratio, of 20–25 [Ribera d'Alcalà *et al.* 2003], is well above the Redfieldian value of 16. Several hypotheses have been proposed to explain this anomaly: i) high nitrate/phosphate ratio in the Adriatic dense waters ventilating the deep layer of the Eastern Mediterranean [Civitarese *et al.*, 1998], ii) significant atmospheric input rich in nitrogen [Herut *et al.*, 1999] and iii) N₂ fixation [Bethoux and Copin-Montegut, 1986, Sachs and Repeta, 1999]. This last hypothesis is further supported by regional geochemical observations including decoupling between phosphocline and nitracline [Bianchi *et al.*, 1999], deep $\delta^{15}\text{N}$ of nitrate significantly lower (2.4–3 ‰) [Pantoja *et al.*, 2002] than ocean values (5–6 ‰) [Sigman *et al.*, 2005] and low $^{15}\text{N}/^{14}\text{N}$ ratio in suspended particulate organic nitrogen in the upper layer [Pantoja *et al.*, 2002].

On the basis of budget calculation, N₂ fixation has been estimated to supply nitrogen to the whole basin from $2.0 \cdot 10^{10}$ mol-N/yr to $12.4 \cdot 10^{10}$ mol-N/yr [Bethoux and Copin-Montegut, 1986], which corresponds to a daily rate of 21.9–178.1 $\mu\text{mol N m}^{-2}$. To date, however, few direct measurements are available for the region. Moreover, these experimental data show high spatial and temporal variability (2.0–7.5 $\text{nmol N l}^{-1}\text{d}^{-1}$, Sandroni *et al.* [2007]; 129 $\text{nmol N l}^{-1}\text{d}^{-1}$, Rees *et al.* [2006]), precluding clarification of the role of this process on a basin scale.

On the other hand, occurrence of N₂ fixation is still debatable, as the depletion of phosphorus in the Eastern Mediterranean should prevent a massive growth of diazotrophs [Krom *et al.*, 2004]. Unbalanced sources of atmospheric and terrestrial origin [Herut *et al.*, 1999] and the lack of significant denitrification [Krom *et al.*, 2004] have been invoked as alternative processes able to maintain the altered N/P ratios.

1.6 Thesis objectives

The study carried out in this thesis aims to contribute to the understanding of the ability of the Mediterranean Sea in absorbing C and N from the atmosphere. Starting from the processes which cause the observed anomaly of nutrient ratios in the Mediterranean Sea, I focused on the role of N₂ fixation in the basin. Indeed, this work presents, for the first time, a basin-wide overview of direct measurements of N₂ fixation. The importance of atmospheric deposition as an alternative process playing a role in the nitrogen cycling is explored through a careful overview of stable isotopic signals in particulate organic and dissolved inorganic nitrogen. One of the first wide datasets for the Mediterranean Sea is presented here. Moreover, the mechanisms behind the high N/P ratio in intermediate and deep water of the Mediterranean are discussed in the framework of the global equilibrium between N sources and sinks that determines the nutrient ratio close to Redfield in the global ocean.

Chapter 2 describes the field measurements carried out. Research site description, sampling strategy and analytical methods are reported here.

Chapter 3 presents the main results obtained. N₂-fixation rates, dissolved inorganic nutrients distribution, isotopic signals in dissolved inorganic and particulate organic nitrogen are presented.

Chapter 4 discusses the role of the N₂ fixation on basin scale taking into account the limiting factors of this process. Atmospheric deposition, as an alternative process explaining deep nitrate excess, is widely analyzed. Consequent implications on primary production are assessed. In order to evaluate sources of the N excess in deep layers, ⁵N/¹⁴N in nitrate is used to evaluate the relevance of nitrogen sources. An estimate of the impact of atmospheric N deposition on the ability of the system to store carbon concludes the chapter.

Finally, the major conclusions of the thesis are summarized in chapter 5.

2. METHODS

In this chapter the sampling strategy and the methods adopted will be described.

2.1 Sampling

Data were collected during the Cruise VECTOR-TRANSMED (28 May to 24 June 2007) carried out on board of Italian research vessels *Urania* and *Universitatis*. Seawater was sampled along the entire water column with a CTD-rosette equipped with Niskin bottles. Ten stations were sampled: in the North Atlantic off the Gibraltar Strait (VA), in the Western (V4, V3, V1 and V2) and Eastern (V6, V7, V8, V9 and V10) Mediterranean Sea (Fig. 2.1). Nutrients were sampled in all stations, whereas N_2 -fixation rates, suspended particulate organic nitrogen (PON), $\delta^{15}N$ -PON, $\delta^{15}N$ -NO₃ were carried out in a sub-set of stations (see map for details).

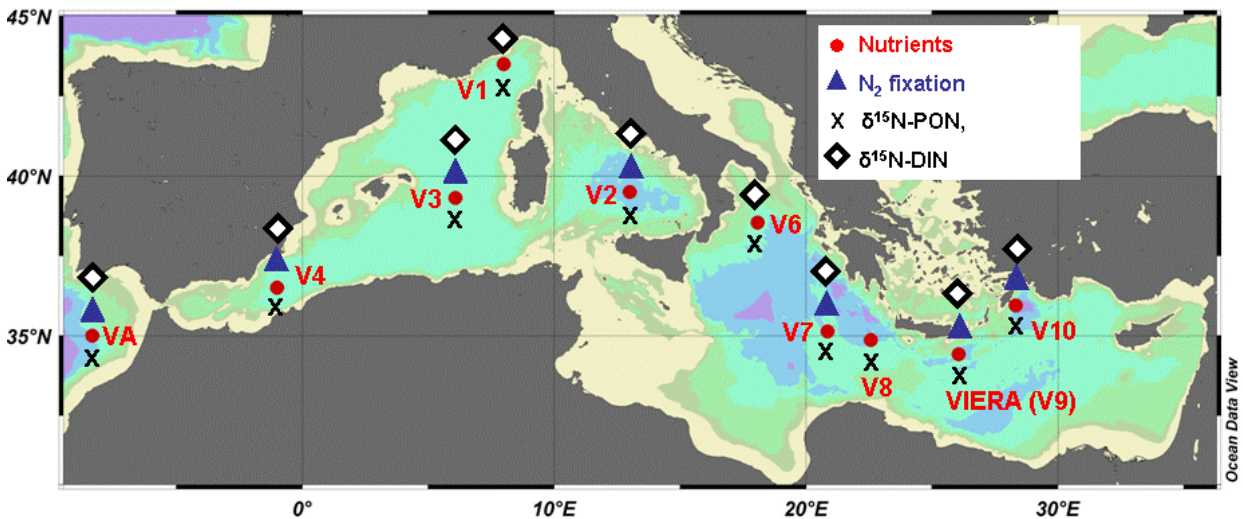


Fig. 2.1. Sampling map of VECTOR-TRANSMED cruise carried out from 28 May to 27 June 2007.

2.2 Physical parameters

The thermoaline properties were measured with a SBE multi-sensor array equipped with a CTD probe for measurements of temperature and conductivity, a fluorometer for fluorescence and a polarographic sensor for dissolved oxygen. Data have been collected

and analyzed by the teams of University of Ancona and OGS–Trieste.

2.3 Dissolved oxygen and inorganic nutrients

Dissolved oxygen was determined following the Carpenter-Winkler titration procedure [Carpenter, 1965]. Samples were collected into volume-calibrated Erlenmeyer flasks (volumes 80 ml) and analyzed directly in the same flask with a DMS 716 Titrino equipped with a dual platinum electrode, for the automatic determination of the final point. The thiosulphate solution was calibrated with standard potassium iodate solution prepared at the lab. The precision was better than 0.2%.

Nitrate plus nitrite (hereafter nitrate), soluble reactive phosphate (hereafter phosphate) and silicic acid (hereafter silicate) were collected at discrete depths, filtered on precombusted (450 C, 6 h) GF/F filters and stored at -20 C until the lab analysis. The analyses were carried out within one month following standard colorimetric methods with an ALPKEM autoanalyzer [Grasshoff *et al.*, 1983].

Concerning analysis of very low nutrient concentrations, a brief note needs to be reported. Although freezing nutrient samples for later lab analyses is a procedure widely shared and tested [Kress and Herut, 2001; Dore *et al.*, 1996], some authors [Krom *et al.*, 2005] have underlined that comparison between phosphate standard colorimetric measurements conducted directly on-board and on frozen samples gives similar and consistent results only at concentrations higher than 0.02 μM . According to that study, at lower concentrations, frozen data are scattered and the best suitable technique should be on-board analysis with a colorimetric segmented flow analytical system, using a long path-length (2 m) liquid waveguide capillary cell (LWCC) [Woodward and Rees, 2002].

The detection limit of our method, was 0.02 μM . So we were able to establish with a deeper detail the variability of the nutrients in the upper layer.

2.4 N₂ fixation

N₂ fixation rates were determined by direct ¹⁵N₂ assimilation [Montoya *et al.*, 2004, 1996]. Samples were collected in 6 replicates using 4.6-litres Nalgene bottles, equipped with a silicon septum cap, at three optical levels (50, 1 and 0.1% of the surface PAR) generally corresponding at 10, 50 m and at the depth of the deep chlorophyll maximum (DCM, 90-110 m). Four replicates were enriched with 4.6 ml of ¹⁵N₂ (Cambridge Isotope Laboratories Inc., 99.90 atom%) with a gas-tight syringe. One replicate (control) was incubated without addition of ¹⁵N₂. The last replicate (blank) was immediately filtered without any treatment. Before the gas-labelled injection, every bottle was controlled and air bubbles were accurately removed. Samples were incubated 24 h on deck, using 3 thermostated incubators screened with neutral density filters, in order to simulate in situ light conditions. Size fractionation of diazotrophs was also carried out. After the incubation, two replicates enriched with ¹⁵N₂ were filtered on pre-combusted (450 C, 6 h) GF/F filters, whereas other two replicates were screened on 10 µm polycarbonate filter to remove the largest phytoplankton prior filtration on GF/F filters. Every step of the experiments was conducted in dim light conditions to avoid phytoplankton photodamage. Enriched and natural samples were always treated separately.

N₂ fixation rates were calculated according to Montoya *et al.* [1996]. ¹⁵N-enrichment of PON in the amended samples was calculated with respect to the control samples. The precision of ¹⁵N measurement in 16 additional enriched replicates, incubated in the same station, was ±0.0001% (±0.3 ‰). For this reason, we considered significant only N₂ fixation data calculated by ¹⁵N enrichments exceeding two times this standard deviation (> 0.0002 atom%). As reported by Montoya *et al.* [1996] the detection limit can be calculated from the following equations:

$$V(T^{-1}) = \left(\frac{1}{\Delta t} \right) \left(\frac{[PN]_{\Delta}}{[PN]_f} \right) = \left(\frac{1}{\Delta t} \right) \left(\frac{A_{PN_f} - A_{PN_0}}{A_{N_2} - A_{PN_0}} \right)$$

$$\rho(M \cdot L^{-3} \cdot T^{-1}) \approx \frac{V}{2} [PN]_f$$

where Δt is the duration of the experiment, A_{N_2} , is the ^{15}N enrichment of the N_2 available for fixation; APN_0 , the ^{15}N enrichment (%) of particulate N at the beginning of the experiment; APN_f , the ^{15}N enrichment of particulate N at the end of the experiment; and $[PN]_f$, the concentration of particulate N at the end of the experiment, V the specific rate of uptake of N_2 by particles in the experimental bottle, and ρ the volumetric rate of N_2 fixation. Standard deviation for A_{PN} was 0.002 atom%, the average substrate enrichment (A_{N_2}) was 8.98 atom%, the duration of the experiment was 24 h, the A_{PN0} was on average 0.3667 atom%, so considering an average PN_f of 0.368 nM, the resulting V was $9.67E-06$ (h^{-1}) and ρ was $1.78E-06$ nM/h.

Comparison between blanks and controls showed a small but systematic increase of PON and $\delta^{15}N$ -PON in the controls (mean difference of 0.0005 ± 0.0004 atom%, $1.47 \pm 0.9\%$). This small variations might likely be due to a slight isotopic fractionation during the incubation.

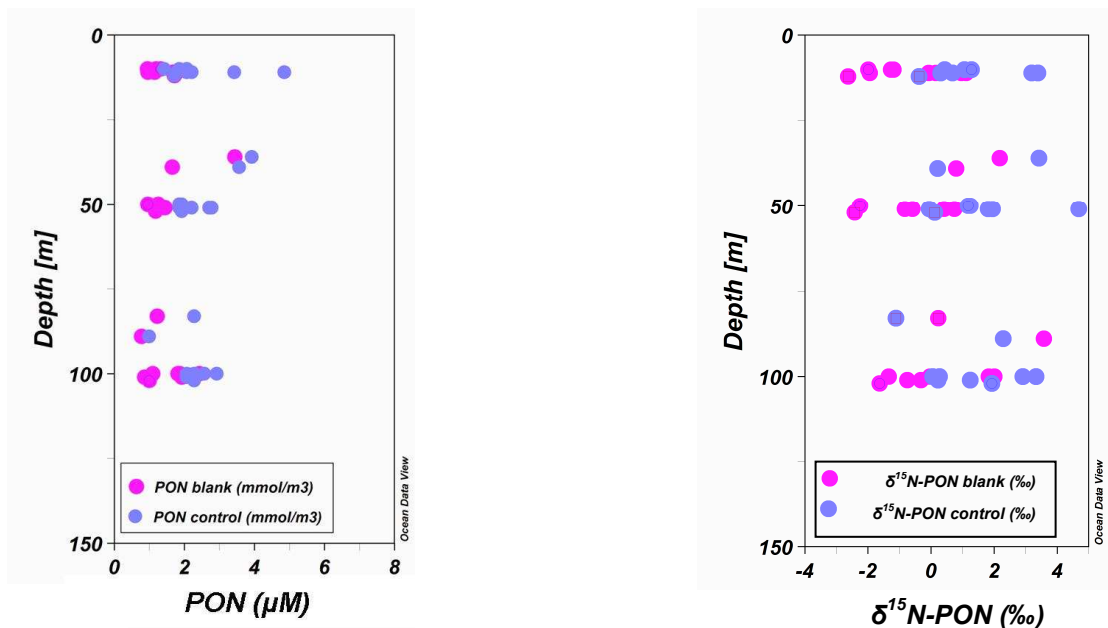


Fig. 2.2. Comparison between mass (left) and natural isotopic enrichment (right) of PON blank (pink) filtered after the collection and PON control (blue), the latter, incubated in the same conditions of the $^{15}N_2$ enriched samples. Effect of the incubation is evidenced by the slightly but constant increase of mass and enrichment in the controls.

2.5 Suspended PON and $\delta^{15}\text{N}$ -PON

Seawater for determination of suspended PON and $\delta^{15}\text{N}$ -PON was collected in all stations, in the layer 0-300 m typically at depths of 0, 40 or 50, 100, 150 200, 300. Generally, in the upper layer, the sampling depths overlapped with N_2 fixation measurements.

Immediately after collection, 8 to 10 litres of seawater were filtered through a precombusted Whatman GF/F (25 mm diameter). After filtration, samples were dried at 60 C and stored at -20 and analyzed in lab within few months.

Analysis of PON were determined integrating the areas under the mass peaks 28, 29 and 30 after calibration with standard material (IAEA- $(\text{NH}_4)_2\text{SO}_4$) of known stoichiometry. This technique [Owens and Rees, 1989] has the relevant advantage to carry out simultaneous measurements of isotope ratio and PON from the same sample. Coefficient of variation for PON analysis was better than 5%. Reproducibility on stable isotopic ratio in PON was $\pm 0.2\%$. Blank contributions (i.e., tin cups and/or filters) were negligible.

2.6 $\delta^{15}\text{N}$ - NO_3^-

Seawater for determination of $\delta^{15}\text{N}$ - NO_3^- was collected along the entire water column, typically at depths of 0, 40 or 50, 100, 200, 300, 500, 1000, 1500, 2000, 2500, 3000m and deeper according to the bottom. Stations sampled were VA, V4, V3, V1, V2, V6, V7, VIERA and V10. Depending on the nitrate concentration, volume collected ranged between 1 and 2 litres. Samples were filtered through a precombusted 25 mm GF/F glass fiber filter, poisoned with 1-2 ml/l of HCl (6M) and stored in HDPE bottles, in dark conditions. Bottles were previously washed with HCl (10%) and rinsed with bi-deionised

water and before being filled, rinsed with sample water three times. Lab analysis were performed within few months after the sampling.

Nitrate was extracted for isotopic measurement following the “passive ammonia diffusion” method of *Sigman et al.* [1997]. According to the nitrate concentration, an appropriate amount of sample (200-800 ml) containing approximately 1-5 μM of NO_3^- was boiled with MgO till a reduction of the volume to 40-50 ml. The basic condition and the high temperature were essential to raise the pH over 9.7 and removing any traces of ammonia naturally present in seawater. After the boiling phase, Devarda’s alloy and diffusion packets were added. Diffusion packet were made with a 10 mm diameter precombusted GF/D filters (Whatman #1823010), acidified with 25 ml of H_2SO_4 (2M) and sandwiched between two 25 mm diameter porous hydrophobic Teflon membranes (10 μm pore size). Devarda’s alloy triggers the reduction of nitrate to ammonia, transferred out the seawater by gas-phase diffusion and trapped by the sulphuric acid on the glass fiber. As reduction of nitrate to ammonia takes place slowly, incubation of a minimum of 7 days at 65 C, followed by 7 additional day on a shaker table, were necessary to reach the complete conversion in ammonium sulphate. After the shaking (14-18 days, totally), diffusion packets were removed and dried in a dessicator for 2 days, after which each GF/D was packed into a tin (or silver) capsule and then analyzed with EA-IRMS as described below. In every phase of the procedure (before boiling, before incubation and after shaking) pH was checked, so if necessary, samples were buffered with NaOH (10%) or HCl (10%) in order to keep the pH always between 10 and 11.

In every day of analysis, two blanks (bi-deionised water plus NaCl and reagents) and two standards (250 ml of a 12 μM NO_3^- solution) were run together with the samples. For the overall analysis, blanks were always lower than 0.3 μmol NO_3^- . The standard deviation of standards was 0.47‰ (n=16). The replicate error of samples was estimated to be within $\pm 0.5\%$. The overall recovery was between 85 and 120% for standards and samples.

2.7 Elemental and isotopic analysis

As already mentioned in the introduction, on the Earth, nitrogen exists in two stable isotopes, the most common ^{14}N (99.634% by atoms) and the relatively scarce ^{15}N (0.366% by atoms). In literature the isotopic ratio is expressed with the 'δ' notation:

$$\delta (\text{‰}) = [(R_x/R_{st})-1]*1000$$

where R_x is the heavy to light isotope ratio in the sample, and R_{st} in the standard. The standard used for $\delta^{15}\text{N}$ is the atmospheric N_2 (0 ‰).

The elemental and isotopic composition of PON and NO_3^- was determined at the Istituto di Scienze Marine (ISMAR-CNR) labs in Trieste using standard elemental analyzer isotope ratio mass spectrometry (EA-IRMS) techniques [Owens and Rees, 1989]. Natural and enriched ($^{15}\text{N}_2$ added) samples were handled separately in every step of the sampling to avoid possible contamination. Instrument used for analyses was an Europa ANCA/SL elemental analyzer coupled with an Europa 20/20 IRMS. For isotopic analyses, ammonium sulphate (PDZ-Europa Ltd) calibrated against IAEA-N-1 was used as working standard. Precision of standards replicate was $\pm 0.2 \text{ ‰}$ ($n=22$). Long-term performance and stability of the instrument measurements was monitored with daily analysis of peptone (Merck) ($\delta^{15}\text{N}=+5.73\pm 0.55\text{‰}$, $n=24$).

3. RESULTS

In this section major results about N_2 fixation rates in six stations of the Mediterranean Sea, and one in the Atlantic Ocean are presented. Data are coupled to a large survey of the isotopic signature of dissolved inorganic nitrogen in the intermediate and deep layer, and particulate organic nitrogen in the upper layer. A general background of the physical-chemical properties of the basin in June 2007 introduces the chapter.

3.1 Physical and nutrient fields

Temperature and salinity displayed a clear longitudinal gradient both in the upper and in the bottom layers, with colder and fresher waters in the Atlantic and western basin and warmer and saltier in the eastern one (Fig. 3.1).

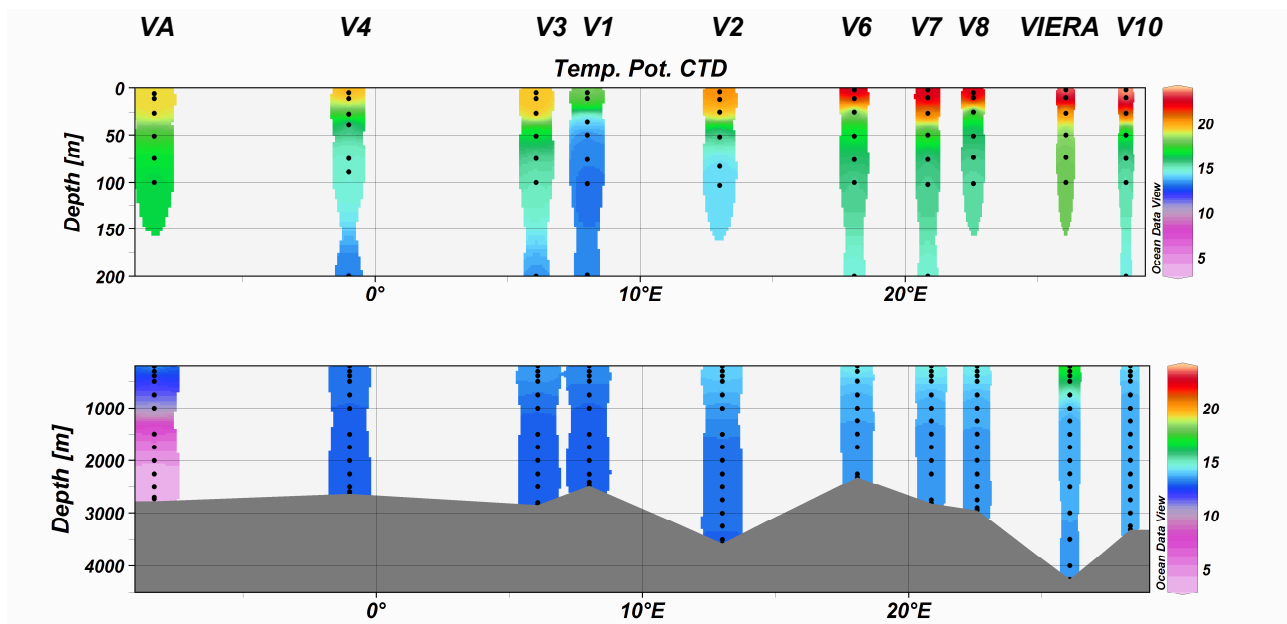


Fig. 3.1. Section of potential temperature across the Mediterranean Sea and the Atlantic Ocean in the upper (0-200 m) and the bottom (200–bottom) layers.

In the Mediterranean stations, different water masses could be identified: Atlantic Water (AW) at the surface in the western basin, Levantine Surface Water (LSW) in the Levantine Basin, Levantine Intermediate Water (LIW) all over the basin, Western

Mediterranean Deep Water (WMDW) and Eastern Mediterranean Deep Water (EMDW) both below 1000 m depth.

Following the anti-estuarine circulation, the surface AW entering from the Gibraltar Strait separated in different branches during its eastward path. The Atlantic salinity signature (maximum of 36.5) observed in the Algero-Provençal basin (V4 and V3), started to disappear in the Tyrrhenian Sea (V2) and it was no more detectable in the Levantine, where high salinity values (39.34) were reported at the surface (Fig. 3.2).

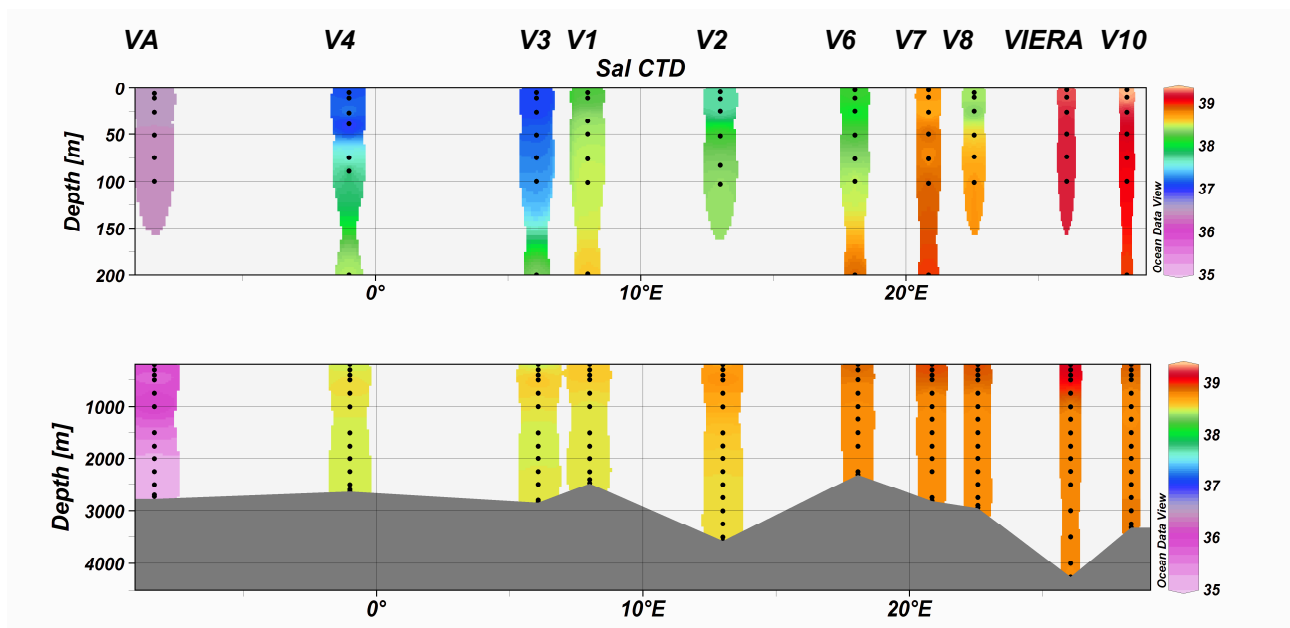


Fig. 3.2. Section of salinity across the Mediterranean Sea and the Atlantic Ocean in the upper (0–200 m) and the bottom (200–bottom) layers.

The Levantine Intermediate Water (LIW), characterized by the maximum of salinity in the 200–600m layer, was recognizable in every Mediterranean station (Fig. 3.3, left) excluding the VIERA. The subsurface peak of the LIW salinity decreased moving from east to west and was still detectable in a layer below 1000 m at the Atlantic station VA.

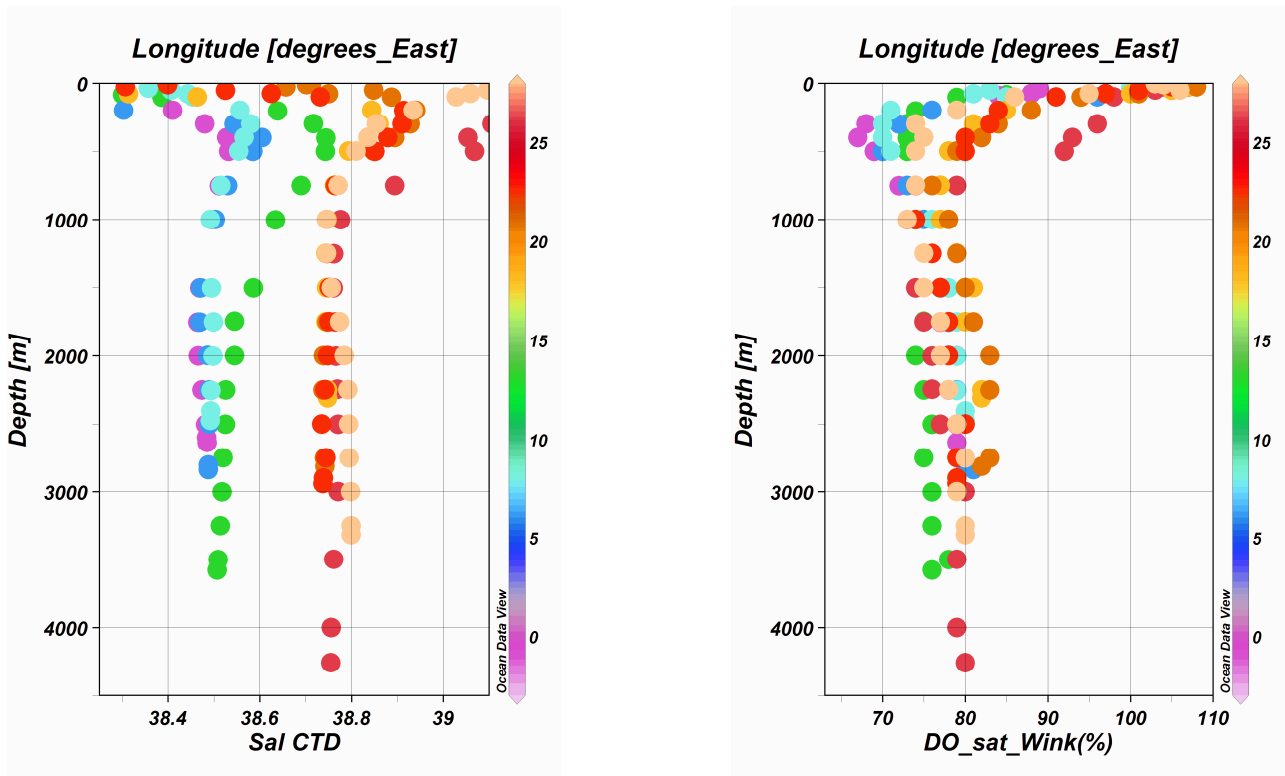


Fig. 3.3. Depth profiles of salinity (left) and percentage of oxygen saturation (right). Note that range of salinity is restricted from 38.25 to 39.25 in order to highlight the core of Levantine Intermediate Water. Thus, the whole VA, and surface values of VIERA are not included in the salinity plot.

An opposite trend to the salinity was observed in the oxygen saturation (Fig. 3.3, right and Fig. 3.4). In the western stations, values rapidly decreased from surface to reach minimum values in the core of the LIW. Eastern stations displayed minima of oxygen saturation in a deeper layer, the so called transitional zone (800-1200 m), where the oldest waters of the basin resides. Moving longitudinally westward, the LIW showed a progressive oxygen consumption corresponding to an increase of nitrate and phosphate concentrations.

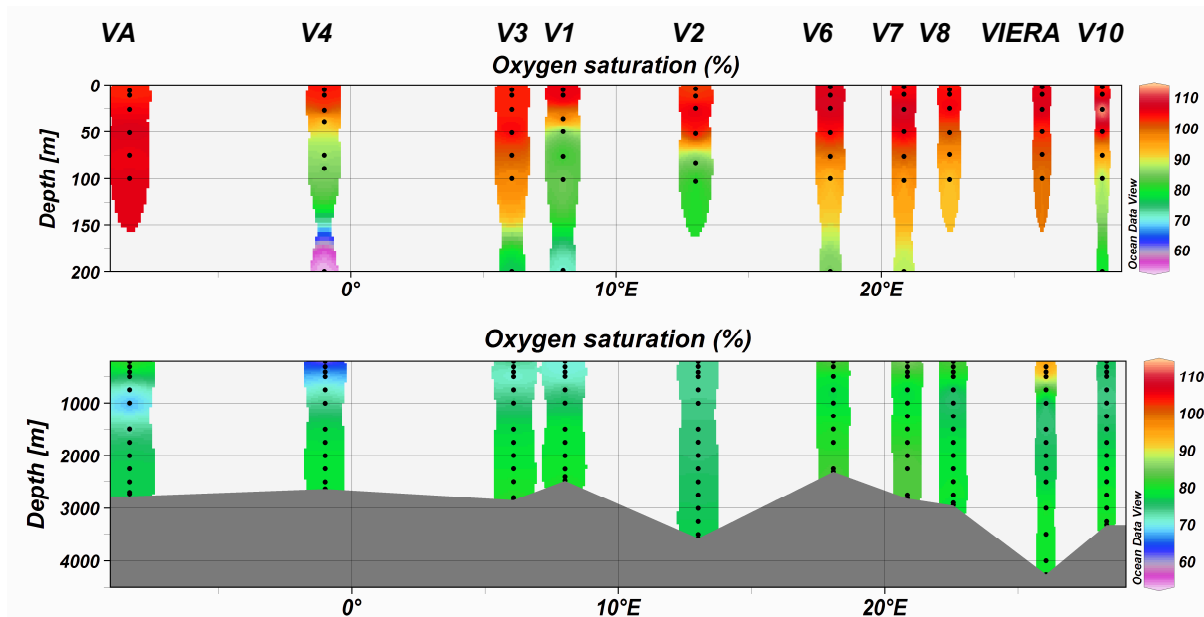


Fig. 3.4. Section of oxygen saturation across the Mediterranean Sea and the Atlantic Ocean in the upper (0-200 m) and the bottom (200–bottom) layers.

Seasonal stratification induced by the spring warming was detectable all over the basin, (Fig. 3.1). The mixed layer depth varied from west to east, being shallower in the Levantine basin. Within the thermocline, the temperature decreased rapidly from 19-24 C to 14-15 C. In the deeper layers temperature never decreased below 13-14 C in the Mediterranean (> 500 m), and reached minimum values (3 C) in the Atlantic.

Different contribution of fresh water content and evaporation rate determined the density fields (Fig. 3.5). The marked difference of density (Fig. 3.5) between Atlantic and Mediterranean waters determined the vertical structure of the water column at Gibraltar (V4), where Atlantic water entering from the Strait overlaid the exiting Levantine Intermediate Water at the subsurface layer.

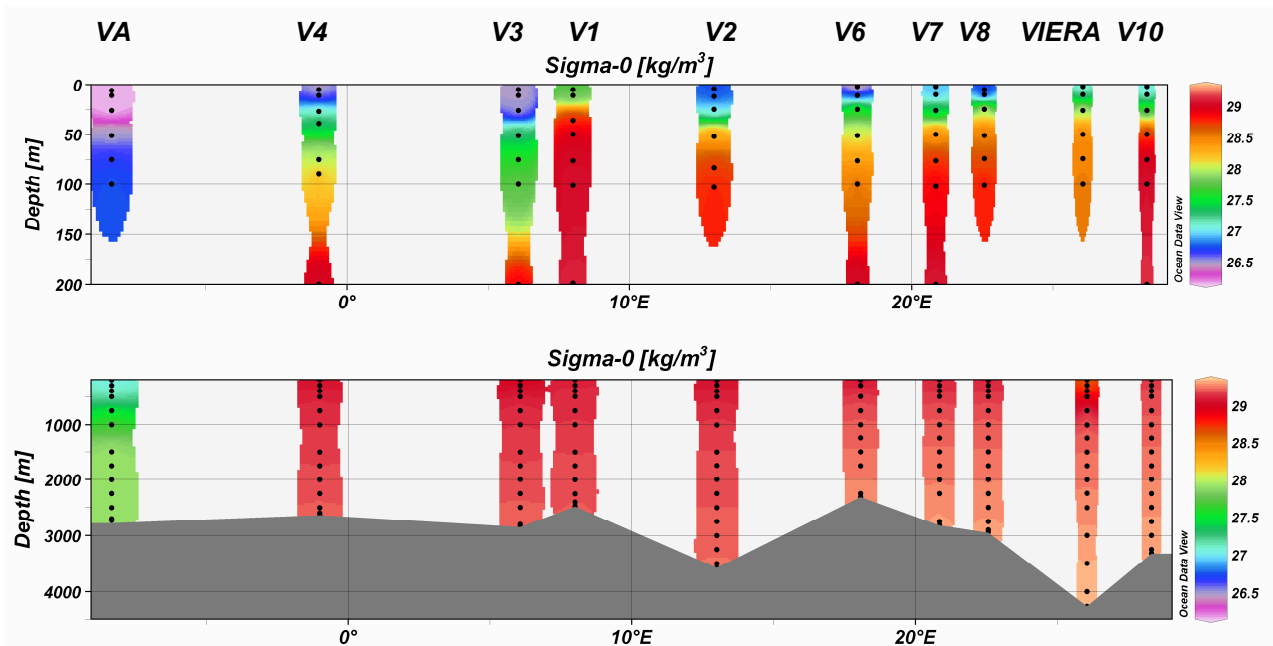


Fig. 3.5. Section of density across the Mediterranean Sea and the Atlantic Ocean in the upper (0-200 m) and the bottom (200–bottom) layers.

Above the DCM, nitrate and phosphate were almost depleted in all stations (Fig. 3.6 and Fig. 3.7). The nitrate concentrations ranged from 0.04–0.60 μM , while phosphate level was generally $< 0.05 \mu\text{M}$, except at the station V4 where values of 0.09-0.22 μM were observed.

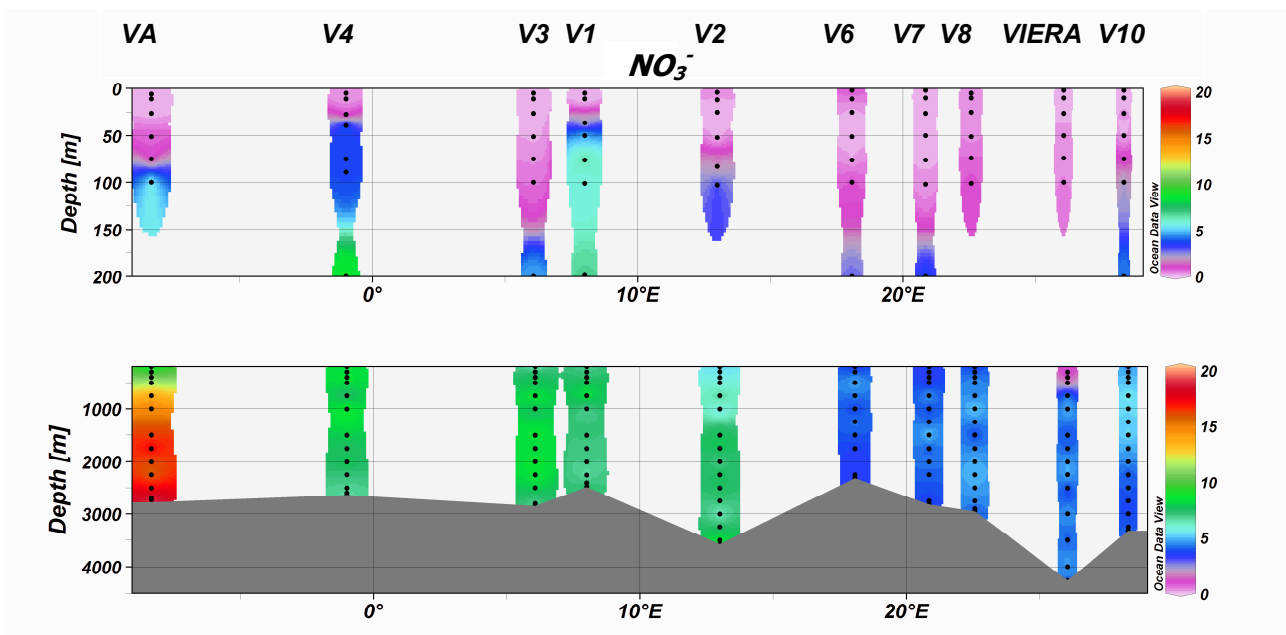


Fig. 3.6. Section of nitrate across the Mediterranean Sea and the Atlantic Ocean in the upper (0-200 m) and the bottom (200–bottom) layers.

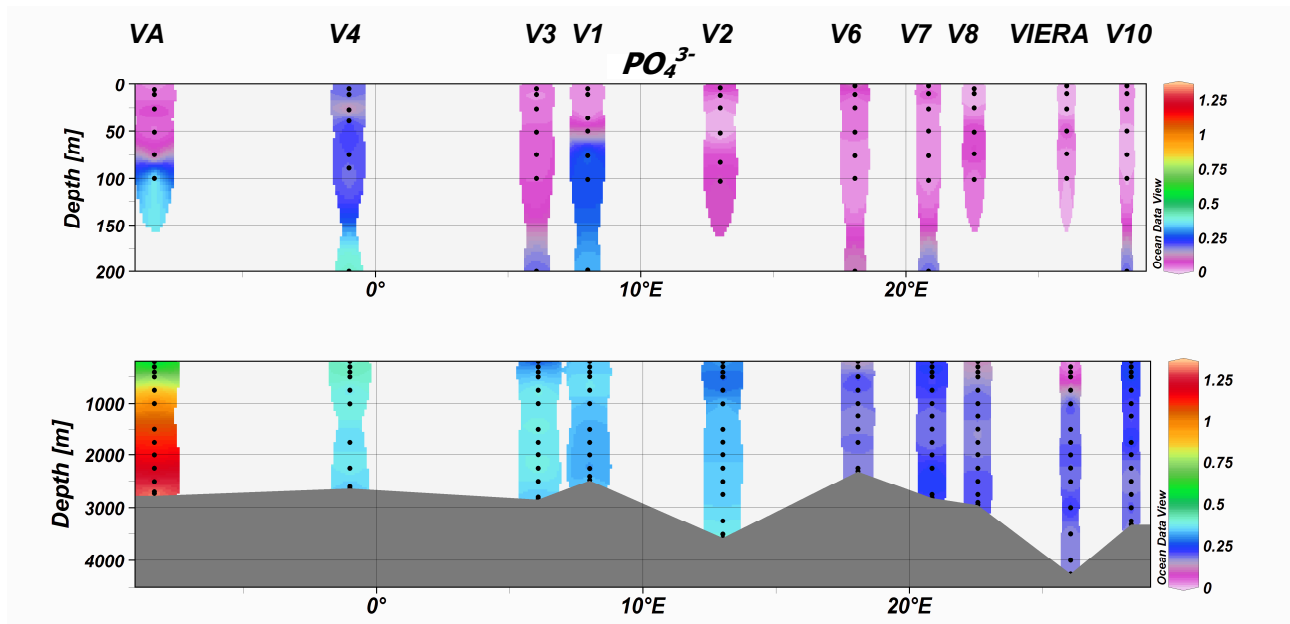


Fig. 3.7. Section of phosphate concentrations across the Mediterranean Sea and in the Atlantic Ocean in the upper (0-200 m) and at the 200-bottom layers.

The average nutrient concentrations in the three main layers of the water column are shown in Fig. 3.8. Atlantic (VA) displayed an increasing concentrations along the water column for both nitrate and phosphate. At this site, the highest values were recorded. Both Western and Eastern Mediterranean showed a weaker increase⁴ of concentrations with depth. A clear west-east decreasing concentrations were evident, with nutrient levels in the western basin almost two times higher than what observed in the eastern one. At the surface (0-100 m), phosphate varied between 0.02 and 0.09 μM, and nitrate between 0.37 and 1.03 μM. In the intermediate layer, phosphate and nitrate varied respectively in the range 0.17-0.35 and 3.60-7.42 μM in the Mediterranean, while the Atlantic station was completely out of such ranges (0.69 μM of PO_4^{3-} , 10.97 μM of NO_3^-). Finally, in the Mediterranean, nutrient concentration ranges in the deep layer (0.19-0.34 μM for PO_4^{3-} , 4.23-7.56 μM for NO_3^-) did not diverge substantially from what recorded in the intermediate layer. On the contrary, the Atlantic deep layer was more enriched in nutrients (0.87 μM for PO_4^{3-} , and 16.27 μM for NO_3^-) than the intermediate layer.

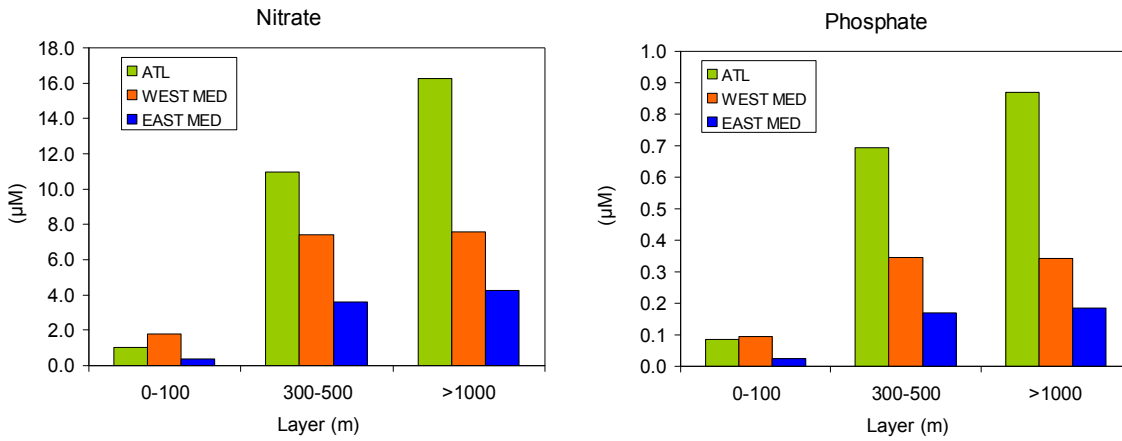


Fig. 3.8. Average nitrate (left) and phosphate (right) concentrations in Atlantic station (VA), Western (V1, V2, V3, V4) and Eastern Mediterranean (V6, V7, V8, VIERA, V10) in the upper (0-100 m), intermediate (100-500 m) and deep layers (>1000m).

Nitrate/phosphate ratio in the Mediterranean ranged between 5 and 30. The slope of the regression line was 21, as shown in Fig. 3.9. At station VA, below the euphotic zone, the N:P ratio was around 15.

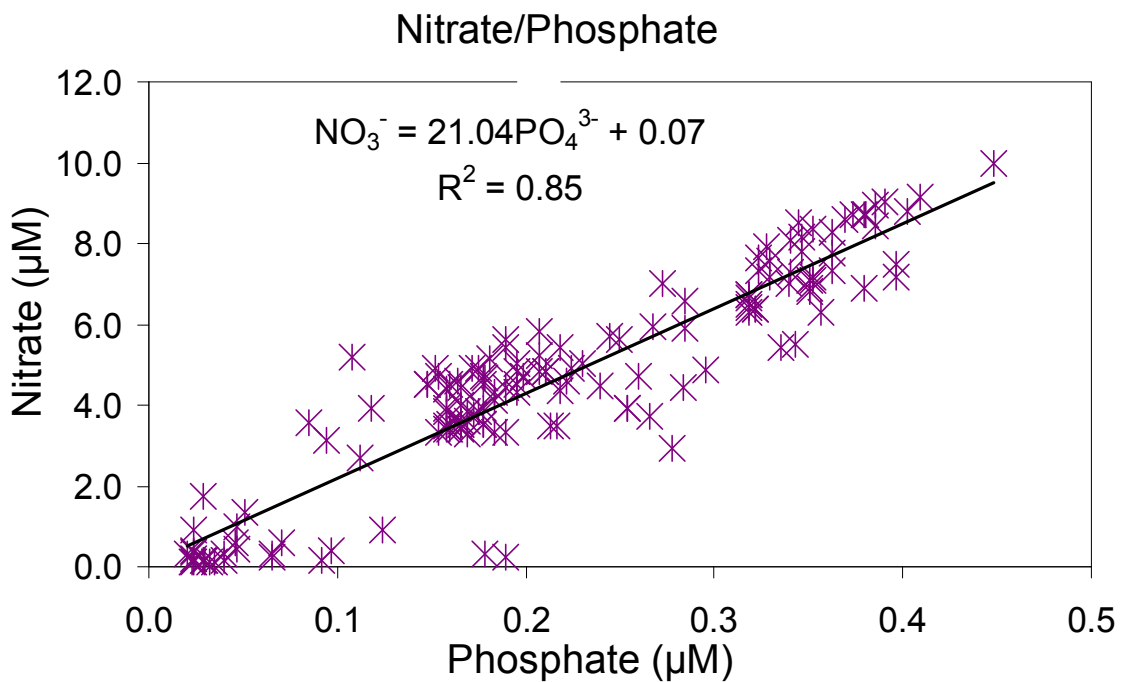


Fig. 3.9. Nitrate to phosphate ratio for all data in the Mediterranean Sea. Note that Atlantic station is not included in the calculation.

RESULTS

As reported above for nutrients, physical and chemical properties of the water masses allow to identify three different blocks: Western Mediterranean, Eastern Mediterranean and Atlantic (Fig. 3.10, Fig. 3.11).

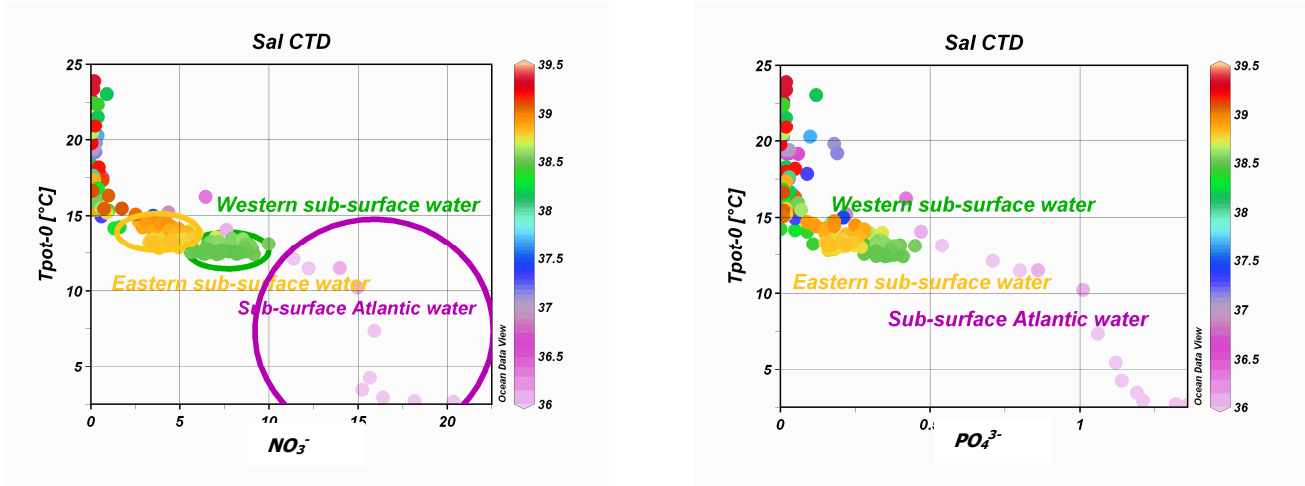


Fig. 3.10. Potential temperature versus nitrate (left) and phosphate (right) concentrations. Salinity is evidenced by the colour bar. Sub-surface water masses of the Eastern, Western Mediterranean and Atlantic ocean are clearly identified.

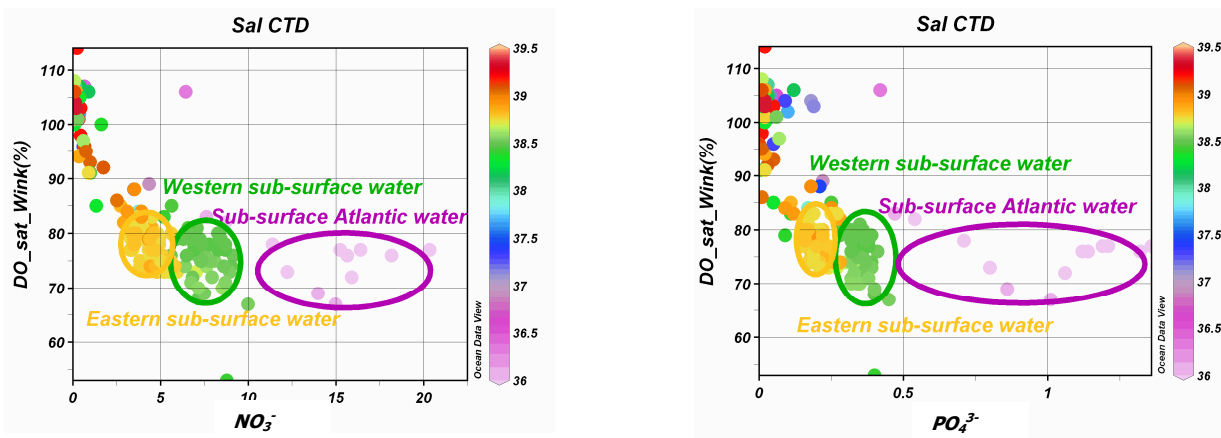


Fig. 3.11. Oxygen saturation versus nitrate (left) and phosphate (right) concentrations. Salinity is evidenced by the colour bar. Sub-surface water masses of the Eastern, Western Mediterranean and Atlantic ocean are clearly defined.

For this reason, hereafter the analysis will focus on the different features in stations grouped by sub-basins.

An overall interpretation of the general patterns observed in the sampled areas will be given in the Discussion chapter. In order to highlight peculiar features, that will be

mentioned in the next chapter, physical and chemical characteristics of selected key stations will be described in detail.

Atlantic station (VA)

The Atlantic site differed greatly from the Mediterranean stations. Physical and biogeochemical parameters displayed wider ranges throughout the water column. Here, minimum values of salinity were recorded. The thermocline was deeper, and the oxygenated mixed layer thicker with respect to the Mediterranean stations. Along the water column, a discontinuity in temperature, salinity and oxygen was detected in the layer 750-1000 m. The slight increase of salinity (36.05), temperature (11 C) and the minimum of oxygen saturation probably indicate the signature of a Mediterranean anticyclonic eddy (Meddy).

The nutrients profile follows the physical structures. The upper layer was completely depleted in nitrate and phosphate down to 100 m, where the concentrations increased at the Deep Chlorophyll Maximum (DCM) depth, (see the fluorescence maximum in Fig. 3.18 in the next paragraph). Nitracline and phosphocline were located at the same depth.

Alboran station (V4)

The Alboran station was strongly affected by lateral water injections coming both from the Atlantic and the Mediterranean interior. The complex dynamics of this area was evident by the step-like salinity profile with a shallow mixed layer at 40 m. This layer reflects the Atlantic inputs, as temperature, oxygen and salinity are very close to the values observed out of Gibraltar Strait. In the layer 300-500 m, the abrupt decrease of oxygen saturation associated to a maximum of salinity and inorganic nutrients reflect the

presence of the LIW and, likely, the oxygen utilization due to intense biological activity in that area.

Nitrate was almost absent down to 40 m, following the salinity step-like profile below the mixed layer, while phosphate displayed relatively high concentration levels in the upper layer. The two DCM observed at 40 and 100 m corresponded to a double nutricline (see Fig. 3.18 in the next paragraph).

Ligurian station (V1)

The V1 was the most eutrophic station sampled during the TRANSMED cruise. Shallowest DCM depth (40 m) and highest chlorophyll concentration were observed here (Fig. 3.18). While in subsurface layer physical and chemical profiles did not differ from the other western stations, a larger availability of nutrients and PON was observed in the euphotic zone.

Tyrrhenian station (V2)

The Tyrrhenian station showed intermediate characteristics between the Western and the Eastern Mediterranean. DCM depth and nutrient concentrations were close to values recorded in the eastern basin, while temperature, salinity and oxygen saturation levels reflected the influence of AW.

Ierapetra station (VIERA)

In the eastern basin, the VIERA station presents a strong homogeneity in salinity down to 500 m depth, due to the Ierapetra anticyclonic gyre often observed in this region. Both temperature and salinity profiles showed a deep penetration of sub-surface water. At 500 m depth, temperature and salinity values displayed strong deviations from the surrounding stations (differences of 3-4 C and 0.20, respectively). The presence of the anticyclonic gyre induced an homogeneity of water properties and a downwelling of the

isopycnals at the center of the gyre. Nutrients and oxygen were approximately constant down to 500 m, and displayed a strong discontinuity below the mixed layer.

3.2 $\delta^{15}\text{N-NO}_3^-$, $\delta^{15}\text{N-PON}$ suspended

$\delta^{15}\text{N-NO}_3^-$ values ranged between -1.42 and 2.03‰. Vertical profiles (Fig. 3.12) do not show a clear relationship with the water masses. In the euphotic zone, where highest variations are typically recorded, nitrate concentrations were often very low, below the detection limit of the nitrate diffusion method ($\sim 1\mu\text{M}$).

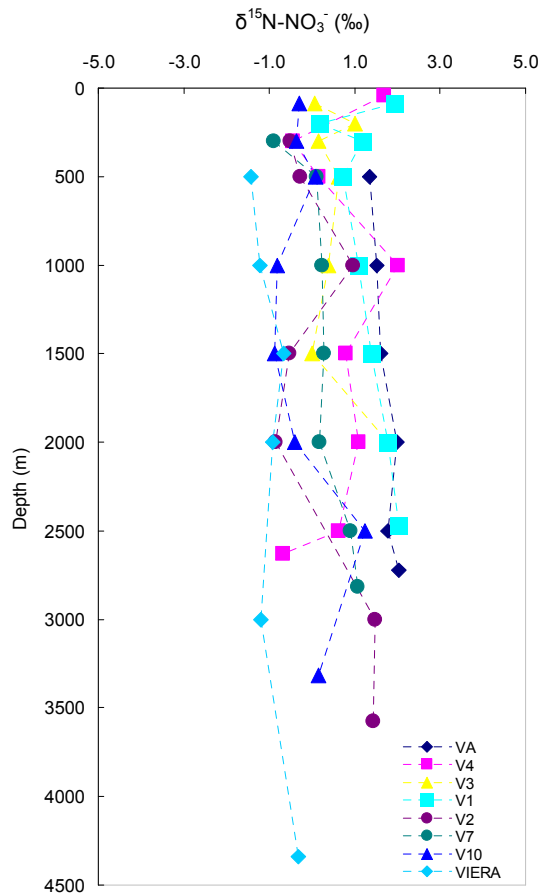


Fig. 3.12. Vertical distributions of $\delta^{15}\text{N-NO}_3^-$ along the water column, in all stations.

In two (V4, V1) of the three stations, where samples within the euphotic layer were available, a slight enrichment in $\delta^{15}\text{N-NO}_3^-$ corresponding to nitrate decrease was evident,

moving from depth to surface (Fig. 3.13).

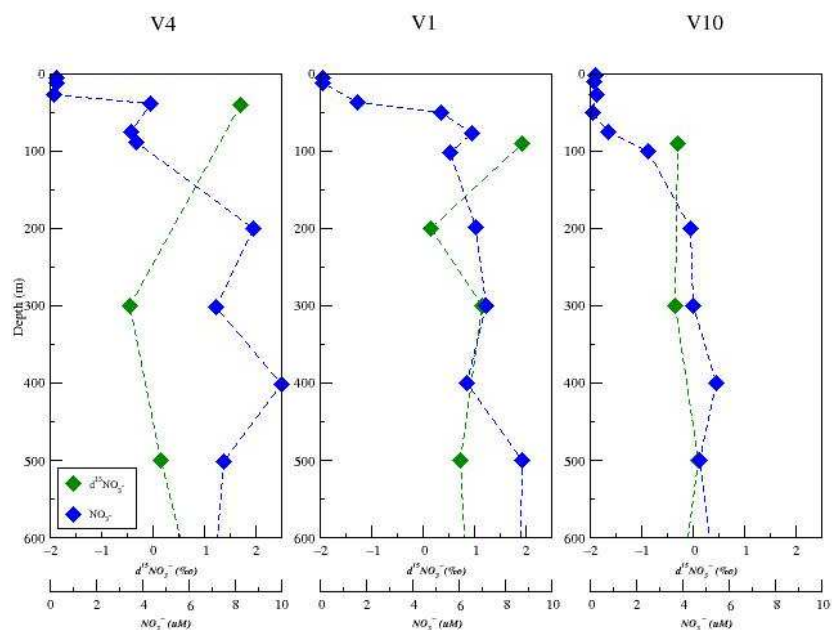


Fig. 3.13. Vertical profile in the first 600 m of the stations V4, V1 and V10.

The comparison between NO_3^- and $\delta^{15}\text{N-NO}_3^-$ (Fig. 3.14, Fig. 3.15, and Fig. 3.16) shows that heavier isotopic values were recorded in the Atlantic Ocean (median=1.69‰) and lighter in the Eastern Mediterranean (median=-0.34 ‰) corresponding to higher and lower nitrate concentrations, respectively. However, the correlation ($R^2=0.34$, Fig. 3.14) between NO_3^- and $\delta^{15}\text{N-NO}_3^-$ was too low in order to infer a dependence between the two parameters.

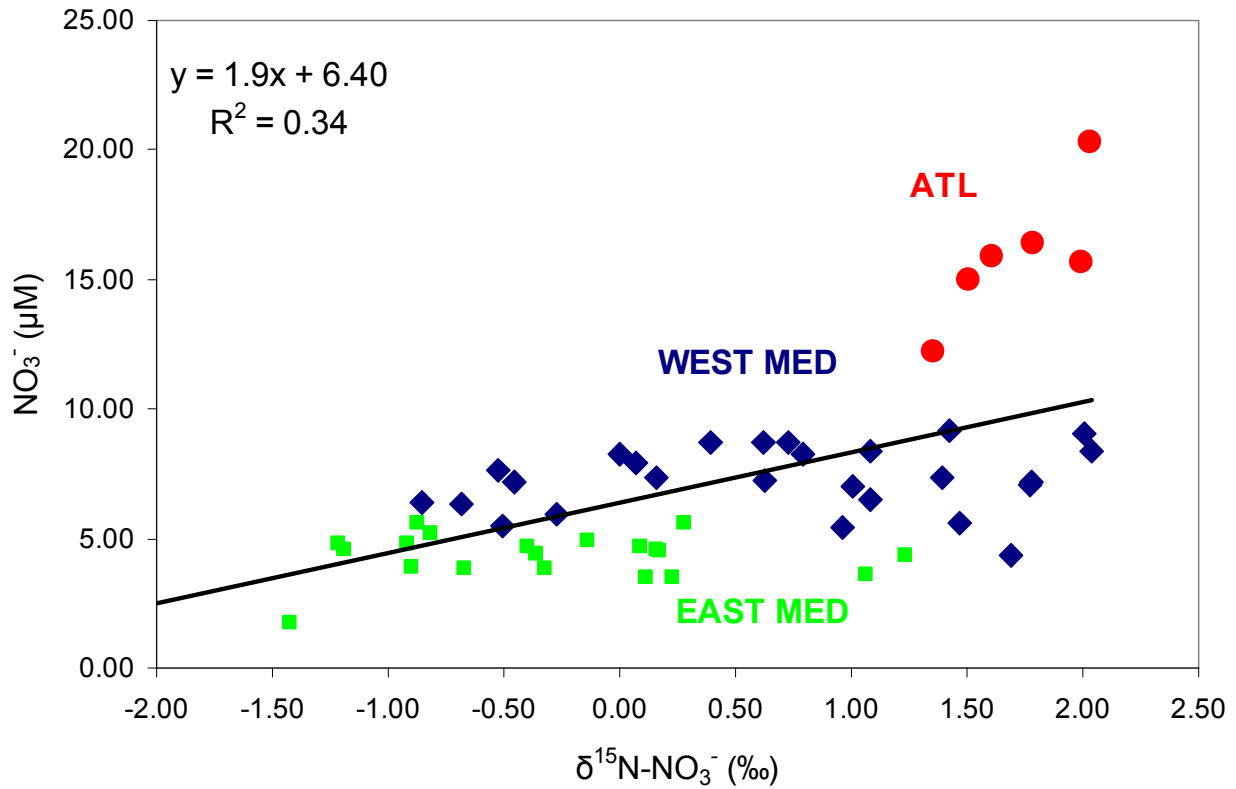


Fig. 3.14. Comparison between $\delta^{15}\text{N-NO}_3^-$ and NO_3^- . Stations are grouped in sub-basins.

$\delta^{15}\text{N-NO}_3^-$ data from Eastern Mediterranean and Atlantic stations presented lower vertical variability (if we exclude station V6 were only 2 data were available), in comparison with the western stations (Fig. 3.15).

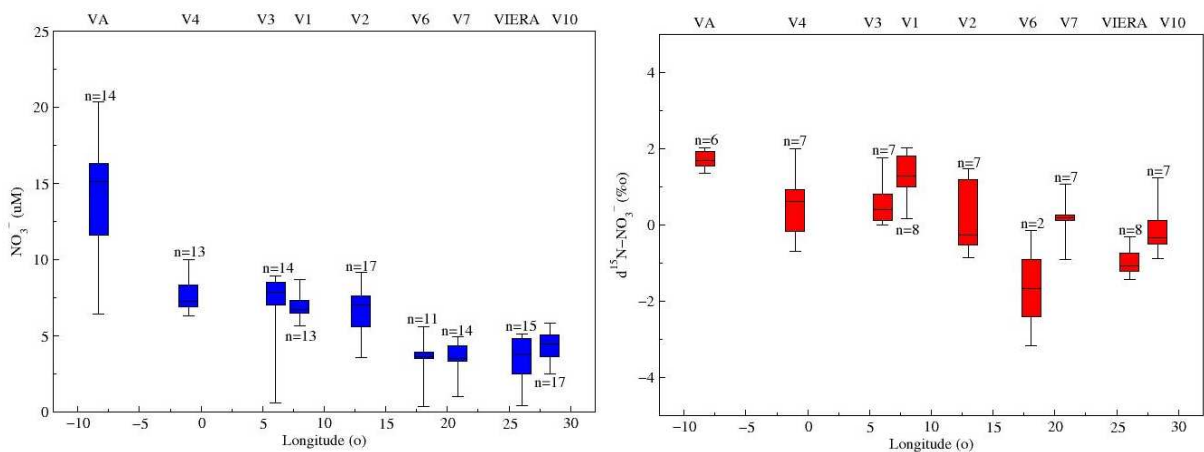


Fig. 3.15. Box plot of NO_3^- (left), and of $\delta^{15}\text{N-NO}_3^-$ (right) for all stations. Samples refer to depth below 300 m.

On basin scale, a longitudinal W-E decreasing trend is visible (Fig. 3.16).

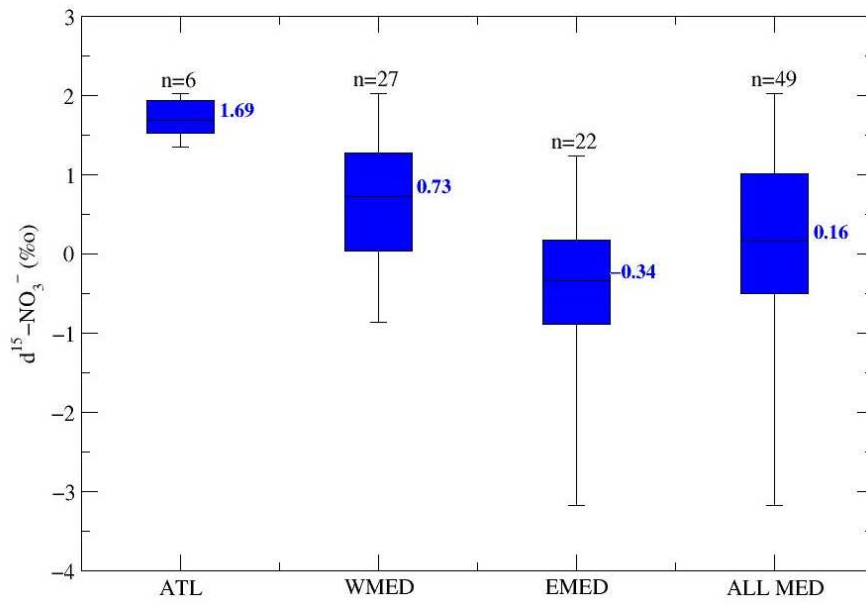


Fig. 3.16. Box plot of $\delta^{15}\text{N-NO}_3^-$ samples deeper than 300 m in specific areas.

$\delta^{15}\text{N-PON}$ ranged between -2.20 and 7.17 ‰. In almost all stations, $\delta^{15}\text{N-PON}$ displayed lowest values in the layer 0-100 m (Fig. 3.17).

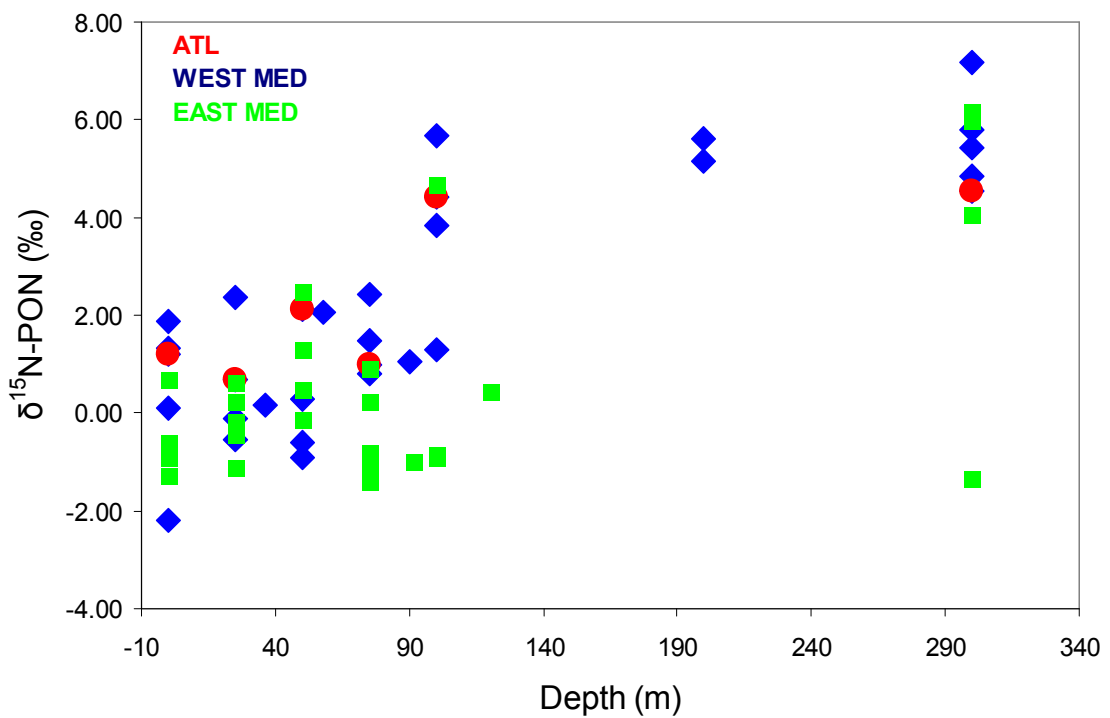


Fig. 3.17. Vertical profiles of all $\delta^{15}\text{N-PON}$. Data are subset for different areas.

Corresponding to the base of the DCM, an inflexion of $\delta^{15}\text{N-PON}$ is often evident (Fig. 3.18). This depth often coincides with the base of the euphotic zone (see fluorescence profile) and the starting point of the nitracline. The PON vertical profiles show, as expected, higher concentration in the upper layer. In the eastern stations the decrease below the euphotic layer is not evident like in the western stations. This feature will widely discussed in the following chapter.

As mentioned above, $\delta^{15}\text{N-NO}_3^-$ data were very scarce in the surface layer, thus it was not possible to provide a relationship between $\delta^{15}\text{N-PON}$ and $\delta^{15}\text{N-NO}_3^-$. However, at stations V4, V1 and V10, where surface $\delta^{15}\text{N-NO}_3^-$ was measurable, $\delta^{15}\text{N-NO}_3^-$ was similar to $\delta^{15}\text{N-PON}$ (Fig. 3.13).

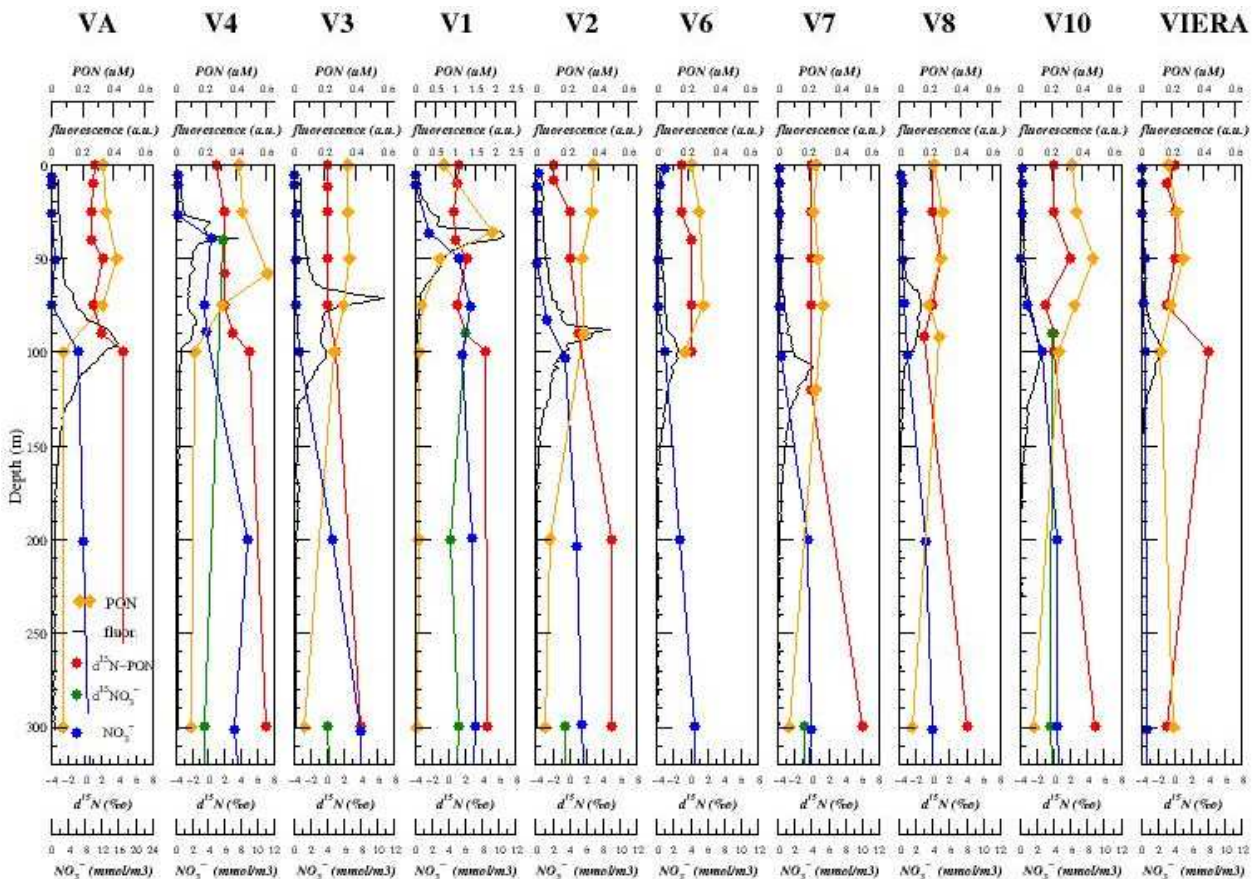


Fig. 3.18. Depth profiles of fluorescence, NO_3^- , PON, $\delta^{15}\text{N-PON}$ and $\delta^{15}\text{N-NO}_3^-$ in all stations sampled, in the upper 300 m. Note that at the station V1 the fluorescence and PON scales are different from the other stations.

3.3 N₂ fixation rates

N₂ fixation rates were generally low, ranging from 0.009 to 0.300 nmoles N l⁻¹d⁻¹ (Figure 3a). Of 84 samples, 26 (31%) were below the detection limit of the method.

The highest N₂ fixation rates were measured at VA (0.300±0.115 nmoles N l⁻¹d⁻¹). At this station only, seawater was pre-filtered on 100 µm Nitex-net. N₂ fixation might thus be underestimated as the contribution of larger cyanobacteria is not included. Maximum values were found at 10 and 50 m depth, with DCM values undetectable. In contrast, rates in the Mediterranean were uniformly lower (0.052±0.031 nmoles N l⁻¹ d⁻¹) and scarcely varying with depth and longitude.

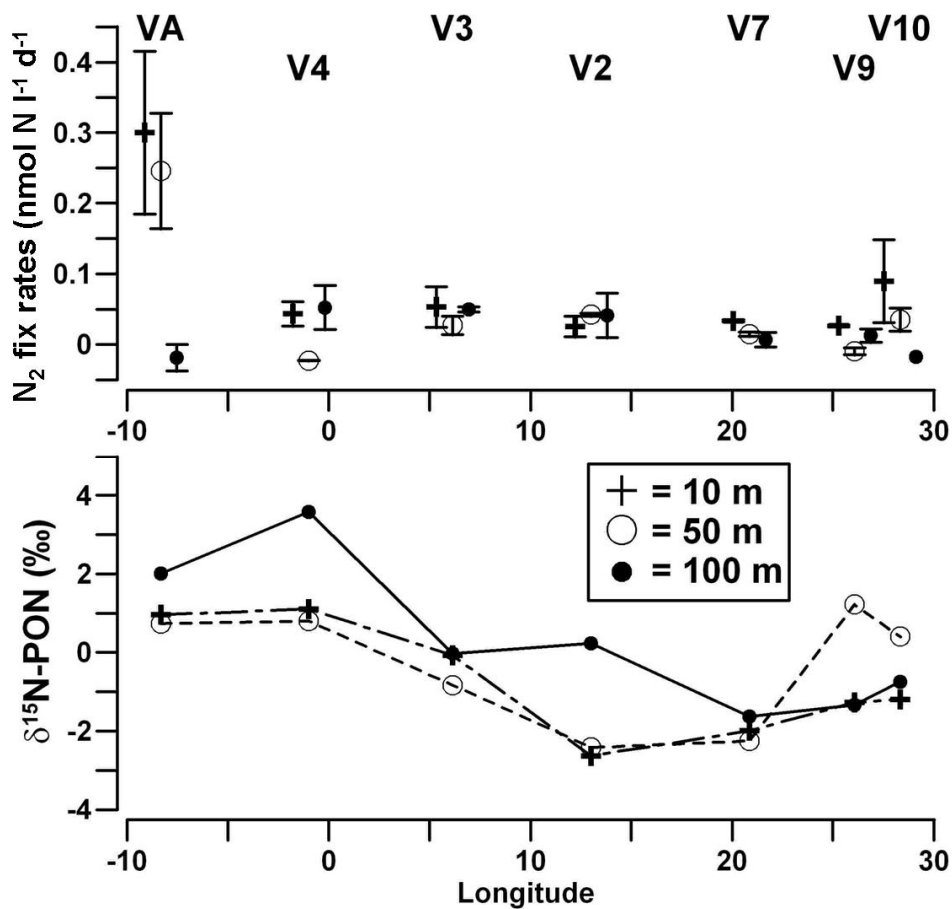


Fig. 3.19. N₂ fixation rates (average value and error bars = 2σ; n = 2) (upper graph), and δ¹⁵N-PON of suspended particulate organic matter in blank samples (lower graph). Station V9 coincided with VIERA.

At VA the integrated value of N₂ fixation rate was 17.6 $\mu\text{mol N m}^{-2} \text{d}^{-1}$, with a range of 1.0–4.0 $\mu\text{mol N m}^{-2} \text{d}^{-1}$ at the Mediterranean stations (Table 1). The range of contribution of the fraction <10 μm to N₂ fixation in the western stations and at coastal stations V10 was 18–46%. At stations V7 and V9, where N₂ fixation values were particularly low, the importance of the small fraction increased to 57-65%.

Natural $\delta^{15}\text{N}$ -PON ranged between -2.10 and +4.11‰ (Figure 3b), with a general decreasing trend from west to east. The major differences between deeper (100 m) and shallower (10 and 50 m) data were found at stations V4 and V2. The integrated values of total PON in the layer 0-100 m ranged from 26.1 to 61.2 mmol N m^{-2} , with the highest values at station V4 (Table 1). The fraction >10 μm always prevailed in the PON pool (77-80%).

Station	PON (TOT)	PON (<10 μm)	N ₂ -fix (TOT)	N ₂ -fix (<10 μm)
	mmol N m^{-2}	mmol N m^{-2} (%)	$\mu\text{mol N m}^{-2} \text{d}^{-1}$	$\mu\text{mol N m}^{-2} \text{d}^{-1}$ (%)
	26.1*	N.A.	17.6*	N.A.
V4	61.2	47.5 (78)	2.5	0.5 (18)
V3	48.2	37.3 (77)	3.7	1.7 (46)
V2	44.2	34.0 (77)	3.3	1.1 (32)
V7	38.0	30.3 (80)	1.6	0.9 (57)
V9	36.2	28.4 (78)	1.0	0.7 (65)
V10	53.9	41.4 (77)	4.0	1.0 (25)

*Size fraction <100 μm

Tab. 3.1. Integrated values of suspended particulate organic nitrogen (PON) and N₂ fixation (N₂-fix) rates in the euphotic layer for the total PON and the fraction <10 μm .

3.4 Conclusions

The biogeochemical peculiarities observed in the Mediterranean Sea, namely the west-east decreasing gradient in nutrients concentration, high nitrate/phosphate ratios in subsurface waters, and light $^{15}\text{N}/^{14}\text{N}$ ratios in PON and DIN, are confirmed by the present study.

Depletion of nutrients was overall present in the Mediterranean surface waters. Phosphate was often below the detection limit in the Eastern Mediterranean.

Values of $\delta^{15}\text{N-NO}_3^-$ in subsurface waters (> 300m) ranged between -1.42 and 2.03‰, with highest values in the Atlantic Ocean. A decreasing gradient was observed moving from west to east.

$\delta^{15}\text{N-PON}$ varied between -2.20 and 7.17 ‰ and showed a strong vertical variability. Lower values were observed in the surface layer, where nitrate concentration decreased.

Very low N_2 fixation rates (0.052-0.031 nmols $\text{N l}^{-1}\text{d}^{-1}$) were observed in all sub-regions of the Mediterranean, in contrast to the higher values measured in the North Atlantic (0.300-0.115 nmols $\text{N l}^{-1}\text{d}^{-1}$).

4. DISCUSSION

In this section, starting from the results described in the previous chapter, some of the crucial questions on nitrogen cycle in the Mediterranean Sea will be discussed.

Data produced during this study are to a certain extent new for the basin. Most of the parameters have already been measured before, but not on a synoptic basin scale and not at the same time. This allowed to better link the different processes governing the nitrogen dynamics in the basin, and to make some inferences on the interplay between P and N cycle.

This last chapter of the thesis is far from being an exhaustive discussion of all the implications and possible scenarios following the experimental results. Rather, it will address the key questions at the basis of the study, discussing how the data improve our understanding of the processes and, more important, formulating new testable hypotheses for future research. These key questions can be summarized as follow: i) why N_2 fixation is not occurring in the basin (in June 2007)?; ii) how to explain the low isotopic signature in dissolved and particulate nitrogen? And which are the processes behind the east-west isotopic gradient?; iii) which are the reasons for the high N:P ratio in intermediate and deep layers?; iv) how these altered ratios impact the ocean's ability to sequester carbon?

4.1 N_2 fixation

In the Atlantic Ocean, depth-integrated rates of N_2 fixation reported in this work are in agreement with other studies in the region [Voss *et al.*, 2004]. In contrast, values reported for the Mediterranean do not confirm the hypotheses that attribute to N_2 fixation a relevant role in supplying new nitrogen [Bethoux and Copin-Montegut, 1986; Sachs and Repeta, 1999; Pantoja *et al.*, 2002]. Previous studies attempting to directly quantify N_2 fixation in the Mediterranean have been spatially limited, thus no areal estimates have ever been

provided. *Rees et al.* [2006] reported a mean N_2 fixation rate of $129 \pm 9 \text{ nmol N l}^{-1} \text{d}^{-1}$ for few stations in an anticyclonic eddy in the Levantine basin during a phosphorus fertilization experiment. *Sandroni et al.* [2007] found much lower rates ($0\text{-}17 \text{ nmol N l}^{-1} \text{d}^{-1}$) and high seasonal variability at the DYFAMED station in the North-Western Mediterranean. Molecular identification and expression of *NifH* genes have been found by *Man-Aharonovich et al.*, [2007] off the Israeli coast. A high diversity of diazotrophs, with a dominance of the cyanobacterium *Richelia intracellularis* associated with the diatom *Hemiaulus hauckii*, has been observed by *Zeev et al.* [2008]. However, the *Richelia-Hemiaulus* abundance and N_2 fixation rates ($\sim 1.1 \text{ nmol N l}^{-1} \text{d}^{-1}$) reported there were similar to what observed in areas without any diazotroph bloom. So, if we exclude data from *Rees et al.* [2006], who presented rate measurements that are among the highest values reported for the world ocean, all the other measurements reported for the Mediterranean are quite low. Indeed, there is good reason to believe that the conditions under which Rees' incubations were carried out were not perfectly under control, resulting in anomalous high rates. Moreover, the samples were collected essentially from one single station.

Measurements carried out in this study established an average areal rate of $2.68 \text{ } \mu\text{mol N m}^{-2} \text{d}^{-1}$, corresponding to a nitrogen input for the whole basin of $2.45 \cdot 10^9 \text{ mol-N/yr}$, which is significantly lower than the estimate proposed by *Bethoux and Copin-Montegut*, [1986].

In order to explain the reasons behind the low rates observed, it is important to focus on the factors controlling N_2 fixation. In the World Ocean, phosphorus and iron deficiency are known to limit N_2 fixation [*Falkowski and Davis*, 2004; *Mills et al.*, 2004]. In addition, as N_2 fixation is a high energy consuming process, depletion of dissolved inorganic nitrogen seems to be an essential condition for the diazotroph growth. Below, on the basis of the present and previous studies, different roles played by these three factors are discussed.

As the Mediterranean Sea has been described as a phosphate-depleted basin [Krom *et al.*, 1991; Ribera d'Alcalà *et al.*, 2003], phosphorus has often been considered a limiting nutrient for N₂-fixers. In this study, we measured variable phosphate concentrations in the euphotic layer but, as the values were often close to the detection limit (0.02 µM), it is not easy to determine a relationship between N₂ fixation rates and phosphate concentrations. Another study carried out in the Mediterranean has also reached similar conclusions [Rees *et al.*, 2006; Sandroni *et al.*, 2007]. Dissolved organic phosphorus (DOP) has been considered an alternative source of phosphorus for N₂-fixers [Mather *et al.*, 2008] but Ibello *et al.* [2009] showed that even in the nutrient-depleted surface water with DOP levels always higher than phosphate (70% of TDP), N₂ fixation was still very low.

The role of iron, the other possible limiting factor, remains uncertain for the Mediterranean. It is known that Saharan dust deposition transfers abundant iron to the Mediterranean [Guerzoni *et al.*, 1999], but particular conditions, such as spring blooms and absence of dust inputs, may also lead to extreme iron depletion [Sarhou and Jeandel, 2001].

Iron was not measured during the TRANSMED cruise, so no information can be provided about that. The only information available comes from satellite observations that confirms the absence of relevant Saharan dust deposition events over the sampling areas during the cruise (Volpe, personal communication). This could suggest a possible lack of iron in the surface waters, but it is relevant to mention that Statham and Hart, [2005] found, in the Eastern Mediterranean, elevated Fe-concentrations in the mixed layer in two seasons (June and September) and attributed them to Saharan dust, even in the absence of dust storms.

Finally, a high nitrate concentration in the marine environment is considered another inhibitory factor for some diazotrophs [Holl and Montoya, 2005]. Indeed, in this study low nitrate concentrations are reported, as well as the absence of a systematic relationship

between nitrate concentrations and N₂ fixation. In addition, the highest N₂ fixation rates observed in the Atlantic (VA) were not associated with different nitrate/phosphate or TDN/TDP ratios because at this station nutrient ratios were within the Mediterranean Sea ranges (5-30 for nitrate/phosphate and 35-82 for TDN/TDP), as reported by *Ibello et al.* [2009].

4.2 P-limitation: cause or consequence of N₂ fixation?

P-limitation in the Mediterranean Sea, observed and theorized for the first time by *Krom et al.*, [1991], could be determined by diazotroph blooms, occurring after the classical phytoplankton bloom. After the first step, indeed, surface waters would remain enriched in phosphorus and poor in nitrogen, creating exceptional positive conditions for N₂ fixation occurrence, and triggering the system to switch from N-limitation to P-limitation [*Mills et al.*, 2004]. This scenario could have reasonably happened in the Mediterranean, but the results presented here showed very low N₂ fixation rates, not only in phosphate depleted areas, where the lack of P could easily explain diazotroph inhibition, but also in sites where phosphate was still available. It follows that, concerning the specific situation observed in June 2007, N₂ fixation should not be invoked as a consequence of the extensive P depletion observed in the basin. Although this study has focused on the impact of N₂ fixation only for one season (late spring), it is possible to speculate that N₂ fixation covers a limited importance for the biogeochemistry of the Mediterranean and does not explain the P-limitation often reported in literature.

An alternative mechanism to justify P-limitation is connected to elevated and constant rates of N atmospheric deposition all over the year. Other oligotrophic areas have showed an important impact of atmospheric deposition on primary production [*Owens et al.*, 1992]. In the Mediterranean, except the areas where deep convection occurs, (limited to 3 sites:

Gulf of Lyon, South Adriatic and Rhodes Gyre), the annual productive cycle is more similar to tropical areas, with an annual production constantly low [Moutin and Raimbault, 2002; Turley *et al.*]. Primary production is based on the nutrients injected into the surface layer by the winter mixing and on nutrients recycled within the euphotic zone. The excess of N in surface waters due to the atmospheric deposition and the mixing waters leads to a P-limited production. The lack of inorganic P, observed many times in the euphotic zone [Krom *et al.*, 1991], can depend on the export of sinking particles containing P and/or on a multiple recycling of organic P, which would lead to an accumulation of refractory DOP.

In the Mediterranean Sea, P-limitation, probably connected to N atmospheric deposition and multiple reuse of P, results from a complete utilization of P compared to other N-limited areas. Therefore, the resulting primary production leads to an increase of the organic N, and eventually to a $\text{NO}_3^-:\text{PO}_4^{3-}$ ratio in the nutrients bulk exceeding the elemental Redfield proportions. Due to this mechanism, the Mediterranean ecosystem results particularly efficient in transferring all the P from the surface to depth, relatively more efficient than other oceanic regions.

In conclusion, P-limitation is neither the cause nor the consequence of N_2 fixation. The hypothesis proposed here is that the observed low nitrogen fixation rates could be explained by high rates of nitrogen atmospheric deposition. As a consequence, ecological competition between small phytoplankton fed by nitrate/ammonium and diazotrophs is won by the former.

4.3 Low $^{15}\text{N}/^{14}\text{N}$ ratios in dissolved and particulate compounds

In oceanic environments, particulate organic nitrogen (PON) is mainly derived from phytoplankton, micro-zooplankton, bacteria and detritus. The nitrogen isotopic signature of PON in suspended matter depends on the isotopic fractionation associated with its

formation, and in turn upon the isotopic composition of inorganic form of dissolved nitrogenous sources available to the phytoplankton.

Present results of $\delta^{15}\text{N-PON}$ (Fig. 4.1) substantially confirm previous values reported by *Pantoja et al.*, [2002] (-0.3 to +2.4 ‰). The values in the western stations reported by the authors were higher compared to those in the present study. However, it is relevant to know that most of their western samples were collected in the Alboran Sea, characterized by significant vertical and horizontal dynamics and where the influence of the Atlantic water is stronger with respect to the western stations considered in the present work.

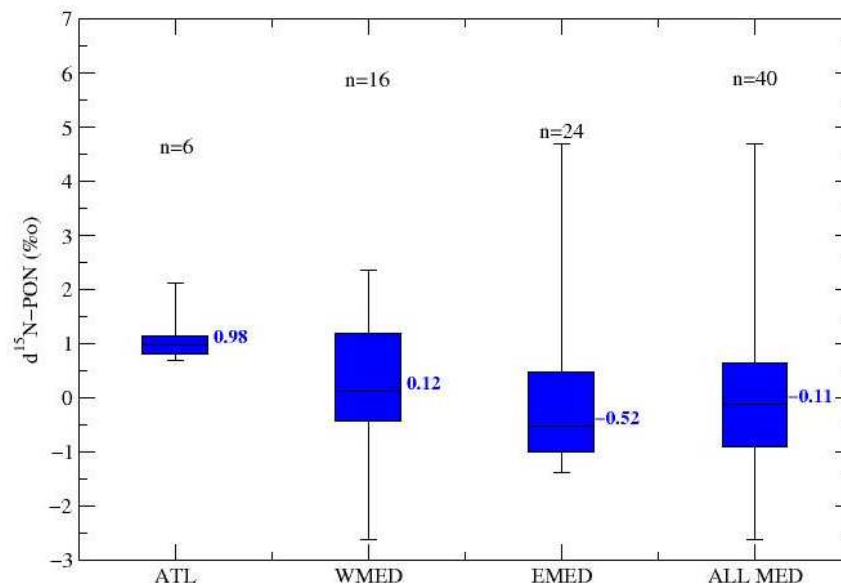


Fig. 4.1. Boxplot of $\delta^{15}\text{N-PON}$ values in the layer 0 m-nutricline.

Moreover, the $\delta^{15}\text{N-NO}_3^-$ values presented here are generally lower than the data reported by *Pantoja et al.* [2002]. As already observed for $\delta^{15}\text{N-PON}$, the discrepancy can be easily explained by the different sampling areas chosen in the two studies. The average value reported for the western basin by *Pantoja et al.*, [2002] referred to 8 stations (61 samples) all located in the Alboran Sea, and highly influenced by Atlantic water inputs. In addition, the resulted average of 3.4‰ was calculated taking into account both deep and surface samples, possibly affected by phytoplankton fractionation processes.

Since the low nitrate surface concentration, the selected data for this study came generally from below the euphotic layer, in four stations characterized by different water mass dynamics and nutrients concentration. For the Eastern Mediterranean only few data were previously reported (4 by *Pantoja et al.* [2003] and 2 from *Sachs and Repeta* [1999]), so the present data collection can be considered more representative than the few fragmented datasets reported in the literature.

$\delta^{15}\text{N-NO}_3^-$ (‰)					
	ATL	WMED	EMED	ALL MED	Reference
Mean (s.d.)	n.a.	n.a.	-0.7 (0.1)	n.a.	<i>Sachs and Repeta. 1999</i> ^a
<i>n</i>	n.a.	n.a.	2	n.a.	
Mean (s.d.)	n.a.	3.4 (0.5)	2.5 (0.1)	n.a.	<i>Pantoja et al. 2002</i> ^b
<i>N</i>	n.a.	61	4	n.a.	
Mean (s.d.)	1.71 (0.27)	0.65 (0.86)	-0.37 (0.97)	0.19 (1.03)	<i>this study</i> ^a
<i>N</i>	6	27	22	49	

Tab. 4.1. Comparison of mean and standard deviation (s.d.) values of $\delta^{15}\text{N-NO}_3^-$ available in the Mediterranean Sea. Data refer to different layers: ^a deep samples, ^b upper and deep samples, n.a. stands for not available.

A west-east decreasing gradient is evident both in $\delta^{15}\text{N-NO}_3^-$ and in $\delta^{15}\text{N-PON}$. *Pantoja et al.* [2002] attributed the west-east gradient to the rates of N_2 fixation, varying in the two sub-basins. As mentioned above, present results show low N_2 fixation rates in the whole Mediterranean and again low $\delta^{15}\text{N-PON}$ values, higher in the west than in the east.

How to explain this spatial variability? If low or no N_2 fixation is occurring, why the isotopic signals are so light?

Low $^{15}\text{N}/^{14}\text{N}$ ratio in suspended PON can result from a fractionation process and/or low $^{15}\text{N}/^{14}\text{N}$ in the nitrogen sources. During the assimilation of N by living organisms a fractionation can occur leading to a light isotope enrichment in the product (i.e.: phytoplankton). If no fractionation takes place, the product shows the same signature of the source.

PON synthesis is essentially connected to primary production, and results from the following processes: i) assimilation of deep nitrate, after the winter mixing (new

production); ii) assimilation of atmospheric nitrogen deposited on the sea surface (new production); iii) assimilation of ammonium excreted by zooplankton in the euphotic zone (regenerated production). Each of these processes uses a different nitrogen source (deep nitrate, deposited atmospheric N, ammonium). As observed in the present work, deep nitrate isotopic signature varies between -1.42 and 2.03‰ , whilst values for N from atmospheric deposition reported by *Mara et al.* [2009] for the Eastern Mediterranean are around -3.1‰ . Ammonium data for the Mediterranean are not available, but they are expected to be lower than the isotopic signature of zooplankton, ranging between 2 and 3 ‰, as reported by [*Koppelman et al.*, 2003]. Fig. 4.2 synthesizes schematically the isotopic signature of the sources involved in PON synthesis.

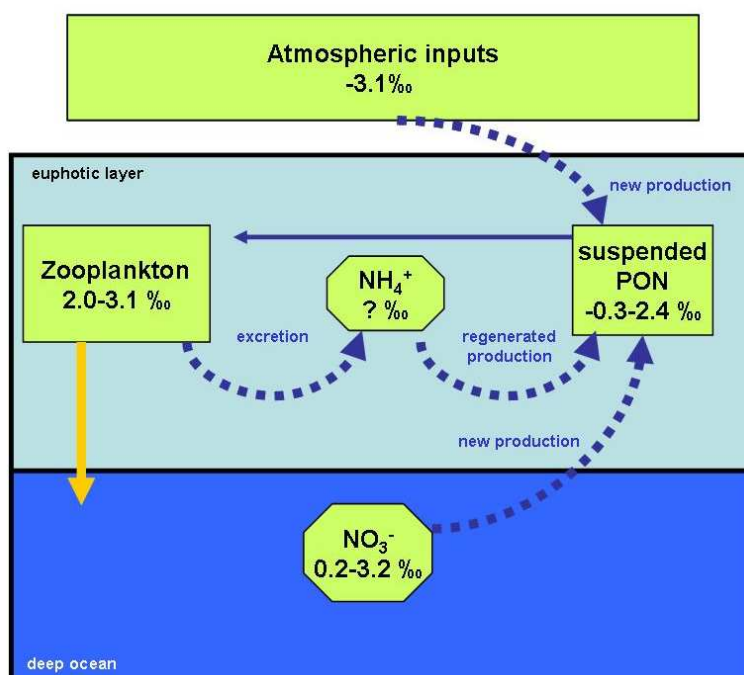


Fig. 4.2. Scheme of $\delta^{15}\text{N}$ nutrient sources and suspended PON available for the Mediterranean Sea. Assimilation processes determining isotopic enrichment of PON are indicated by arrows. Note that data relative to atmospheric deposition inputs [*Mara et al.*, [2009] and zooplankton [*Koppelman et al.*, 2003] refer to the Eastern Mediterranean. Analogous data for the western basin are not available. Data relative to isotopic signature of deep nitrate and suspended PON refer to data of present study and *Pantoja et al.* [2002]

It is expected that, depending on the relative importance of the processes producing PON, its isotopic signature should vary within the ranges above mentioned.

From the comparison of the $\delta^{15}\text{N}$ -PON median above the nitracline (defined as $\text{NO}_3^- = 1 \mu\text{M}$) and the $\delta^{15}\text{N}$ - NO_3^- average below the nitracline (300-500m), it seems that nitrate plays a relevant role in feeding phytoplankton, as evident by the patterns in Fig. 4.3. Isotopic signature of nitrate below the nitracline should represent the value not affected by biological fractionation occurring above the nutricline. These $\delta^{15}\text{N}$ - NO_3^- values are, at least in theory, the same isotopic values of nitrate entering the euphotic layer and used by phytoplankton for the assimilation.

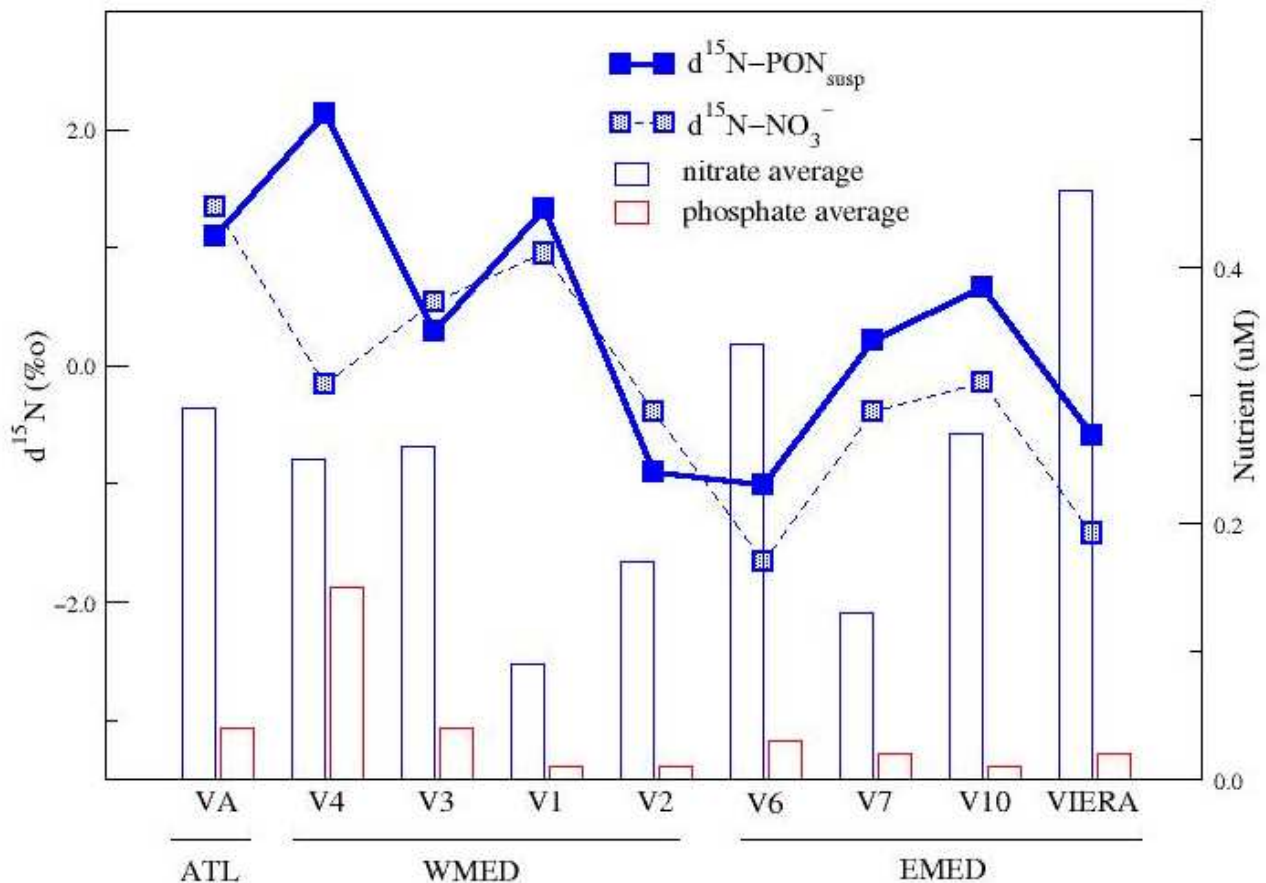


Fig. 4.3. Spatial variation of $\delta^{15}\text{N}$ -PON (blue continuous line), ^{15}N - NO_3^- (blue dashed line), nitrate (blue bar) and phosphate concentrations (red bar). $\delta^{15}\text{N}$ -PON values are the median in the zone above nitricline (defined as $\text{NO}_3^- = 1 \mu\text{M}$). $\delta^{15}\text{N}$ - NO_3^- values are the average of data below the nitricline, between 300 and 500 m. Nitrate and phosphate are the average concentrations above the nitricline.

Two different situations can be described: $\delta^{15}\text{N-NO}_3^-$ equal or slightly higher than $\delta^{15}\text{N-PON}$, and $\delta^{15}\text{N-NO}_3^-$ lower than $\delta^{15}\text{N-PON}$.

The former case, where the isotopic signatures of deep nitrate are equal or slightly higher than PON (stations VA, V3, V2), indicates that almost no fractionation is occurring, as showed by the signature of the source similar to the product. In this case, production would be based essentially on nitrate, thus new production would be an important fraction of the total PP. The slightly lower $\delta^{15}\text{N-PON}$ values at the station V2 could indicate both fractionation of nitrate or assimilation of ammonia. When nitrate is fractionated, phytoplankton uptakes first the light ^{14}N , producing a light PON and leaving a heavy substrate (NO_3^-), enriched in $\delta^{15}\text{N}$. The regenerated production, based on the assimilation of light ammonia excreted by zooplankton, will generate, analogously, a light isotopic PON. This process can be particularly relevant in the oligotrophic waters of the Mediterranean, as regenerated production greatly exceeds new production [Estrada, 1996].

The second case, where isotopic signature of nitrate is lower than PON, is observed in all the 4 eastern stations and at V4, in the Alboran Sea. Fluorescence profiles, smoothed chlorophyll peaks at the DCM and PON concentrations (Fig. 3.18) suggest that active production in these stations is low or absent. Phosphate concentrations are very close or below the detection limit, suggesting that production has been probably blocked by the lack of phosphate. PON would thus represent the residuals of a previous production based on nitrate or recycled ammonium, but not active anymore. The enzymatic reactions involved in the heterotrophic degradation of PON leave a substrate enriched in heavier isotope of ^{15}N .

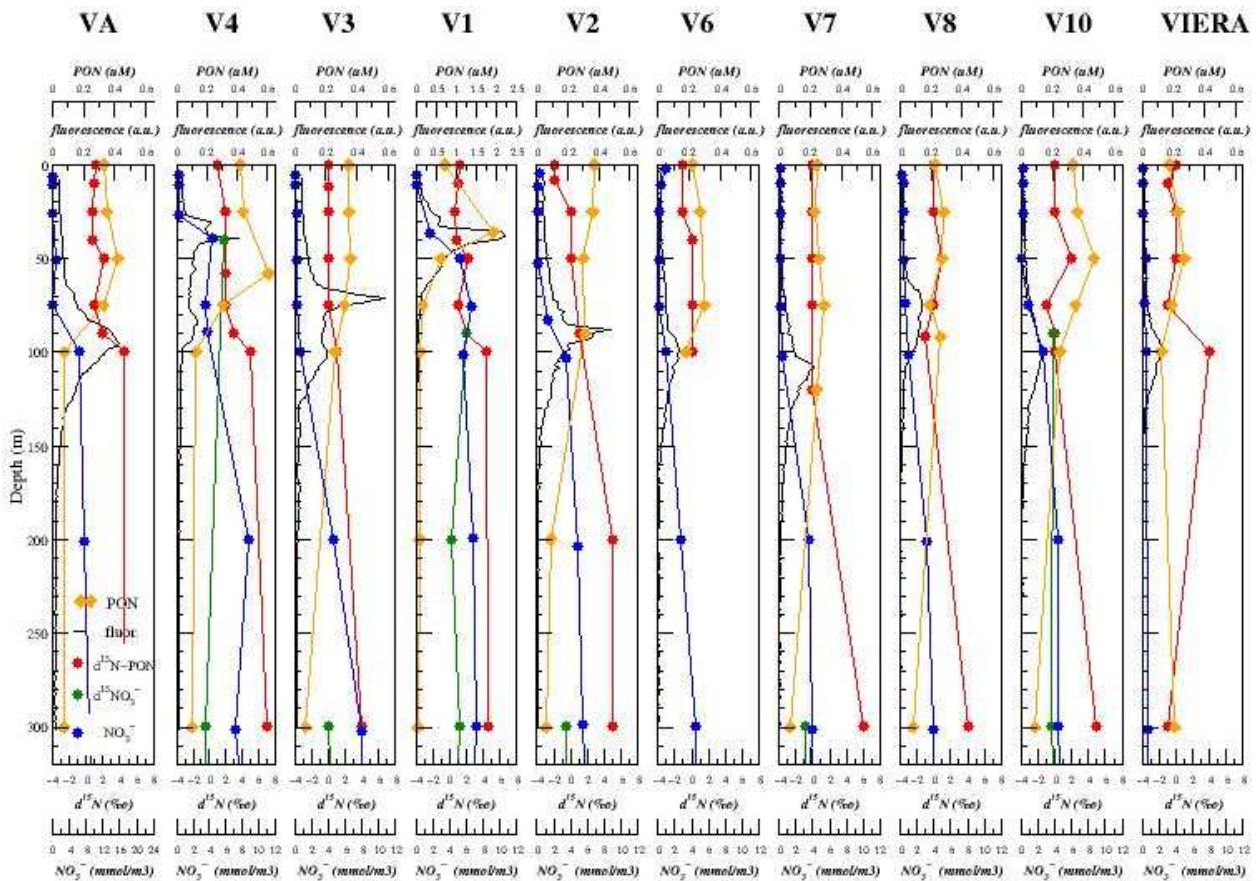


Fig. 3.18. Depth profiles of fluorescence, NO_3^- , PON, $\delta^{15}\text{N}$ -PON and $\delta^{15}\text{N}$ - NO_3^- in all stations sampled in the upper 300 m. Note that at the station V1 the fluorescence and PON scales are different from the other stations. The figure has been already shown in the chapter of Results. Here it is reported to facilitate the comprehension of the discussion.

This hypothesis is further confirmed by a peculiar feature observed in many western stations, showing a decreasing of $\delta^{15}\text{N}$ -PON in the euphotic zone from the DCM to the surface. The decrease of $\delta^{15}\text{N}$ -PON with respect to deeper samples, exactly at the DCM where both light and nutrients are not limiting, indicates an active production of fresh phytoplankton, in the euphotic zone. Heavier $\delta^{15}\text{N}$ -PON values in the layer below, coupled to a slight vertical profile, confirm an ongoing mineralization and absence of active production.

This reasoning does not fit with the station in the Alboran Sea (V4), that displays still high nutrient concentrations in the euphotic zone and a peak in the chlorophyll, indicating an active production of phytoplankton. As described in the chapter of Results, this station

is characterized by strong horizontal and vertical dynamics, so maybe the approximation that the nitrate below the euphotic layer displays the same signature of the nitrate entering the euphotic layer (adopted in the all other stations) could be inadequate here. In addition, the very shallow nitracline (29 m) observed at V4 and the $\delta^{15}\text{N-NO}_3^-$ (1.69 ‰) measured below the nitracline (40 m), higher than the values below (-0.2 ‰), suggest that the nitrate entering the euphotic layer could have a heavier isotopic signature.

However, the linear regression between the median values of $\delta^{15}\text{N-PON}$ in the euphotic layer above the nutricline and the $\delta^{15}\text{N-NO}_3^-$ value below the nutricline shows a high correlation index ($R^2=0.71$) suggesting that isotopic signature of deep nitrate affects substantially the PON formed in the euphotic zone.

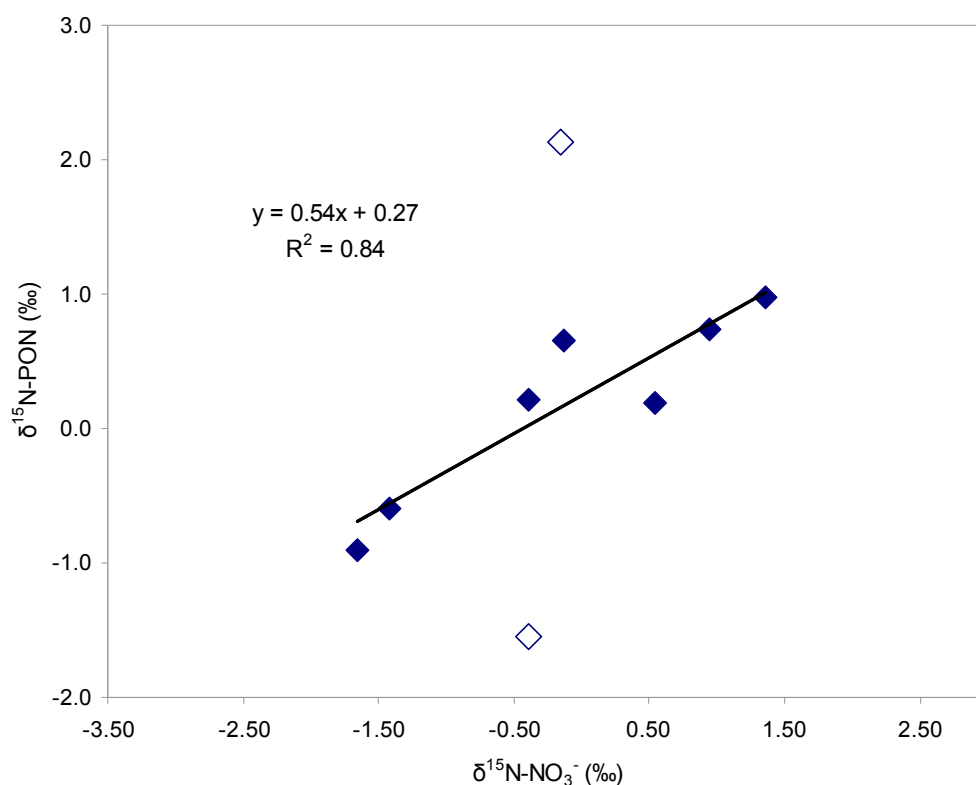


Fig. 4.4. Linear regression of $\delta^{15}\text{N-NO}_3^-$ average values below the nutricline (layer 300-500 m) and the median values of the $\delta^{15}\text{N-PON}$ in suspended matter above the nutricline. Empty diamonds represent the two stations V4 and VIERA excluded from the linear regression.

The east-west gradient in suspended $\delta^{15}\text{N-PON}$ has to be attributed to the isotopic signature of the deep nitrate and to its longitudinal variation.

A complete understanding of the impact of biological processes occurring in the euphotic zone to the deep nitrate would be clarified by measurements of sinking PON below the euphotic layer. Indeed, differently from suspended PON, sinking PON is the fraction of PON that contributes to the export of organic matter from the upper layer and affects the isotopic signature of the deep nitrate. A complete explanation of the east-west gradient of the isotopic signature of the deep nitrate cannot be provided with the present measurements. However, as it will be widely discussed below, the contribute of atmospheric deposition transferred by sinking PON, can affect nitrate signature on multidecadal time scale. Different intensity of the export rates such as different amount of atmospheric deposition can affect the transfer of the light atmospheric signature to the nitrate into the deep layers.

4.4 Evaluation of nitrogen sources

Despite dramatic variations of $\delta^{15}\text{N-NO}_3^-$ in the surface ocean caused by incomplete nitrate uptake and remineralization, nitrate assimilation has little effect on the global distribution of N isotopes. Complete nitrate utilization in most of the surface ocean and virtually complete remineralization in the subsurface prevents the accumulation of isotopically distinct non-nitrate pools. Dissolved organic nitrogen, the only other pool of appreciable size, has a very similar isotopic composition to that of nitrate where it has been measured (Sargasso Sea, *Knapp et al.*, 2005) and therefore can have a limited impact in this regard. In the global ocean, indeed, nitrate concentrations in the deep sea are so high that the small amount of low $\delta^{15}\text{N}$ organic matter supplied from the nutrient-rich surface has only a minimal effect on the $\delta^{15}\text{N}$ of deep ocean nitrate [*Sigman et al.*, 1999].

A different situation can be described for the Mediterranean. While average concentrations of nitrate in the global oceans vary between 30 and 40 μM , in the

Mediterranean nitrate values are almost one order of magnitude lower, varying between 3 and 9 μM . Thus, even though the impact of the $\delta^{15}\text{N}$ -PON sinking derived by an incomplete nitrate uptake and remineralization cannot be relevant, on a longer time scale the inputs of a very light external source of N assimilated by the phytoplankton would lower the total isotopic signature for an important fraction.

As suggested by *Sachs and Repeta* [1999], it is possible to assume that deep nitrate is a temporal and spatial integration of primary production in the basin for many decades. In the Mediterranean deep waters, the nitrate is the main reservoir of reactive nitrogen of the basin and its residence time ranges between 30 and 120 years [*Roether and Schlitzer*, 1991; *Roether et al.*, 1998]. This implies that nitrate should reflect the remineralization of sinking particles fed by surface N sources, that are atmospheric deposition, terrestrial inputs and surface Atlantic Water entering at Gibraltar Strait. Therefore, the isotopic signature of deep nitrate can be used to provide a rough evaluation of the importance of these N sources.

If we exclude N_2 fixation, the nitrogen sources are Atlantic Water (AW), atmospheric deposition (AI) and terrestrial inputs (TI). Due to the lack of available data, some simplifications need to be made. The isotopic signature of AW is $\sim 5\text{‰}$ [*Pantoja et al.*, 2002]. TI present many uncertainties but, referring to the paper by *Mara et al.* [2009] and references therein, we took a value of 5‰. AI have been measured by *Mara et al.* [2009] for the Eastern Mediterranean (-3.1‰). We took the same value for the Western basin since isotopic values for AI are not available.

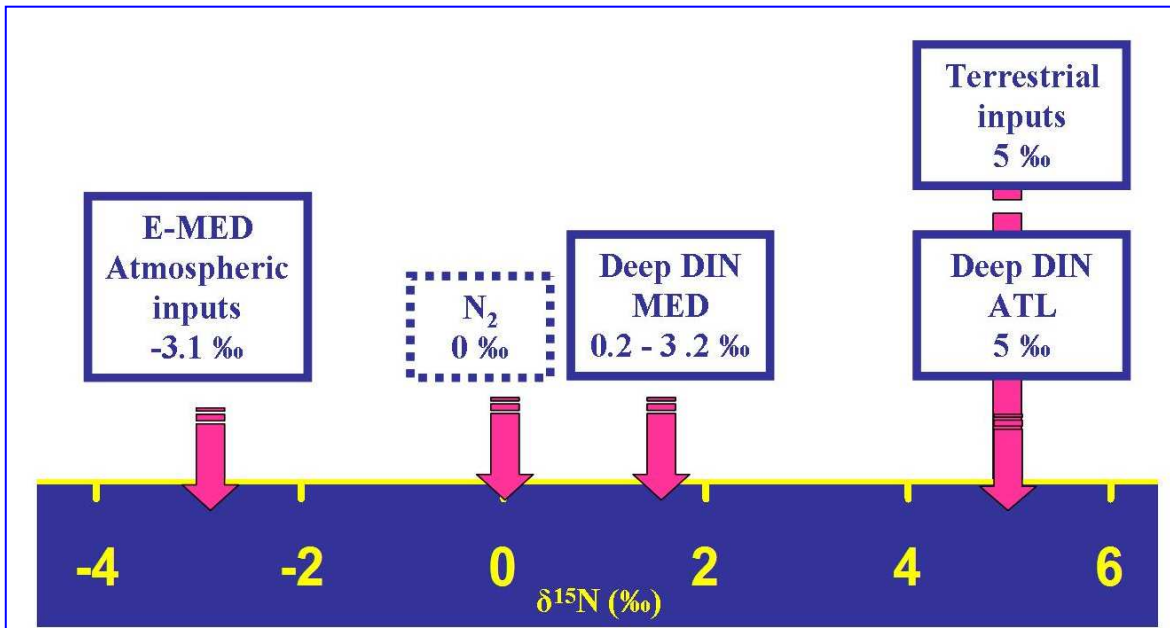


Fig. 4.5. Scheme of the isotopic signature of the nitrogen sources of the Mediterranean Sea.

Using these values gives

$$x \cdot \delta^{15}\text{N}_{\text{AW+TI}} + (1-x) \cdot \delta^{15}\text{N}_{\text{AI}} = \delta^{15}\text{N-NO}_3^-_{\text{deepMED}}$$

where x and $(1-x)$ are the fractions of nitrate inputs of AW+TI and AI, respectively, and $\delta^{15}\text{N}_{\text{AW+TI}}$, $\delta^{15}\text{N}_{\text{AI}}$ and $\delta^{15}\text{N-NO}_3^-_{\text{deepMED}}$ are the isotopic signature of AW+TI, AI and deep Mediterranean nitrate, respectively. $\delta^{15}\text{N-NO}_3^-_{\text{deepMED}}$ ranges between 0.2 and 3.2‰ putting together present results and data from *Pantoja et al.* [2002]. It is thus estimated that AI fraction varies between 22 and 60% of the total nitrate entering the Mediterranean Sea. Although approximate, the lower value of the range fits reasonably well with the estimate proposed by *Ribera d'Alcalà et al.* [2003], where AI accounts for 17–43% of the total N entering the basin. However the lack of data about the isotopic signature in the Western Mediterranean can partially determine an overestimate of the highest range. Indeed, it is important to underline that the results coming out from this calculation cannot provide a detailed view of the atmospheric deposition inputs in different areas of the basin. Starting from the poor available data ($\delta^{15}\text{N}_{\text{AI}}$ only from the Eastern basin), the only possible attempt is to evaluate, on average, the intensity of the atmospheric deposition inputs on basin scale.

4.5 N:P ratio anomaly and carbon sequestration

The results of N₂ fixation rates and the atmospheric deposition estimates reported above, have highlighted that the N excess characterizing the deep layer of the Mediterranean Sea should be ascribed to atmospheric deposition rather than to N₂ fixation. Here, it will be discussed how much new primary production is based on atmospheric deposition, and if the excess of N in the ocean can result in a carbon sequestration.

The primary production (PP) based on atmospheric deposition would constitute a new input of C to the system, because by using external N, the organic matter synthesis will demand new C, lowering the CO₂ partial pressure in the ocean and recalling new inorganic C from the atmosphere [*Michaels et al.*, 2001; *Michaels*, 2003]. The low primary production rates and the pronounced oligotrophy of the Mediterranean should not induce to think that the basin is a C source for the atmosphere. Although regenerated production seems to play an important role in the total PP [*Selmer et al.*, 1993; *Van Wambeke and Bianchi*, 1985] and organic export from euphotic zone is rather low [*Boldrin et al.*, 2002], on a longer time scale, the new primary production based on atmospheric deposition can be important in terms of absorbing new C from the atmosphere [*Owens et al.*, 1992].

Firstly, in order to estimate the C potentially transferable from the atmosphere to the sea, it is necessary to clarify how it is possible to alter the N:P ratios in the deep nutrient pool if the main mechanism transferring N to the deep layers is the Redfieldian biological pump. In most oceans, where deep nutrient are in Redfield equilibrium (NO₃⁻:PO₄³⁻~16), surface waters are characterized by the lack of nitrogen together with the presence of a residual concentration of phosphorus. This state represents the equilibrium of the system in terms of nutrient proportions. In other words, when mineralization rates are equal to assimilation rates, the N and P bulks in deep layer present a ratio approximately of 16, and

in the surface layers nitrate is consumed and phosphate is still present. The Mediterranean Sea (in particular its eastern basin) presents a different situation, with the deep nitrate in higher proportion with respect to the Redfield ratio of 16, and the surface layer P depleted with a residual level of N. In the Mediterranean Sea, the extra N provided by the atmospheric inputs consumes all the surface P in producing organic matter, subsequently transferred to depth through the classical Redfieldian biological pump mechanism. The residual P (typical of the ocean surface) in the Mediterranean is utilized for this extra new production. In addition, the faster recycling of P with respect to N makes P available for its “multiple utilization” in the production of organic matter. In this way, the organic matter produced is relatively enriched in N through a process where N and P still are in Redfieldian proportions.

This different situation could represent a next step, following the classical basic state (N-limitation). This means that, when atmospheric deposition is acting, N depletion in surface layer cannot be reached and the phosphorus, that in other ocean remains unused because of the lack of N, here can allow organic matter synthesis and vertical transfer of N (and so C) into the deep layers according to the classical biological pump mechanisms. According to this hypothesis, it is not expected that assimilation and mineralization rates diverge significantly from the classical ones observed in other oceans. Therefore the present Mediterranean nutrient ratios would reflect just a different equilibrium reached starting from different initial conditions.

Interestingly, while high N:P ratios have been extensively documented, there is no evidence of deviation of inorganic C:N ratio from its Redfield ratio of 6-7. Not many data are reported in this regard, but *Copin-Montégut*, [2000] referred that at DYFAMED station (Western Mediterranean) DIC:DIN ratio were close to Redfield in the upper layer, just after an intrusion of deep water.

It is possible to estimate the excess of C requested by the sea system due to the multiple utilization of P, as the difference

$$C_{\text{excess}} = C_N - C_P$$

Where C_N is the N based new production converted in C amount, and C_P is, similarly, the P based new production converted in C amount. Obviously, the C_{excess} is the direct consequence of the high N:P ratio in the Mediterranean.

In the calculation reported in Tab. 4.2, nitrate and phosphate bulks have been derived from data reported in the review paper of *Ribera d'Alcalà et al.* [2003].

	WMED	EMED	ALL MED
Nitrate bulk	1.22+07	1.12E+07	2.34E+07
Phosphate bulk	5.86E+05	4.28E+05	1.01E+06
C_N bulk	7.33E+07	6.71E+07	1.40E+08
C_P bulk	6.22E+07	4.53E+07	1.07E+08
$C_{\text{excess}} = C_N - C_P$	1.11E+07	2.18E+07	3.03E+07

Tab. 4.3. Nutrients bulks of Eastern and Western Mediterranean Sea and corresponding C bulk. All data are in Mmols. Original data for nutrients from *Ribera d'Alcalà et al.* [2003].

The excess of N traduced in terms of C absorption accounts for 1.11E+07 Mmols of C for the Western Mediterranean, 2.18E+07 for the Eastern Mediterranean for a total amount of 3.03E+07 Mmols of C, equal to 23% of total carbon absorbed.

If the excess of N in the intermediate and deep waters would be explained only by atmospheric deposition - that is, assuming that all N falling at surface is totally assimilated by phytoplankton - it is possible to provide an estimate of the C inputs to the system. Starting from the atmospheric deposition rates reported by from *Guerzoni et al.* [1999] for the western basin and by *Krom et al.* [2004] for the eastern basin, the highest amount of carbon absorbed by the Mediterranean could be equal to 7.40E+05 Mmols/yr.

4.6 Conclusions

Lack of N₂ fixation observed in late spring could be related, ultimately, to the deposition of N from the atmosphere, that makes the Mediterranean relatively more enriched in N than in P. The lack of phosphate and the approximate constant supply of N by atmospheric deposition could explain the low rates observed. N₂ fixation rates reported here establish an average areal rate of 2.68 $\mu\text{mol N m}^{-2}\text{d}^{-1}$, corresponding to a nitrogen input for the whole basin of 2.45 - 109 mol-N/yr, significantly lower than the estimates proposed by other studies.

Low ¹⁵N/¹⁴N ratio in dissolved and particulate nitrogen observed already in the Mediterranean can be explained by the effect of the atmospheric deposition on a long time scale. The west-east gradient observed both in the $\delta^{15}\text{N-PON}$ and in $\delta^{15}\text{N-NO}_3^-$ has to be attributed to different amounts of atmospheric deposition in the two sub-basins. In this regard, more research is requested to assess the isotopic signature to the N inputs in the Western Mediterranean.

P-limitation, often observed in the Eastern Mediterranean and occasionally in the western basin, would be the consequence of an almost constant supply of external N. Switching from N to P limitation (or co-limitation) would depend on the intensity of N deposition.

On a multi-decadal time scale, the enrichment in nitrate due to the external inputs can increase the ability of the basin in absorbing CO₂ even if primary production and export below the euphotic zone are rather low.

5. GENERAL CONCLUSIONS

The biogeochemical anomalies observed in the Mediterranean Sea - high nitrate/phosphate ratios in subsurface waters, low $^{15}\text{N}/^{14}\text{N}$ ratio in particulate and dissolved nitrogen - have suggested a significant occurrence of N_2 fixation. This study presents, for the first time, a basin-wide overview of direct measurements of N_2 fixation compared to the North Atlantic. Very low rates (0.052-0.031 nmols $\text{N l}^{-1}\text{d}^{-1}$) were observed in all sub-regions of the Mediterranean, in contrast to the higher values measured in the North Atlantic (0.300-0.115 nmols $\text{N l}^{-1}\text{d}^{-1}$). N_2 fixation rates reported here establish an average areal rate of 2.68 $\mu\text{mol N m}^{-2}\text{d}^{-1}$, corresponding to a nitrogen input for the whole basin of 2.45 - 109 mol-N/yr, significantly lower than the estimates proposed by other studies. Lack of correlation between phosphorus concentrations and N_2 fixation rates allows to hypothesise that ecological competition between small phytoplankton fed by nitrate/ammonium and diazotrophs is won by the former.

Low $^{15}\text{N}/^{14}\text{N}$ ratio of particulate organic and dissolved inorganic nitrogen suggests that other light N sources, such as atmospheric deposition fuels the Mediterranean ecosystem.

Considering the isotopic signatures of the deep nitrate of the Mediterranean, atmospheric deposition inputs and Atlantic Water inflow, a rough estimate of the amount of nitrate from atmospheric deposition ranges between 22 and 60% of the total nitrate entering the Mediterranean Sea.

Large amount of N inputs from the atmosphere can explain both P-limitation and anomalous N/P ratio in deep layers. The external inputs of N, determining the excess of N in the marine system could ultimately fuel new production and enhance Mediterranean ability in carbon sequestration. Further studies to evaluate the C pathways in dissolved and particulate organic matter will permit to clarify and quantify the effective ability of the basin in storing carbon on a long time scale.

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APPENDIX

A manuscript summarizing some of the main results of the work carried out in this thesis has been submitted to the international Journal *Geophysical Research Letters*. The draft is attached below.

First basin-wide experimental results on N₂ fixation in the open Mediterranean Sea

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Abstract

The Mediterranean Sea presents several biogeochemical anomalies compared to the global ocean. An unbalanced N budget, high nitrate/phosphate ratios in subsurface waters and low ¹⁵N/¹⁴N ratios in particulate and dissolved nitrogen suggest a significant occurrence of N₂ fixation. This study presents, for the first time, a basin-wide overview of direct measurements of N₂ fixation, with values in the North Atlantic for comparison. Very low N₂ fixation rates (0.052 ± 0.031 nmols N l⁻¹d⁻¹) were observed in all sub-regions of the Mediterranean, in contrast to the higher values measured in the North Atlantic (0.300 ± 0.115 nmols N l⁻¹d⁻¹). Higher phosphorus (inorganic or organic) concentrations were not associated with higher N₂ fixation rates. Low ¹⁵N/¹⁴N ratios in particulate organic nitrogen (from -2.10 to +4.11‰), associated with low N₂ fixation rates, suggest that other N sources, such as atmospheric inputs, fuel the Mediterranean ecosystem.

1. Introduction

Due to the anti-estuarine circulation at the Gibraltar Strait the Mediterranean Sea presents a pronounced oligotrophy, with a clear decreasing gradient in nutrient concentration from west to east. An intriguing biogeochemical characteristic is the anomalous nutrient ratios in intermediate and deep layers, whereas the nitrate/phosphate ratio, range 20–25 [Ribera d'Alcalà *et al.* 2003], is well above the ocean Redfieldian value of 16. Several hypotheses have been proposed to explain this anomaly: i) high nitrate/phosphate ratios in the Adriatic dense waters feeding the deep layer of the Eastern Mediterranean [Civitarese *et al.*, 1998]; ii) significant atmospheric input rich in nitrogen [Herut *et al.*, 1999]; and iii) N₂ fixation [Bethoux and Copin-Montegut, 1986; Sachs and Repeta, 1999][Sachs and Repeta, 1999]. This last hypothesis is further supported by regional geochemical observations, including (i) decoupling between the phosphocline and the nitracline [Bianchi *et al.*, 1999], (ii) $\delta^{15}\text{N}$ of deep nitrate significantly lower (2.4–3 ‰) [Pantoja *et al.*, 2002] than ocean values (5-6 ‰ [Sigman *et al.*, 2005] and (iii) the low isotopic signature of suspended particulate organic nitrogen in the upper layer [Pantoja *et al.*, 2002].

On the basis of budget calculations it has been estimated that N₂ fixation supplies nitrogen to the whole basin at rates from 2.0×10^{10} mol-N/yr to 12.4×10^{10} mol-N/yr [Bethoux and Coupin-Montegut, 1986], which corresponds to a daily rate of 21.9-178.1 $\mu\text{mol N m}^{-2}$. To date, however, few direct measurements are available for the region. Moreover, these experimental data show high spatial and temporal variability (2.0–7.5 $\text{nmol N l}^{-1}\text{d}^{-1}$, Sandroni *et al.* [2007]; 129 $\text{nmol N l}^{-1}\text{d}^{-1}$, Rees *et al.* [2006]), precluding clarification of the role of this process on a basin-wide scale.

On the other hand, the occurrence of N₂ fixation is still debatable as the depletion of phosphorus in the Eastern Mediterranean should prevent a massive growth of diazotrophs [Krom *et al.* 2004]. Unbalanced sources of atmospheric and terrestrial origin [Herut *et al.* 1999] and lack of significant denitrification [Krom *et al.* 2004] have been invoked as alternative processes able to maintain the altered N/P ratios. This study aims to contribute

to the understanding the role of N₂ fixation in the Mediterranean Sea on the basis of a basin-scale overview. The importance of atmospheric deposition as an alternative process playing a role in the nitrogen cycle of the area is also explored.

2. Methods

The VECTOR-TRANSMED cruise was carried out from 28 May to 24 June 2007. Seven stations were sampled: in the North Atlantic (VA) and in the Western (V4, V3, V2) and Eastern (V7, V9, V10) Mediterranean (Figure 1).

Seawater was sampled with a CTD-rosette equipped with Niskin bottles. Nitrate, phosphate, total dissolved nitrogen (TDN) and total dissolved phosphorus (TDP) were collected at 0, 10, 25, 50, 75 and 100 m depth, filtered on precombusted (450 °C, 6 h) GF/F filters and stored at –20 °C until analysis. Nitrate and phosphate were analyzed following standard colorimetric methods with an ALPKEM autoanalyzer [Yilmaz and Tugrul, 1998]. TDN and TDP were determined following the UV photo-oxidation method of Walsh [1989]. Samples for $\delta^{15}\text{N}$ -PON (particulate organic nitrogen) determinations were collected on precombusted GF/F filters by on-board filtration of 5-9 liters of seawater.

N₂ fixation rates (nmoles N l⁻¹d⁻¹) were determined according to Montoya *et al.* [1996]. Samples were collected in six replicates using 4.6-l Nalgene bottles, at three optical levels (50, 1 and 0.1% of the surface photosynthetic available radiation), generally 10 m, 50 m and the deep chlorophyll maximum (DCM; 90-110 m). Four replicates were enriched with 4.6 ml of ¹⁵N₂ (Cambridge Isotope Laboratories Inc.; 99.90 atom%) with a gas-tight syringe. One replicate (control) was incubated without added ¹⁵N₂. The last replicate (blank) was immediately filtered without any treatment. Samples were incubated for 24 h on deck, and screened at appropriate light and temperature (thermostatically controlled) in order to simulate *in situ* conditions. Size fractionation of diazotrophs <10 μm was also

carried out. Each step was conducted in dim light conditions to avoid phytoplankton photodamage. Natural and enriched samples were always handled separately.

^{15}N -enrichment of PON was calculated with respect to the control samples. Isotopic enrichment (^{15}N -atom%) due to N_2 fixation was determined by continuous-flow isotope ratio mass spectrometry (PDZ-Europa 20-20) [Owens and Rees, 1989], with a precision $<0.04\%$ CV (0.3671 ± 0.0001 ^{15}N -atom%).

PON and natural $\delta^{15}\text{N}$ -PON samples were analyzed on the same instrument, with ammonium sulphate as working standard calibrated against IAEA-N-1 (precision 1 std = 0.21% , $n=10$ replicate samples). Long-term performance [Werner & Brand, 2001] was monitored daily with peptone (Merck). Comparison between blanks and controls showed similar values of ^{15}N -atom% (mean difference 0.0005 ± 0.0004 atom%).

3. Results

3.1. Nutrient distribution

Nitrate and phosphate were severely depleted above the DCM at all stations (Figure 2). The nitrate range was $0.04\text{--}0.60\ \mu\text{M}$ while phosphate was generally $< 0.05\ \mu\text{M}$, except at station V4 where values of $0.09\text{--}0.22\ \mu\text{M}$ were found. Both nutrients showed large increases at DCM depth at stations VA, V4 and V2, their profiles remaining homogeneous in the other cases. Vertical profiles of TDP ($0.06\text{--}0.57\ \mu\text{M}$) and TDN ($5.28\text{--}13.32\ \mu\text{M}$) reflected the inorganic nutrient trend. However, dissolved organic nitrogen and phosphorus strongly prevailed in the layer above the DCM, being respectively 95% and 70% of the total dissolved pools. At station VA, depth-integrated nutrient quantities above DCM showed a nitrate/phosphate ratio of 11 and TDN/TDP ratio of 36. At the Mediterranean stations, ratio ranges were 5-30 and 35-82, respectively.

3.2. N_2 fixation and suspended $\delta^{15}\text{N}$ -PON

N_2 fixation rates were generally low, 0.009–0.300 nmoles $N\ l^{-1}d^{-1}$ (Figure 3a). Of 84 samples, 26 (31%) were below the detection limit of the method.

The highest N_2 fixation rates were measured at VA ($\leq 0.300 \pm 0.115$ nmoles $N\ l^{-1}d^{-1}$). At this station only, seawater was pre-filtered on 100- μm Nitex net. N_2 fixation might thus be underestimated because the contribution of larger cyanobacteria was not included. Maximum values were found at 10 and 50 m depth, with DCM levels undetectable. In contrast, rates in the Mediterranean were uniformly lower (0.052 ± 0.031 nmoles $N\ l^{-1}d^{-1}$) and scarcely varied with depth and longitude. At VA the integrated value of the N_2 fixation rate was $17.6\ \mu mol\ N\ m^{-2}d^{-1}$, with a range of 1.0–4.0 $\mu mol\ N\ m^{-2}d^{-1}$ at the Mediterranean stations (Table 1). The range of contribution of the fraction $<10\ \mu m$ to N_2 fixation in the western stations and at coastal stations V10 was 18–46%. At stations V7 and V9, where N_2 fixation values were particularly low, the importance of the small fraction increased to 57–65%.

Natural $\delta^{15}N$ -PON ranged between -2.10 and +4.11‰ (Figure 3b), with a general decreasing trend from west to east. The major differences between deeper (100 m) and shallower (10 and 50 m) data were found at stations V4 and V2. The integrated values of total PON in the layer 0–100 m ranged from 26.1 to 61.2 $mmol\ N\ m^{-2}$, with the highest values at station V4 (Table 1). The fraction $>10\ \mu m$ always prevailed in the PON pool (77–80%).

Table 1. Integrated values of suspended particulate organic nitrogen (PON) and N_2 fixation (N_2 -fix) rates in the euphotic layer for the total PON and the fraction $<10\ \mu m$.

Station	PON (TOT) <i>mmol N m⁻²</i>	PON ($<10\ \mu m$) <i>mmol N m⁻² (%)</i>	N_2 -fix (TOT) <i>$\mu mol\ N\ m^{-2}d^{-1}$</i>	N_2 -fix ($<10\ \mu m$) <i>$\mu mol\ N\ m^{-2}d^{-1}$ (%)</i>
VA	26.1*	N.A.	17.6*	N.A.
V4	61.2	47.5 (78)	2.5	0.5 (18)
V3	48.2	37.3 (77)	3.7	1.7 (46)
V2	44.2	34.0 (77)	3.3	1.1 (32)
V7	38.0	30.3 (80)	1.6	0.9 (57)
V9	36.2	28.4 (78)	1.0	0.7 (65)
V10	53.9	41.4 (77)	4.0	1.0 (25)

*Size fraction <100 μm

4. Discussion

In the Atlantic Ocean, our depth-integrated rates of N_2 fixation are in agreement with other studies of the region [[Voss *et al.*, 2004]. In contrast, values reported for the Mediterranean do not confirm the hypotheses that attribute to N_2 fixation a relevant role in supplying new nitrogen [Bethoux and Copin-Montegut, 1986; Sachs and Repeta, 1999; Pantoja *et al.*, 2002]. Previous studies attempting to directly quantify N_2 fixation in the Mediterranean have been limited in coverage, so no areal estimates have ever been provided. Rees *et al.* [2006] reported a mean rate of $129 \pm 9 \text{ nmol N l}^{-1} \text{d}^{-1}$ for few stations in an anticyclonic eddy of the Eastern basin during a phosphorus fertilization experiment. Sandroni *et al.*, [2007] found much lower rates ($0\text{-}17 \text{ nmol N l}^{-1} \text{d}^{-1}$) and high seasonal variability in one single station (DYFAMED) in the north-west Mediterranean. Molecular identification and expression of *NifH* genes have been investigated by Man-Aharonovich *et al.* [2007] off the Israeli coast. A high diversity of diazotrophs, with a dominance of the cyanobacterium *Richelia intracellularis* associated with the diatom *Hemiaulus hauckii*, has been observed [Zeev *et al.* 2008]. However, the *Richelia*–*Hemiaulus* abundance and N_2 fixation rates ($\sim 1.1 \text{ nmol N l}^{-1} \text{d}^{-1}$) reported there were similar to no-bloom diazotroph areas. Our direct measurements established an average areal rate of $2.68 \mu\text{mol N m}^{-2} \text{d}^{-1}$, corresponding to a nitrogen input for the whole basin of $2.45 \times 10^9 \text{ mol-N/yr}$, significantly lower than the results in these three studies.

In order to explain this contradictory scenario it is important to focus on the factors controlling N_2 fixation. In the world ocean, phosphorus and iron deficiency are known to limit N_2 fixation [Falkowski and Davis, 2004.; Mills *et al.*, 2004]. In addition, as N_2 fixation is a high energy consuming process, depletion of dissolved inorganic nitrogen seems to be

an essential condition for the diazotroph growth. Below, on the basis of the present and previous studies, we discuss how these three factors seem to play different roles.

As the Mediterranean Sea has been described as a phosphate-depleted basin [Krom *et al.*, 1991] [Krom *et al.*, 1991, Ribera d'Alcalà *et al.*, 2003], phosphorus has often been considered a limiting nutrient for N₂-fixers. In this study we measured variable phosphate concentrations in the euphotic layer but, as the values were often close to the detection limit (0.02 µM), it is not easy to determine a clear dependence between N₂ fixation rates and phosphate concentrations. The other studies carried out in the Mediterranean have also reached a similar conclusion [Sandroni *et al.*, 2007; Rees *et al.*, 2006]. Dissolved organic phosphorus (DOP) has been thought an alternative source of phosphorus for N₂-fixers [Rees *et al.*, 2006] but the present study shows that, even in the nutrient-depleted surface water with DOP always higher than phosphate (70% of TDP), N₂ fixation was still very low.

The role of iron, the other possible controlling factor, remains uncertain for the Mediterranean. Experiments conducted in the Eastern Mediterranean have shown no relevant increase in N₂ fixation after iron enrichment [Rees *et al.*, 2006]. However, the authors did not provide any information about the background concentration of iron during the experiment. It is known that Saharan dust deposition transfers abundant iron to the Mediterranean [Guerzoni *et al.*, 1999] but particular conditions, such as spring blooms and absence of dust inputs, may also lead to extreme iron depletion [Sarhou and Jeandel, 2001]. In our case, satellite observations confirmed the absence of relevant Saharan dust deposition events over the sampling areas during the cruise (Volpe, personal communication). For these reasons, switching between episodic inputs of dust and depletion conditions could be the mechanism responsible for both diazotroph blooms and low N₂ fixation rates.

Finally, a high nitrate concentration in the marine environment is considered another inhibitory factor for some diazotrophs [Holl and Montoya, 2005]. In contrast, in this study we reported low nitrate concentrations and the absence of a systematic relationship between nitrate and N_2 fixation ($R^2 = 0.002$). In addition, the highest N_2 fixation rates, observed in the Atlantic (VA), were not associated with different nitrate/phosphate or TDN/TDP ratios because at this station nutrient ratios lay within the Mediterranean Sea value ranges (5-30 for nitrate/phosphate and 35-82 for TDN/TDP).

However, while N_2 fixation showed no relevant spatial variability through the Mediterranean, natural $\delta^{15}N$ -PON values displayed a clear decreasing gradient from west to east. This feature, already reported by *Pantoja et al.* [2002], has suggested the occurrence of N_2 fixation, increasing eastward with the increasing oligotrophy. If most PON is organic matter fixed during the days prior to sampling, low $\delta^{15}N$ values in oligotrophic environments may be attributed to any or all of three different processes: i) abrupt and intense N_2 fixation events; ii) strong impact of ammonium-based phytoplankton production; and iii) relevant atmospheric and terrestrial inputs (ATI).

Concerning regenerated production, *Altabet* [1988] suggests that preferential removal of ^{15}N by sinking matter and the concurrent uptake of regenerated ammonium by phytoplankton could result in a low $^{15}N/^{14}N$ ratios in suspended PON. The effect of these food-web processes could be particularly relevant in the oligotrophic waters of the Mediterranean, where regenerated production greatly exceeds new production [Estrada, 1996].

Finally, the Mediterranean is a semi-enclosed basin, surrounded by industrialized countries, where significant natural and anthropogenic discharges of atmospheric and terrestrial nitrogen can influence the marine environment. As proposed by *Sachs and Repeta*, [1999], we can assume that deep nitrate is a temporal and spatial integration of primary production in the basin for many decades. Deep nitrate should thus reflect the

remineralization of sinking particles fed by surface N coming from atmospheric and terrestrial inputs and surface Atlantic Water entering at the Gibraltar Strait. The isotopic signature of deep nitrate can therefore be used to provide a rough estimate of the N inputs.

If we exclude N₂ fixation, the nitrogen sources are Atlantic Water (AW), atmospheric inputs (AI) and terrestrial inputs (TI). Due to the lack of available data, some simplifications need to be made. The isotopic signature of AW is ~ 5‰ [Pantoja *et al.*, 2002]. TI present many uncertainties but, referring to the paper by Mara *et al.*, [2009] and references therein, we took a value of 5‰. AI have been measured by Mara *et al.*, [2009] for the East Mediterranean (-3.1‰). We took the same value for the western basin as isotopic values for AI are not available. Using these values gives

$$x \cdot \delta^{15}\text{N}_{\text{AW+TI}} + (1-x) \cdot \delta^{15}\text{N}_{\text{AI}} = \delta^{15}\text{N-NO}_3^-_{\text{deepMED}}$$

where x and $(x-1)$ are the fractions of nitrate inputs of AW+TI and AI, respectively, and $\delta^{15}\text{N}_{\text{AW+TI}}$, $\delta^{15}\text{N}_{\text{AI}}$ and $\delta^{15}\text{N-NO}_3^-_{\text{deepMED}}$ are the isotopic signature of AW+TI, AI and deep Mediterranean nitrate, respectively. $\delta^{15}\text{N-NO}_3^-_{\text{deepMED}}$ ranges between 2.4 and 3‰ [Pantoja *et al.*, 2002]. Calculated thus, the AI fraction varies between 24 and 32% of the total nitrate entering the Mediterranean Sea. Although approximate, this averaged estimate fits reasonably well with the budget proposed by Ribera d'Alcalà *et al.* [2003], where AI accounts for 17–43% of the total N entering the basin.

5. Conclusions

To date, a complex scenario emerges from the information available on N₂ fixation in the Mediterranean Sea. This study provides the first spatially extensive direct measurements of N₂ fixation, but low rates reported may not be representative of the whole annual cycle

due to the high temporal variability of this process. Different results could be possible during late summer, when oligotrophic conditions are more established and/or after Saharan dust deposition events. On the other hand, the present results also suggest that N_2 fixation may not be such a relevant process in the biogeochemical cycle of nitrogen. Other processes, such as regenerated production and/or atmospheric deposition and terrestrial discharges, could be more important in determining the anomalies of the isotopic signal and shaping the nitrogen biogeochemistry of the Mediterranean Sea.

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Figure captions

Figure 1. Map of stations of Cruise TRANSMED (May-June 2007).

Figure 2. Inorganic and total dissolved nitrogen and phosphorus profiles in the euphotic zone. Shaded areas indicate the Deep Chlorophyll Maximum (DCM) layer.

Figure 3. (a) N_2 fixation rates (average value and error bars = 2σ ; $n = 2$), and (b) natural isotopic abundance in suspended particulate organic nitrogen in blank samples.

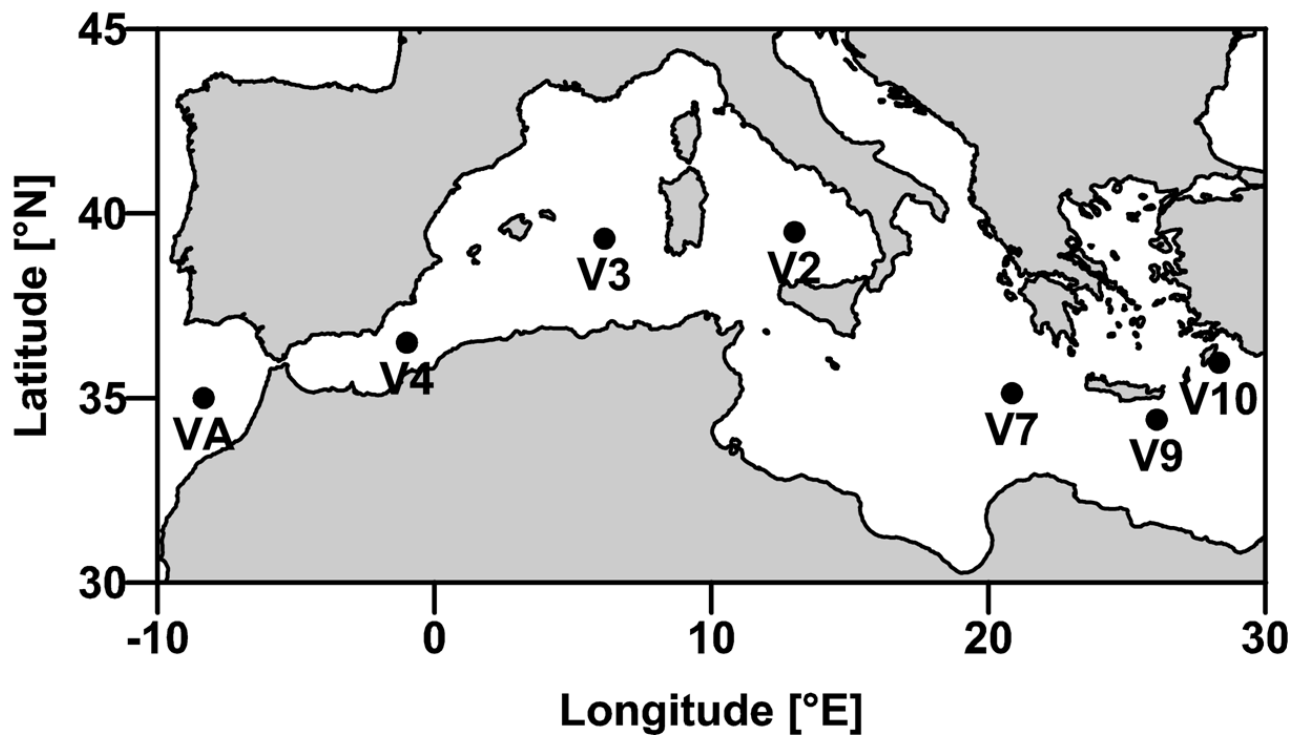


Fig. 1.

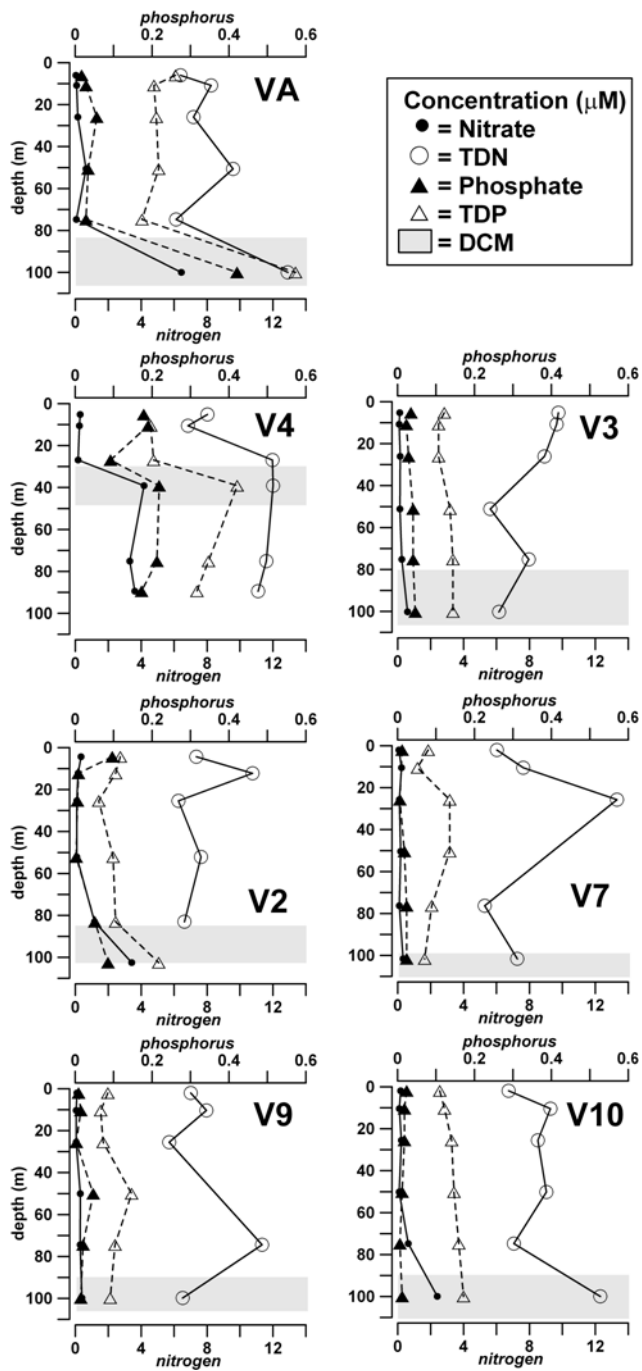


Fig. 2.

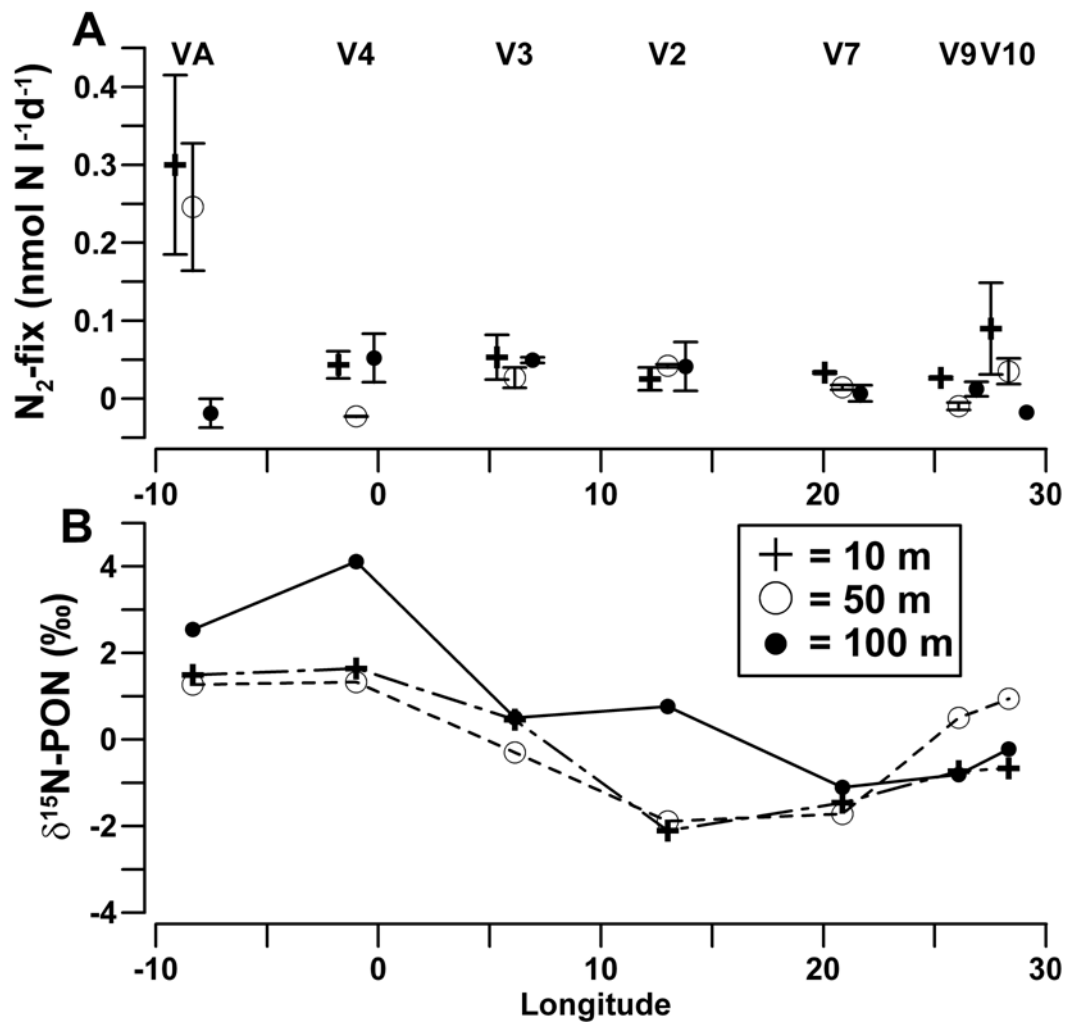


Fig. 3.