

## A Review on Marine N<sub>2</sub> Fixation: Mechanism, Evolution of Methodologies, Rates, and Future Concerns

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Received 4 June 2019; Revised 8 August 2019; Accepted 10 September 2019

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**Abstract** – Investigations on marine N<sub>2</sub> fixation have gained momentum since 1960s with eventual establishments of relevant methodologies to identify species involved and quantify the rates. The evolution of various methodologies to understand N<sub>2</sub> fixation and to estimate its rates were underpinned by the constant efforts of pioneers in the ocean biogeochemical research field. Those efforts succeeded in introducing various methodologies that include experimental (<sup>15</sup>N<sub>2</sub> bubble method and acetylene reduction method), geochemical (N\* and P\* method), mathematical modelling, and remote sensing techniques. However, the construction of an accurate N budget is still under progress due to inseparable issues associated with each method and difficulties in conducting the experiments onboard on a larger scale. Nevertheless, the contributions by each of the methodologies are significant and helped in forming basic ideas about N<sub>2</sub> fixation activities on a global scale. It is not only important to recognize the contributions made by the formation of various methodologies by marine research pioneers, but also vital to summarize what we have achieved in the marine N<sub>2</sub> fixation research area so far. Hence, this review is an attempt to brief on the various milestones achieved in research on the N<sub>2</sub> fixation mechanism, species involved, evolution of methodologies to estimate N<sub>2</sub> fixation rates, species identification, budgets, and future concerns.

**Keywords** – N<sub>2</sub> fixation, climate change, <sup>15</sup>N<sub>2</sub> labelling, cyanobacteria

### 1. Introduction

Biogeochemical cycling in a healthy ecosystem is perplexingly interrelated to the availability of bioavailable nitrogen (N) due to its potential role in building and maintaining life components. Understanding N cycling has always been one of the major focuses of marine research due to its complexity and enigmatic pathways. Despite the fact that molecular N accounts for 78% of the earth's atmospheric composition, the accessibility to reactive N pool by organisms is critically low. The two possible natural sources of the bioavailable N are the biological N<sub>2</sub> fixation and lightning which are highly dependent on unique environmental conditions (Capone et al. 1997; Howarth 1998). The role of marine N<sub>2</sub> fixation as a significant source of new N in the ocean has been recognized since the 1960s (e.g. Dugdale and Goering 1967; Carpenter and Price 1977; Capone and Carpenter 1982; Carpenter and Romans 1991; Gruber and Sarmiento 1997; Capone et al. 1998; Lipschultz and Owens 1996, Lipschultz et al. 2002). The N fixed by marine prokaryotes in the global ocean is approximately 106–120 Tg N y<sup>-1</sup> (Gruber 2004; Gruber and Galloway 2008). However, the possibility of N<sub>2</sub> fixation evolving as a potential source of bioavailable N mainly depends on favorable environmental conditions such as warm and stratified water columns with sufficient nutrient concentrations (iron, manganese, phosphorous etc.).

Apart from these physico-chemical conditions, the abundance

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of diazotrophs, the species which can convert  $N_2$  molecules in to  $NH_4^+$ , is the primary factor to determine the availability of biologically fixed N. The cycling of other elements like carbon (C) and phosphorous (P) in marine biological systems is highly dependent on N flux and, hence, it plays an important role in C sequestration and P cycle indirectly. Since most marine environments are N deficient the potential  $CO_2$  uptake has always been a matter of concern until the introduction of the Haber-Bosch process which is considered one of the major mile stones in the green revolution era. The commercial production of N compounds has changed the whole picture of the N cycle by introducing bioavailable N to terrestrial as well as marine ecosystems, while open oceans remained to be N deficient (Meybeck 1982; Sañudo-Wilhelmy et al. 2001; Bauer et al. 2013; Regnier et al. 2013). Rivers, lakes, estuaries, and coastal waters are the main victims of elevated usage of N-fertilizers, which has introduced excess N loading and caused high dissolved inorganic and organic nitrogen concentrations in these ecosystems (Doney 2010; Paerl et al. 2014; Bhavya et al. 2016, 2017).

There have been various studies conducted so far regarding the influx and loss of N in marine ecosystems; however, the formulation of a proper methodology to assess the potential  $N_2$  fixation and loss process took decades. And hence, the precise magnitudes of altered N transformation rates, particularly  $N_2$  fixation rates, are still unclear (Bauer et al. 2013; Regnier et al. 2013). However, those research works those shed light on gave accountability to biological  $N_2$  fixation deserve recognition and praise since they have made a significant contribution towards the understanding of the  $N_2$  cycle. Paying tribute to the efforts undertaken to reveal the mystery of the  $N_2$  fixation process, this review sought to summarize

studies conducted on the  $N_2$  fixation process by giving special emphasis to the species involved, methodologies, rate estimation, budget, and future concerns.

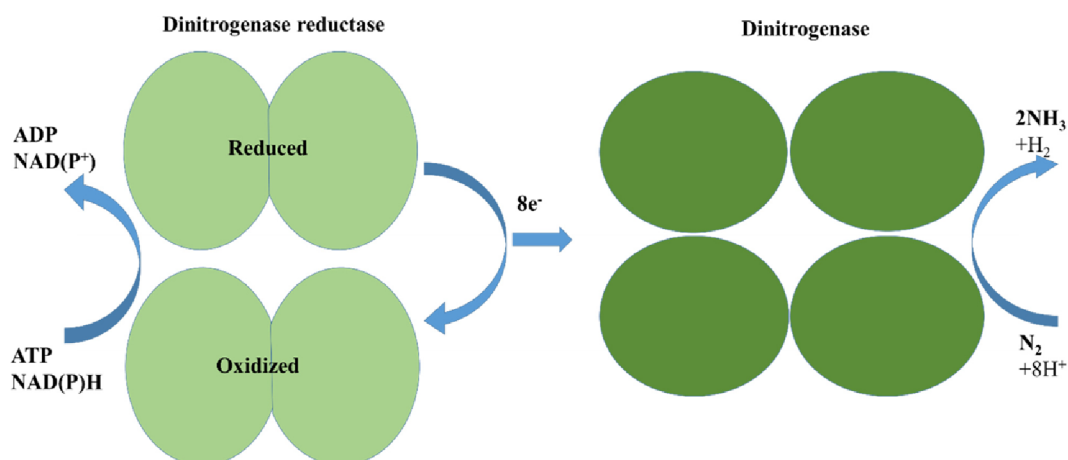
## 2. Mechanism of $N_2$ Fixation

The importance of biological  $N_2$  fixation lies in the fact that it is capable of producing bioavailable N by breaking molecular  $N_2$  at even  $20^\circ C$  and under normal atmospheric pressures whereas the Haber - Bosch process can achieve that only at 300–500 degree Celsius and pressures of 15–26 MPa. Basically,  $N_2$  fixation is a process involving the reduction of  $N_2$  gas to  $NH_3$  where the power to bring about this reduction is supplied by NAD(P)H (Fig. 1). The reduction of  $N_2$  gas involves breaking the triple bond between two nitrogen atoms in a  $N_2$  molecule and this requires a tremendous amount of energy. Under natural conditions the energy required can be higher than 16 ATPs (Eq.1) for some microbes (Hill 1978).



The  $N_2$  fixation process is mediated by a complex enzyme called nitrogenase which is as large as 300 kDa. It is basically a complex that contains two different proteins which are dinitrogenase and dinitrogenase reductase (Postgate 1998). Fig. 1 depicts the  $N_2$  fixation by nitrogenase complex.

The dinitrogenase reductase contains iron (Fe) and either molybdenum (Mo) or vanadium (V) while dinitrogenase is comprised of only Fe. Nitrogenase enzyme is known to have been present in the early stages of the evolution of life and the diazotrophs are thought to be the first cells that evolved on the Earth's surface (Zehr and Pearl 2008).



**Fig. 1.** Nitrogen fixation by the nitrogenase complex in the diazotrophs

### 3. Species of N<sub>2</sub> Fixers

Earlier, in the 1970s, N<sub>2</sub> fixation expeditions were mainly focused on autotrophic cyanobacteria and researchers believed that diazotrophic activity was constrained to the sunlit and oligotrophic layers of the tropical and subtropical oceans (Zehr 2011). The patterns of marine microbial biodiversity and functional activities were unknown to the research world till the introduction of ‘omic’-based techniques (Sunagawa et al. 2015; Carradec et al. 2018). Such studies revealed the existence of various diazotrophic and non-diazotrophic cyanobacterial (bacteria and archaea) species and their high diversity and wide distribution in the oceans (Zehr et al. 1998, 2000; Farnelid et al. 2011; Bombar et al. 2016; Moisander et al. 2017). In general, marine diazotrophs are comprised of both phototrophic and heterotrophic bacteria and archaea. They have distinctive morphological and physiological characteristics and, hence, different optimal growth conditions and mortality factors are expected.

*Trichodesmium erythraeum*, the filamentous colonial diazotrophs, is one of the important N<sub>2</sub> fixers and is commonly known as “sawdust” due to its appearance during blooming (Gandhi et al. 2011; Bhavya et al. 2016; Kumar et al. 2017; Singh et al. 2019). The heterocyst-forming symbionts of diatoms (diatom-diazotroph associations, or DDAs) are also known to be important N<sub>2</sub>-fixers (Jabir et al. 2013). Both *Trichodesmium* and DDAs are commonly distributed through tropical and subtropical regions and are major sources of bioavailable N in oligotrophic regions under warm and stratified conditions (Villareal 1992; Capone et al. 2005; Mulholland and Bernhardt. 2005; Gandhi et al. 2011, Jabir et al. 2013, Bhavya et al. 2016). Under favorable conditions multiple lineages of unicellular N<sub>2</sub>-fixing cyanobacteria are also found to be a significant source of new N globally (Montoya et al. 2004; Moisander et al. 2010; Luo et al. 2012).

The free-living cyanobacterial diazotroph *Crocospaera* (UCYN-B) occasionally “blooms” in the North Pacific Subtropical Gyre (NPSG) (Turk-Kubo et al. 2018); however, the presence of unicellular diazotroph, the uncultivated cyanobacteria group A (UCYN-A), are observed year-round (Thompson et al. 2014; Farnelid et al. 2016; Turk-Kubo et al. 2017). Apart from UCYN lineages, *Trichodesmium* and several DDA lineages are also commonly found in the North Pacific Subtropical gyre (NPSG) region (Letelier and Karl 1996; Church et al. 2005, 2008, 2009; Sohm et al. 2011). NPSG is also observed with the presence of *Richelia* spp.-

*Rhizosolenia* (Het-1) and *Hemiaulus* (Het-2) association and the latter is a significant diazotrophic lineage that also contributes to vertical export following summer blooms (Karl et al. 2002). There have been many reports suggesting the presence of non-cyanobacterial diazotrophs in the NPSG (Gradoville et al. 2017); however, their distributions and significance as a contributor to biological N<sub>2</sub> fixation are poorly known (Turk-Kubo et al. 2014; Bombar et al. 2016; Moisander et al. 2017).

### 4. Evolution of N<sub>2</sub> Fixation Estimation Methodologies

Spatial and temporal distributions of diazotrophs are eventually a function of their growth and mortality rates just like every individual taxa (Zehr et al. 2011). In general, such processes are difficult to measure in natural populations of marine N<sub>2</sub> fixers because of their low abundances and absence of cultivated representatives of many lineages. And hence, the effect of fluctuations in favorable physical conditions and critical nutrient availability on their physiological properties is not well understood yet (Zehr et al. 2011). Researchers in the early periods used natural isotopic composition of <sup>15</sup>N (δ<sup>15</sup>N) in the particulate organic matter to identify the presence of N<sub>2</sub> fixers in marine ecosystems. The inference from these studies suggested that low δ<sup>15</sup>N values (-2 to +2‰) indicate the presence of significant N<sub>2</sub> fixation (Wada and Hattori 1976; Saino and Hattori 1980) since N<sub>2</sub> fixation incorporates the lighter isotopes (<sup>14</sup>N) preferentially into the POM from the atmospheric N<sub>2</sub> pool with δ<sup>15</sup>N values close to 0‰. However, δ<sup>15</sup>N values do not imply the growth rate and mortality rates of individual taxa.

Parallel studies also used the geochemical imprint on nutrients (Gruber and Sarmiento 1997) and stable N isotope distributions (Altabet 2007) to indirectly infer diazotrophic activity. The main drawback of geochemical estimates is that this method uses integrated N<sub>2</sub> fixation signature over large scales of space and time and neglects the small-scale variability inherent in experimental N<sub>2</sub> fixation measurements by smoothing the variations. Such small scale variability is the key to measuring the highly difficult components in natural populations which are the growth and mortality rates of individual taxa. This is particularly applicable for N<sub>2</sub> fixers because of their low abundances and lack of cultivated representatives of many of their lineages. Michaels et al. (1994) observed from Bermuda Atlantic Time-series Studies (BATS) that a decrease in dissolved inorganic carbon (DIC) in the

absence of sufficient  $\text{NO}_3^-$  can possibly lead to  $\text{N}_2$  fixation activity due to deviations from Redfield stoichiometry (Michaels et al. 1994). It is well known that iron (Fe) and P are potential limiting factors of  $\text{N}_2$  fixation. Based on this concept, Michaels et al. (1994) introduced a new idea of the  $\text{N}^*$  parameter, a quasi-conservative tracer which would help in overcoming the temporal and spatial scale constraints of *in situ*  $^{15}\text{N}_2$  uptake measurements. To be precise, the  $\text{N}^*$  parameter indicates the excess of  $\text{NO}_3^-$  as compared to  $\text{PO}_4^{3-}$  while considering the Redfield ratio ( $\text{NO}_3^- : \text{PO}_4^{3-}$  ratio (N:P) = 16:1; Michaels et al. 1996; Gruber and Sarmiento 1997) (Eq. 2).

$$\text{N}^* = (0.87[\text{NO}_3^-] - 16[\text{PO}_4^{3-}] + 2.9) \text{ mmol kg}^{-1} \quad (2)$$

The basic concept of this  $\text{N}^*$  model is that a non-conservative positive gradient in  $\text{N}^*$  (defined as  $\text{N}^*$  when N:P = 16:1,  $> 2.5 \mu\text{mol kg}^{-1}$ ) in excess of mixing implies a net  $\text{N}_2$  fixation and a negative gradient in  $\text{N}^*$  ( $< 2.5 \mu\text{mol kg}^{-1}$ ), implies a net denitrification (Gruber and Sarmiento 1997). The spectra of marine microbial biodiversity and functional activities have been widely investigated (Sunagawa et al. 2015; Carradec et al. 2018) with the use of 'omic'-based techniques which are very useful in distinguishing  $\text{N}_2$  fixers from other microbes. The path breaking entry of polymerase chain reaction (PCR) techniques amplified the investigations on  $\text{N}_2$  fixers by decoding the genetic diversity among the large varieties of diazotrophs in the ocean (Zehr et al. 1998). Molecular studies demonstrated that the ubiquity of unicellular diazotrophic cyanobacteria (UCYN) makes them equally or much more capable of fixing  $\text{N}_2$  than *Trichodesmium* (Falcón et al. 2004; Montoya et al. 2004) under nutrient rich conditions in cold areas of the North Atlantic Ocean (Langlois et al. 2005, 2008; Goebel et al. 2010; Benavides and Voss 2015). Such profound investigations also led to distinctive researches on physiology, autoecology, and distribution of *Trichodesmium* communities (Bergman et al. 2013). Researchers found that *Trichodesmium* communities are nourished under conditions with warm temperatures (24–30°C; Breitbart et al. 2008), low dissolved inorganic nitrogen, and calm stratified waters (Bergman et al. 2013).

The estimation of  $\text{N}_2$  fixation rates has been possible since the establishment of acetylene reduction assays (Stewart et al. 1967, 1968; Hardy et al. 1968). Initially, indirect estimations of  $\text{N}_2$  fixation were based on acetylene reduction assays which rely on the preferential reduction of acetylene ( $\text{C}_2\text{H}_2$ ) to ethylene ( $\text{C}_2\text{H}_4$ ) by *nitrogenase*. A theoretical conversion factor is used to estimate  $\text{N}_2$  fixation rates which assumes that one

molecule of  $\text{N}_2$  is fixed for each 3 molecules of  $\text{C}_2\text{H}_2$  is reduced to  $\text{C}_2\text{H}_4$ ; however, a direct comparison of  $\text{N}_2$  fixation rates with acetylene reduction was not possible (Stewart et al. 1967, 1968; Hardy et al. 1968). The potential indirect effects of  $\text{C}_2\text{H}_2$  on microbial metabolic activities and the consistency of the conversion factor used to extrapolate  $\text{N}_2$  fixation rates were always debatable (Flett et al. 1976; Giller 1987; Hardy et al. 1973). Additionally, cell damage due to shipboard filtration or net tows performed for concentrating the microbial biomass to obtain detectable signals may also lead to underestimation of  $\text{N}_2$  fixation rates.

Modern marine  $\text{N}_2$  fixation estimation using the  $^{15}\text{N}_2$  gas injection method was successfully established by (Nees et al. 1962) and Richard Dugdale, John Goering, and colleagues conducted various  $\text{N}_2$  fixation measurements in the tropical and subtropical Northern Atlantic Ocean (Dugdale et al. 1964; Goering et al. 1966). Montoya et al. (1996) introduced a high precision method of  $\text{N}_2$  estimation using the  $^{15}\text{N}_2$  tracer technique by modifying the  $^{15}\text{N}_2$  gas method by Neess et al. (1962). This method relies on the net incorporation of the  $^{15}\text{N}_2$  tracer into cellular biomass during sample incubation period with added  $^{15}\text{N}_2$  tracer bubble. The basic assumption of this technique is that the  $^{15}\text{N}_2$  bubble injected into the airtight serum sample bottles will be incorporated into the diazotrophic biomass contained in the samples if  $\text{N}_2$  fixation occurs. The incorporated  $^{15}\text{N}_2$  will be detected as elevated  $\delta^{15}\text{N}$  values during the mass spectrometric analysis. Since the  $^{15}\text{N}_2$  technique has a lower detection limit than the acetylene reduction assay, it is more preferred for the  $\text{N}_2$  fixation estimations in the oligotrophic waters (Montoya et al. 1996). However, at a later stage, it was demonstrated that the  $\text{N}_2$  fixation rates based on  $^{15}\text{N}_2$  tracer bubble could be 40% lower due to lack of complete equilibration of the tracer gas in incubation bottles within the incubation period (Mohr et al. 2010; Gandhi et al. 2011; Großkopf and LaRoche 2012). And hence, the  $^{15}\text{N}_2$  bubble experiment underwent further amendments with more elaborative steps such as the addition of tracer as filtered sea water infused with tracer gas and measurement of  $^{15}\text{N}$  content in the tracer infused water using a membrane inlet mass spectrometer (Großkopf and LaRoche 2012).

Remote sensing is another modern non-experimental tool employed to examine the distribution of marine diazotrophs on broad spatial and temporal scales (Hood et al. 2001; Westberry et al. 2005). Particularly, *Trichodesmium* can be detected efficiently at relatively high concentrations using ocean color remote sensing (for example, SeaWiFS).

*Trichodesmium* has a unique optical signal as it possesses phycoerythrin and gas vesicles with a high reflectance (Subramaniam et al. 1999) which allow for the effective identification of its position in the upper water column. The entry of remote sensing techniques to identify N<sub>2</sub> fixers, particularly *Trichodesmium*, was a revolutionary step for the global ecosystem (Capone et al. 1997; Moore et al. 2002), biological and coupled biological-physical models, (Hood et al. 2001; Fennel et al. 2001; Coles et al. 2004; Hood et al. 2004), and algorithms using climatological satellite data (Hood et al. 2001). Such models assisted in improving our understanding of spatial and temporal variabilities and constraints of marine N<sub>2</sub> fixers, and their impact on the global C and N cycles (Fennel et al. 2001; Moore et al. 2002).

## 6. N<sub>2</sub> Fixation Rates and Budget

The availability of estimations of N<sub>2</sub> fixation rates is not splendid due to the lack of proper methodology in earlier periods and difficulty in executing the <sup>15</sup>N<sub>2</sub> tracer techniques in later periods. The geochemical estimates are the main recorded N<sub>2</sub> fixation rates from earlier periods. Initial studies using the basic <sup>15</sup>N<sub>2</sub> tracer method by Richard Dugdale, John Goering, and colleagues in the 1960s reported a substantial <sup>15</sup>N atom % excess values (upto 0.9) in the isolated *Trichodesmium* bloom patches in the northern Atlantic Ocean (Dugdale et al. 1964; Goering et al. 1966). Carpenter and Romans (1991) reported that N<sub>2</sub> fixation rates based on the extrapolation of the *Trichodesmium* blooms that occurred in the tropical North Atlantic Ocean ranged from 714–3571 mmol N m<sup>-2</sup> d<sup>-1</sup>. However, *Trichodesmium*-specific extrapolation could lead to underestimation of N<sub>2</sub> fixation rates since diazotrophic activity is not limited to the *Trichodesmium* species, in fact, other unicellular cyanobacteria can also contribute significantly to the N budget (Montoya et al. 2004; Moisaner et al. 2010; Thompson et al. 2012).

After the implementation of the N\* method, Michaels et al. (1996) calculated a nitrogen excess generation rate of 51.8–89.6 Tg N y<sup>-1</sup> in the top 1000 m of the Sargasso Sea. Further, Gruber and Sarmiento (1997) reported a fixed nitrogen excess rate of 28 Tg N y<sup>-1</sup> for the tropical and subtropical North Atlantic Ocean. According to the N\* method in earlier periods, the nitrogen excess obtained is attributed to N<sub>2</sub> fixation, mainly by *Trichodesmium* (Gruber and Sarmiento 1997). Sipler et al. (2017) reported N<sub>2</sub> fixation rates in the ice-free coastal Alaskan Arctic (3.5–17.2 nmol N L<sup>-1</sup> d<sup>-1</sup>)

and if the Arctic experiences widespread surface waters, N<sub>2</sub> fixation could contribute up to 3.5 Tg N yr<sup>-1</sup> to the total Arctic nitrogen budget. Further low N<sub>2</sub> fixation rates were found in the southern Indian Ocean (24.6–47.1 μmol N m<sup>-2</sup> d<sup>-1</sup>, Shiozaki et al. 2014a), and in cold Bering Sea waters (10 μmol N m<sup>-2</sup> d<sup>-1</sup>, Shiozaki et al. 2017). The Indian Ocean has been subjected to N<sub>2</sub> fixation rate estimations using the direct detection method since the 2010s and provided a significant data set in the presence and absence of *Trichodesmium* blooms (Gandhi et al. 2011; Bhavya et al. 2016; Kumar et al. 2017; Ahmed et al. 2017; Singh et al. 2019). Similarly, there have been significant studies conducted in various parts of the global oceans which are summarized in Table 1.

The N<sub>2</sub> fixation budget constructed is incomplete since the available observational and experimental data are not sufficient to document diazotrophic activities on a global scale due to spatial and temporal constraints. Most N<sub>2</sub> fixation estimates reported are, in general, obtained from bulk unfiltered upper ocean samples, including cyanobacteria and potentially other diazotrophs. Landolfi et al. (2018) attempted to make a N<sub>2</sub> fixation budget by compiling N<sub>2</sub> fixation rates from various oceanic regions by adding depth integrated estimates to existing surface N<sub>2</sub> fixation compilations (MARine Ecosystem DATA) by Luo et al. (2012) (Table 2). They found that the highest N<sub>2</sub> fixation rate so far occurred in the tropical South Pacific (638 ± 1689 μmol N m<sup>-2</sup> d<sup>-1</sup>, 201 vertical sampling profiles combined from Bonnet et al. 2009, 2015, 2017, 2018; Shiozaki et al. 2013, 2014b; Berthelot et al. 2017). These rates were significantly higher than those in the subtropical North Atlantic (182 ± 479 μmol N m<sup>-2</sup> d<sup>-1</sup>, 636 profiles; Großkopf and LaRoche 2012; Singh et al. 2017) and North Pacific (118 ± 101 μmol N m<sup>-2</sup> d<sup>-1</sup>, 272 profiles; Shiozaki et al. 2015a, 2015b, 2017). Surprisingly, the phosphate-rich waters of the eastern South Pacific showed an average N<sub>2</sub> fixation rate of 86 ± 99 μmol N m<sup>-2</sup> d<sup>-1</sup> (213 profiles; Löscher et al. 2014, 2016; Knapp et al. 2016) which is relatively lower in comparison with the above oceanic regions. Regular estimations of N<sub>2</sub> fixation in the global ocean in order to produce a reliable N budget are not feasible. Since such measurements are scant the available data on N<sub>2</sub> fixation remain meagre and highly variable in space and time. However, a few oceans have been subjected to N<sub>2</sub> fixation estimations with respect to spatial, seasonal, or inter-annual variabilities. Among them, the western tropical South Pacific showed a large spatio-temporal variability. Similarly, the North Atlantic Ocean also showed a variability due to the strong spatial and temporal

**Table 1.** Photic N<sub>2</sub> fixation rates estimations from global oceans using different methodologies

Methodology	Depth integrated N <sub>2</sub> fixation rates (μmol N m <sup>-2</sup> d <sup>-1</sup> )	Region	References
Acetylene Reduction Assay	0.05–540	Subtropical Northeast Atlantic	Carpenter and Price (1977)
Acetylene Reduction Assay	85	Subtropical North Pacific Ocean	Karl et al. (1997)
Acetylene Reduction Assay	170	Central Arabian Sea	Capone et al. (1998)
Acetylene Reduction Assay	239	Western tropical North Atlantic	Capone et al. (2005)
Acetylene Reduction Assay	0.001–0.09	Subtropical Northeast Atlantic	Benavides et al. (2011)
<sup>15</sup> N <sub>2</sub> -bubble and Acetelene Reduction Assay	73–90	Tropical Northwest Atlantic	Falcon et al. (2004)
$N^* = [NO_3^-] - 16 \times [PO_4^{3-}] + 2.72$	500–2500	North Atlantic Ocean	Michaels et al. (1996)
$N^* = ([NO_3^-] - 16 \times [PO_4^{3-}] + 2.90) \times 0.87$	197	North Atlantic Ocean	Gruber and Sarmiento (1997)
$DIN_{xs} = [NO_3^-] - 16 \times [PO_4^{3-}]$	70–208	North Atlantic Ocean	Hansell et al. (2004)
$DIN_{xs} = [NO_3^-] - 16 \times [PO_4^{3-}]$	45–259	Sargasso Sea	Bates and Hansell (2004)
$P^* = -[PO_4^{3-}] - [NO_3^-] / 16$	63	North Atlantic Ocean	Deutsch et al. (2007)
$P^* = -[PO_4^{3-}] - [NO_3^-] / 16$	151–178	North Atlantic Ocean	Palter et al. (2011)
$N^* = [NO_3^-] - 14.63 \times [PO_4^{3-}]$	120 ± 9	Sargasso Sea	Singh et al. (2013)
Extrapolation	714–3571	North Atlantic Ocean	Carpenter and Romans (1991)
Extrapolation	160–430	North Atlantic Ocean	Lipschultz and Owens (1996)
<sup>15</sup> N-nitrate and ammonium	4.5–68.1	Tropical Northwest Atlantic	Goering et al. (1966)
<sup>15</sup> N <sub>2</sub> -bubble method	41–93	Sargasso Sea	Orcutt et al. (2001)
<sup>15</sup> N <sub>2</sub> -bubble method	520 ± 160	Eastern North Pacific gyre	Montoya et al. (2004)
<sup>15</sup> N <sub>2</sub> -bubble method	126 ± 47	Timor-Arafura-Coral seas	Montoya et al. (2004)
<sup>15</sup> N <sub>2</sub> -bubble method	3995	Arafura Sea	Montoya et al. (2004)
<sup>15</sup> N <sub>2</sub> -bubble method	850	Western tropical North Atlantic	Capone et al. (2005)
<sup>15</sup> N <sub>2</sub> -bubble method	1.0–13	South China Sea	Chen et al. (2008)
<sup>15</sup> N <sub>2</sub> -bubble method	1.8–182	North Atlantic Ocean	Moore et al. (2009)
<sup>15</sup> N <sub>2</sub> -bubble method	20–310	North Pacific Ocean	Church et al. (2009)
<sup>15</sup> N <sub>2</sub> -bubble method	1.2–298	North Atlantic Ocean	Fernandez et al. (2010)
<sup>15</sup> N <sub>2</sub> -bubble method	28–142	Tropical Northeast Atlantic	Turk-Kubo et al. (2011)
<sup>15</sup> N <sub>2</sub> -bubble method	100–34000	Eastern Arabian Sea	Gandhi et al. (2011)
<sup>15</sup> N <sub>2</sub> -bubble method	56–60	Tropical Northeast Atlantic	Turk-Kubo et al. (2012)
<sup>15</sup> N <sub>2</sub> -bubble method	15–424	Eastern equatorial Atlantic	Subramaniam et al. (2013)
<sup>15</sup> N <sub>2</sub> -bubble method	0–148	Eastern Tropical South Pacific	Dekazemacker et al. (2013)
<sup>15</sup> N <sub>2</sub> -bubble method	up to 800	Off Peru-South Pacific	Loescher et al. (2013)
<sup>15</sup> N <sub>2</sub> -bubble method	0–90	North Pacific Ocean	Schiozaki et al. (2014a)
<sup>15</sup> N <sub>2</sub> -bubble method	24.6–47.1	Arabian Sea	Schiozaki et al. (2014b)
<sup>15</sup> N <sub>2</sub> -bubble method	6.27–16.6	Equatorial and Southern Indian Ocean	Schiozaki et al. (2014)
<sup>15</sup> N <sub>2</sub> -bubble method	0–23	Eastern Tropical South Pacific	Knapp et al. (2016)
<sup>15</sup> N <sub>2</sub> -bubble method	174–238	Southeastern Arabian Sea	Bhavya et al. (2016)
<sup>15</sup> N <sub>2</sub> -bubble method	10	Cold Bering Sea	Schiozaki et al. (2017)
<sup>15</sup> N <sub>2</sub> -bubble method	1140–8405	Eastern Arabian Sea	Kumar et al. (2017)
<sup>15</sup> N <sub>2</sub> -bubble method	1300–2500	Eastern Arabian Sea	Singh et al. (2019)
<sup>15</sup> N <sub>2</sub> -Tracer dissolution method	91 ± 4	Atlantic Ocean	Großkopf et al. (2012)
<sup>15</sup> N <sub>2</sub> -Tracer dissolution method	1739	Arabian Sea-Trichodesmium bloom	Ahmed et al. (2017)

gradients of physical forcing and associated environmental factors (Mouriño-Carballido et al. 2011; Landolfi et al. 2016). The variability observed in the North Pacific Ocean is mainly linked to the changes in seasonal (Böttjer et al. 2017) and

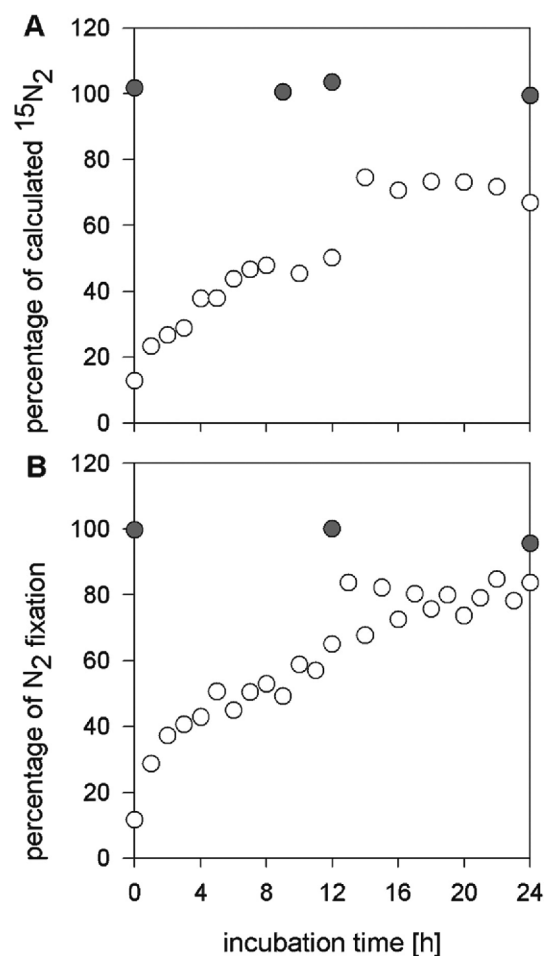
mesoscale activity (e.g. Church et al. 2009). In comparison, the variability in the Northern Indian Ocean, particularly the Arabian Sea, is mainly due to seasonal changes and occurrence of *Trichodesmium* blooms (Gandhi et al. 2011; Bhavya et al.

**Table 2.** The compilation of depth integrated marine N<sub>2</sub> fixation rates from various experimental results quantified by Landolfi et al. (2018)

Oceanic region	N <sub>2</sub> fixation rates ( $\mu\text{mol N m}^{-2} \text{d}^{-1}$ )	Number of sampling profiles
Tropical South Pacific	638 $\pm$ 1689	201
Subtropical North Atlantic	182 $\pm$ 479	636
North Pacific	118 $\pm$ 101	272
Eastern South Pacific	86 $\pm$ 99	213

2016; Singh et al. 2019).

It is highly important to expand the expedition boundaries, beyond the classical understanding about N<sub>2</sub> fixation. Recent studies reported that all diazotrophs are not constrained by light for their nitrogenase activity and they have been seen to be active in various environments such as nutrient-rich, cold, and/or dark ecosystems including coastal upwelling regions (Sohm et al. 2011; Bhavya et al. 2016), eutrophic estuaries (Bhavya et al. 2016), temperate coastal zones (Bentzon-Tilia et al. 2015), and the deep ocean (Hewson et al. 2007; Hamersley et al. 2011). To represent a considerable increase in the potentially active oceanic areas of N<sub>2</sub> fixation it would be necessary to extend the latitudinal limits from the tropics and subtropics to temperate waters and also conduct vertical expansion of estimations to the mesopelagic (200–1,000 m) and bathypelagic (1,000–4,000 m) ocean; however, this would be an enormous undertaking. Although aphotic N<sub>2</sub> fixation rates are conceptually low when compared to surface activity ( $< 1 \text{ nmol N L}^{-1} \text{ d}^{-1}$ ; Moisaner et al. 2017), while considering the volume of the deep ocean it would make a significant addition to the global N<sub>2</sub> fixation budget. A few studies reported that depth-integrated aphotic rates N<sub>2</sub> fixation rates can represent 40–95% of the whole water column diazotrophic activity (Bonnet et al. 2013; Rahav et al. 2013; Benavides et al. 2015). Similarly, N<sub>2</sub> fixation rates on the dark incubation samples also showed significant rates close to that of light incubation samples (Bhavya et al. 2016) and, hence, the nocturnal activities of diazotrophs also should be considered. Mohr et al. (2010) experimentally demonstrated that the injected <sup>15</sup>N<sub>2</sub> gas bubble does not attain equilibrium with the surrounding sample water during the short incubation period. This lack of complete dissolution can lead to a <sup>15</sup>N<sub>2</sub> concentration lower than the expected and underestimation of the actual rates. They also found that the extent of underestimation varies with the incubation time (Fig. 2); however, the amount of injected gas is not seen as a major controlling factor. It is also known that *Trichodesmium* releases recently fixed N in the form of NH<sub>4</sub><sup>+</sup> and dissolved organic nitrogen (Mulholland et



**Fig. 2.** Time-dependence of the equilibration of a <sup>15</sup>N<sub>2</sub> gas bubble with seawater. This figure is obtained from Mohr et al. (2010). Results are presented as a function of the time after bubble injection (white symbols). (A) Measured dissolved <sup>15</sup>N<sub>2</sub> concentrations as percentage of calculated concentration assuming rapid and complete isotopic equilibrium. (B) N<sub>2</sub> fixation rates by *C. watsonii* as percentage of the maximum rate measured during the experiments. For comparison, the addition of <sup>15</sup>N<sub>2</sub>-enriched water to samples yielded a constant <sup>15</sup>N<sub>2</sub> enrichment over 24 h (A, grey symbols) or constant N<sub>2</sub> fixation rates (B, grey symbols)

al. 2004, 2006). Such release can even exceed the net accumulation of N in the biomass (Mulholland and Capone 2001) and might lead to underestimation of the N<sub>2</sub>-fixation

rates measured if the incubation periods are longer. To resolve this issue researchers usually follow an incubation period of 4 hours symmetric to the local noon (Gandhi et al. 2011). To ensure the complete dissolution of  $^{15}\text{N}_2$  gas the sample bottles have usually been subjected to gentle shaking more than 50 times.

To construct an efficient N budget, the gain processes such as fixed nitrogen inputs to the ocean include fluvial inputs, atmospheric deposition and biological  $\text{N}_2$  fixation and loss processes such as denitrification and anaerobic ammonium oxidation (anammox) should be taken into account. The N gain process all together account for up to 187–279  $\text{Tg N y}^{-1}$  and the combination of denitrification (including sediment burial) and anammox account for fixed nitrogen losses up to 260–475  $\text{Tg N y}^{-1}$  (Benavides et al. 2018). However, the incorporation of mesopelagic aphotic  $\text{N}_2$  fixation rates is seen as significant for establishing a balance between N loss and gain processes. To elaborate, the lower end range of  $\text{N}_2$  fixation rates in the mesopelagic zone recorded in the literature is 0.01–0.1  $\text{nmol N L}^{-1} \text{d}^{-1}$  (Moisander et al. 2017) and the estimated volume of the mesopelagic zone is  $2.63 \times 10^{17} \text{m}^3$  (Aristegui et al. 2005). In that case, the mesopelagic  $\text{N}_2$  fixation would range between 13 and 134  $\text{Tg N y}^{-1}$  and the addition of mesopelagic  $\text{N}_2$  fixation to fixed nitrogen inputs creates a dramatic change in the budget from a gross of 183 to a surplus of 114  $\text{Tg N y}^{-1}$  for N gain process (Benavides et al. 2018). However, such extrapolations based on scant observation data can produce ambiguities while demonstrating at the same time that aphotic  $\text{N}_2$  fixation can make a significant contribution toward the N budget (Zehr et al. 2008; Thompson et al. 2012).

In addition, recent findings by Großkopf and LaRoche (2012) proved that the  $\text{N}_2$  fixation rates estimated using the  $^{15}\text{N}_2$  bubble method have been significantly underestimated and the N budget estimated in this manner raises serious concerns. Furthermore, Bombar et al. (2018) claimed that filtration through conventional glass fiber filters in  $^{15}\text{N}_2$  tracer assays may fail to capture all nitrogen-fixing Prokaryotes. Dabundo et al. (2014) reported that there can be a significant amount of N contamination in commercial  $^{15}\text{N}_2$  gas stocks with  $^{15}\text{N}$ -labeled  $\text{NO}_3^-$  and  $\text{NH}_4^+$  and can potentially affect the  $\text{N}_2$  fixation measurements. Taking these factors to account, it is highly important to sort out issues associated with previous methods and minimize the errors in those data using new conventions derived employing newly introduced techniques.

## 7. Anthropogenic Influences and Climate Change

As marine  $\text{N}_2$  fixers are highly sensitive to the physico-chemical variabilities of the environment in which they live, it is obvious that climate change and anthropogenic activities play a major role in altering the activities of marine diazotrophs. Global warming greatly influences Arctic Ocean biogeochemistry and as a result an unusual  $\text{N}_2$ -fixing unicellular cyanobacteria (UCYN-A)/haptophyte symbiosis was found in entirely unexpected environments such as the northern waters of the Danish Strait and the Bering and Chukchi Seas (Harding et al. 2018). The increase in temperature leads to sea ice melt which further lengthens the growing season for phytoplankton and diazotrophs, which was not obvious in the polar oceans previously. Recent research works reported that the UCYN-A have existed in the Arctic as the environment has warmed as a result of climate change and have the potential to begin  $\text{N}_2$  fixation (Harding et al. 2018). On a different note, warmer temperatures in the polar regions can potentially support phytoplankton growth and they tend to remove C from the ocean and ultimately the atmosphere (Thomas et al. 2012); however, they need sufficient bioavailable N to support this. The presence of UCYN-A may provide N requirement for the phytoplankton to perform C sequestration as the influence of global warming takes hold. The  $\text{N}_2$  fixation rate estimates from the Arctic Ocean suggest that UCYN-A can independently contribute approximately 2% of global  $\text{N}_2$  fixation (Harding et al. 2018). The researchers hypothesize that the increase in available N in the Arctic regions in the future could impact biogeochemical cycling in the North Atlantic Ocean and may lead to further shifts in ocean biochemical cycles (Harding et al. 2018).

On the other hand, the anthropogenic  $\text{CO}_2$  added to the atmosphere eventually enhances the oceanic partial pressure of  $\text{CO}_2$  ( $p\text{CO}_2$ ) which, in turn, results in lowering the pH of the sea water, which is known as ocean acidification (Riebesell 2004). Phytoplankton, in general, invest a large amount of energy in a carbon-concentrating mechanism (CCM) by which it can accumulate sufficient  $\text{CO}_2$  by converting  $\text{HCO}_3^-$  to perform photosynthesis (Badger et al. 1998). Hong et al. (2017) reported that ocean acidification inversely affects the *Trichodesmium* in terms of their nitrogenase activity and reduces the global  $\text{N}_2$  fixation rates. They also experimentally demonstrated that there will be a significant decrease in  $\text{N}_2$  fixation by *Trichodesmium* with increasing  $p\text{CO}_2$ , particularly under the iron-limited conditions that prevail in large



oceanic regions (Sohm et al. 2011). Similar effects could be expected on other diazotrophs if they are also sensitive to the higher  $p\text{CO}_2$ ; however, no experiment has been performed to prove this hypothesis.

## 8. Conclusions

In this review, we summarized major research works conducted on biological N<sub>2</sub> fixation in marine environments which includes detailed analysis of the N<sub>2</sub> fixation mechanism, species, evolution of methodologies, and rates in the global ocean regimes. We also discussed the challenges that exist in constructing an N budget since the methodologies used to estimate N<sub>2</sub> fixation rates were methodologically different and were often subjected to qualitative assessments and amendments to achieve better results. Nevertheless, approaches to constrain global rates of N<sub>2</sub> fixation (experimental, geochemical, and model-based estimates) – even though they contain their own uncertainties and biases - have helped to develop an understanding of diazotrophic activities around the globe. However, there are many ambiguities pertaining to the environmental and ecological interactions of diazotrophs, which inhibit us from obtaining robust N<sub>2</sub> fixation rate estimates and predictions. To reduce the current level of uncertainty it is highly important to amalgamate environmental factors and evolving ecological interactions, both in the field and in the laboratory. Such combinations would help in identifying essential characteristics and ecological interactions experimentally and form mechanistic based models to generate N<sub>2</sub> fixation rates close to the ambient values.

## Acknowledgements

This research was a part of the project entitled "Construction of Ocean Research Station and their application" and "Improvements of ocean prediction accuracy using numerical modeling and artificial intelligence technology" funded by the Ministry of Oceans and Fisheries, Korea. This research was also partly supported by a grant from the National Institute of Fisheries Science (NIFS) in the Republic of Korea (grant number: R2019062).

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