Dissolved hydrogen and nitrogen fixation in the oligotrophic North Pacific Subtropical Gyre

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Summary

The production of hydrogen (H\textsubscript{2}) is an inherent component of the biological dinitrogen (N\textsubscript{2}) fixation process with the theoretical stoichiometry predicting an equimolar production of H\textsubscript{2} for every mole of N\textsubscript{2} fixed. However, while the stoichiometry of N\textsubscript{2} fixation can be evaluated in high biomass cultures of diazotrophs, conducting the relevant measurements for a field population is more complex. Independent measurements of N\textsubscript{2} fixation, H\textsubscript{2} consumption, and dissolved H\textsubscript{2} concentrations were performed on surface water samples collected in the oligotrophic North Pacific Ocean to constrain the cycling of H\textsubscript{2} associated with N\textsubscript{2} fixation. The quantity of H\textsubscript{2} consumed by microbial oxidation was equal to 1-7\% of ethylene produced during the acetylene reduction assay and to 11-63\% of $^{15}$N\textsubscript{2} assimilation. Varying abundance of Crocosphaera and Trichodesmium as revealed by nifH gene abundance broadly corresponded with diel changes observed in both N\textsubscript{2} fixation and H\textsubscript{2} oxidation. However no corresponding changes were observed in the dissolved H\textsubscript{2} concentrations which remained consistently supersaturated (147–560\%) relative to atmospheric equilibrium. The results from this field study allow the efficiency of H\textsubscript{2} cycling by natural populations of diazotrophs to be compared to their cultured representatives. The findings indicate that the extent to which dissolved H\textsubscript{2} concentrations correspond to N\textsubscript{2} fixation in the open ocean may depend less upon the species of diazotrophs present in the water column and more upon relevant environmental parameters e.g. light intensity or the presence of other H\textsubscript{2}-metabolizing microorganisms.
In the surface waters of the tropical and subtropical open ocean, dissolved H\textsubscript{2} concentrations typically range from 1–3 nmol l\textsuperscript{-1}, equivalent to 300–900% supersaturation relative to atmospheric equilibrium (Herr \textit{et al.}, 1984; Conrad and Seiler, 1988; Moore \textit{et al.}, 2009). The magnitude of the dissolved H\textsubscript{2} pool is determined by the ‘oceanic H\textsubscript{2} cycle’ which reflects the balance between production and consumption processes. As such, the main source of H\textsubscript{2} is considered to be biological dinitrogen (N\textsubscript{2}) fixation (Scranton \textit{et al.}, 1987; Herr \textit{et al.}, 1984; Moore \textit{et al.}, 2009) whereby N\textsubscript{2} is reduced to ammonia (NH\textsubscript{3}), as shown in Equation 1:

\begin{equation}
\begin{aligned}
\text{N}_2 + 8 \text{H}^+ + 8 \text{e}^- + 16 \text{ATP} &\rightarrow 2 \text{NH}_3 + \text{H}_2 + 16 \text{ADP} + 16 \text{Pi}
\end{aligned}
\end{equation}

where ADP and ATP are adenosine-5’-diphosphate and adenosine-5’-triphosphate respectively, H\textsuperscript{+} is hydrogen ion, e\textsuperscript{−} is electron, and Pi is inorganic phosphorus (Simpson and Burris 1984).

While N\textsubscript{2} fixation is more commonly measured than H\textsubscript{2} production, it is unwise to use the theoretical stoichiometry predicted in Eq. 1 to provide an estimate of H\textsubscript{2} production associated with nitrogenase activity. This is due to several inherent issues associated with H\textsubscript{2} cycling linked to N\textsubscript{2} fixation, as listed below:

(i) Measurements of H\textsubscript{2} production alongside measurements of N\textsubscript{2} fixation are always less than the equimolar stoichiometry predicted in Equation 1 (Schubert and Evans, 1976; Wilson \textit{et al.}, 2010). This is because all diazotrophs contain uptake hydrogenases that re-assimilate a variable portion of H\textsubscript{2} released during N\textsubscript{2} fixation to conserve energy (Burns and Hardy, 1975, Tamgnini \textit{et al.}, 2007).
(ii) Rates of net H$_2$ production by diazotrophs appear to be highly species-specific. Laboratory-maintained cultures of two diazotrophs, *Crocosphaera* and *Trichodesmium* produce H$_2$ at approximately 1 and 25% of their respective rates of N$_2$ fixation, as measured by the acetylene reduction (AR) assay (Wilson *et al.* 2010). The comparatively high rates of net H$_2$ production by *Trichodesmium* are a consequence of the cells fixing N$_2$ during the day-time as the supply of photosynthetically-derived energy and reductant decreases the need to re-assimilate the H$_2$ as an energy source, resulting in an increase of net H$_2$ production (Wilson *et al.*, 2012b). By comparison, *Crocosphaera* fixes N$_2$ during the dark period restricting the supply of cellular energy to nitrogenase from the respiration of photosynthetically-fixed carbon (Waterbury *et al.*, 1988; Berman-Frank *et al.*, 2007). This causes a greater demand for the energy and reductant produced from oxidizing H$_2$ and therefore decreases the net H$_2$ production (Wilson *et al.*, 2010).

(iii) Field measurements of N$_2$ fixation can be conducted using the $^{15}$N$_2$ assimilation technique or the AR assay. The $^{15}$N$_2$ tracer technique is considered to be a measure of net N$_2$ fixation (Montoya *et al.*, 1996; Mulholland *et al.*, 2004). The AR assay measures total nitrogenase activity by quantifying the reduction of acetylene (C$_2$H$_2$) to ethylene (C$_2$H$_4$) and therefore represents an indirect assay of N$_2$ fixation (Burris, 1975). Because H$_2$ production would be expected to scale on gross N$_2$ fixation (Eq. 1), the AR assay could represent a better correlative measurement to comparing N$_2$ fixation and H$_2$ cycling.

Due to the issues listed above, to define the role of N$_2$ fixation in the global H$_2$ cycle (*e.g.* Price *et al.*, 2007) it is imperative to conduct field measurements of both N$_2$ fixation and H$_2$ production. In this study, simultaneous measurements of N$_2$ fixation, biological H$_2$ consumption, and dissolved H$_2$ concentrations were conducted in the surface waters of
the open ocean where diazotrophs are present. Results are presented of the diazotrophic community composition (as measured by \textit{nifH} gene abundance and diversity), rates of net and gross \textsubscript{N}2 fixation (as measured by \textsuperscript{15}N\textsubscript{2} tracer assimilation and AR assay, respectively), \textsubscript{H}2 concentrations, and \textsubscript{H}2 oxidation rates (using \textsuperscript{3}H\textsubscript{2} as a tracer).

Quantitative interpretation of the field data is aided by the recent measurement of net \textsubscript{H}2 production and \textsubscript{N}2 fixation in laboratory cultures of diazotrophs to infer the relative contribution of the representative marine \textsubscript{N}2 fixing microorganisms to the oceanic \textsubscript{H}2 cycle.

\textbf{Results and discussion}

\textit{Sampling overview}

The oceanographic cruise was located approximately 250 km north of Oahu, Hawaii in the North Pacific Subtropical Gyre (NPSG) and occurred between 6 and 21 September 2011. The sampling stations were occupied along north-western edge of an anticyclonic eddy spanning a total distance of 90 km and the subsequent westward section of the cruise track which spanned 80 km. Vertical profiles of dissolved \textsubscript{H}2 were conducted daily alongside biogeochemical and hydrographic measurements. Biological rate measurements of \textsubscript{N}2 fixation and \textsubscript{H}2 consumption were conducted at 3 sampling stations: Station (Stn) 3, 7, and 13 which were sampled on the 7, 9, and 18 September 2011, respectively. Descriptions of the hydrographic conditions and biogeochemical properties of the water column are available in the accompanying Supplementary Information and also online at http://hahana.soest.hawaii.edu/cmorebiolincs/biolincs.html.
Dissolved $H_2$ concentrations

Dissolved $H_2$ concentrations were super-saturated with respect to atmospheric equilibrium in the upper 75 m of the water column (Fig. 1). Overall, dissolved $H_2$ concentrations in the surface mixed layer (0–45 m) ranged from 0.5–1.9 nmol l$^{-1}$, with an average concentration of 0.83 nmol l$^{-1}$, equivalent to 250% supersaturation. On four separate occasions the concentrations of dissolved $H_2$ in the mixed layer exceeded 1 nmol l$^{-1}$ (Fig. 1). The concentrations of $H_2$ measured in surface seawater during this cruise are consistent with measurements in other marine environments (e.g. the Atlantic, Mediterranean, and Pacific Ocean) revealing a persistent supersaturation of dissolved $H_2$ in the near-surface seawater (Conrad and Seiler 1988, Herr et al., 1984, Moore et al., 2009, Scranton et al., 1982). At depths exceeding 75 m a progressive depletion in $H_2$ concentrations was observed with values approaching undersaturation with respect to atmospheric equilibrium by a depth of 100 m. Vertical profiles of $N_2$ fixation in the NPSG measured on previous occasions (Church et al., 2009, Grabowski et al., 2008) similarly show a decrease at 75 m, providing indirect evidence that the dissolved $H_2$ is derived from nitrogenase activity.

$N_2$ fixation

$N_2$ fixation rate measurements, determined by both the $^{15}N_2$ tracer assimilation and the AR assay, were conducted at Stn 3, 7 and 13. The overall temporal pattern of $N_2$ fixation changed between the stations from an initial prevalence during the night-time, to a subsequent dominance during the day-time. Specifically, rates of $^{15}N_2$ assimilation during the night-time (0.22 nmol l$^{-1}$ h$^{-1}$) exceeded the day-time (0.08 nmol l$^{-1}$ h$^{-1}$) at Stn 3
(Fig. 2A). In contrast, at Stn 13, rates of $^{15}$N$_2$ assimilation in whole seawater were highest (0.26 nmol l$^{-1}$ h$^{-1}$) during the day-time, compared to the rates during the night-time (0.04 nmol l$^{-1}$ h$^{-1}$) (Fig. 2C). No significant difference was observed between the day-time and night-time measurements of N$_2$ fixation at Stn 7. At all sampling stations, the rate of $^{15}$N$_2$ assimilation in whole seawater samples exceeded the comparative rates in the accompanying <10 µm size fractionated seawater samples. Comparison of the <10 µm size fraction across the three stations reveals low variability in the rate of $^{15}$N$_2$ assimilation (0.04–0.06 nmol l$^{-1}$ h$^{-1}$) during the day-time. In contrast, night-time rates of $^{15}$N$_2$ assimilation for the <10 µm size fraction varied by an order of magnitude, decreasing from 0.14 nmol l$^{-1}$ h$^{-1}$ at Stn 3, to 0.01 nmol l$^{-1}$ h$^{-1}$ at Stn 13 (Fig. 2A-C).

AR was measured on whole seawater samples and a significant increase in C$_2$H$_4$ concentrations was always detected during the 3–4 h incubations (Fig. 2D-F). The rates of C$_2$H$_4$ production support the $^{15}$N$_2$ assimilation measurements with higher rates during the night-time (2.9 nmol l$^{-1}$ h$^{-1}$) compared to the day-time (1.8 nmol l$^{-1}$ h$^{-1}$) at Stn 3. Furthermore, at Stn 13, the diel pattern of C$_2$H$_4$ production changed with day-time (3.3 nmol l$^{-1}$ h$^{-1}$) exceeding night-time (0.4 nmol l$^{-1}$ h$^{-1}$) (Fig. 2F). Overall, the ratio of C$_2$H$_4$ to $^{15}$N$_2$ assimilation varied from 9–22 which exceeds the theoretical ratio of 3:1 (Capone 1993) by 3–7 fold. It should be noted that the theoretical ratio of 3:1 is based on the difference between 2 hydrogen ions required to reduce C$_2$H$_2$ to C$_2$H$_4$ and 6 hydrogen ions needed to reduce N$_2$ to 2NH$_3$. The reasons for the discrepancies between the theoretical and observed ratios have previously been discussed (e.g. Graham et al., 1980) and focus mainly on the excretion of N from the cell and the role of H$_2$. There is insufficient data in this study to contribute to this discussion, however we do note from our work and the
relevant literature that there is increased discrepancy in the C$_2$H$_4$:¹⁵N$_2$ assimilation ratio in field measurements compared to culture-based analyses. Furthermore there is a lack of experimental testing on the effect of key environmental parameters on the C$_2$H$_4$:¹⁵N$_2$ assimilation ratio e.g. light intensity or nutrient concentrations (Mague et al., 1977).

**Diazotroph community structure**

Representative N$_2$ fixing microorganisms in the open ocean include: (i) the filamentous, non-heterocystous cyanobacterium *Trichodesmium*, (ii) the heterocystous cyanobacteria (e.g. *Richelia* and *Calothrix*) that form symbioses with eukaryotic algae, and (iii) unicellular cyanobacteria including Group A (termed UCYN-A) and Group B (e.g. *Crocosphaera*) (Mague et al., 1977; Carpenter and Romans 1991; Zehr et al., 2001).

The analysis of *nifH* gene abundances revealed Group B was the most abundant diazotroph for the first two sampling occasions (Stn 3 and 7), with 4.3 x 10$^5$ and 1.3 x 10$^6$ gene copies l$^{-1}$. At the third sampling site (Stn 13), *nifH* gene copies of Group B decreased to 2.9 x 10$^4$ gene copies l$^{-1}$, in contrast to *Trichodesmium* *nifH* gene copies which increased to a maximum of 1.6 x 10$^6$ gene copies l$^{-1}$ (Fig. 2). The shift from a Group B-dominated to a *Trichodesmium*-dominated diazotroph community between Stn 3 and 13, respectively, could help account for the change in the pattern of N$_2$ fixation. The unicellular *Crocosphaera* fixes N$_2$ in the dark and rates of N$_2$ fixation were highest during the night-time when *Crocosphaera* gene copies were most abundant. Two other groups of diazotrophs were present at lower abundances throughout the cruise; UCYN-A *nifH* abundance ranged from 1.6 x 10$^3$ to 1.9 x 10$^5$ gene copies l$^{-1}$ and the total
heterocystous cyanobacterial gene copies were the lowest of all \textit{nifH} gene groups measured with a maximum abundance of $6.2 \times 10^3$ gene copies l$^{-1}$ at Stn 13.

\textit{Microbial consumption of H$_2$}

Biological $^3$H$_2$ oxidation was measured during the day and night-time, alongside N$_2$ fixation rate measurements at Stn 3 and 13. Overall, the rates of biological $^3$H$_2$ oxidation ranged from 15 to 42 pmol H$_2$ l$^{-1}$ h$^{-1}$ (Table 1). At Stn 3, night-time rates of biological $^3$H$_2$ oxidation (25 pmol H$_2$ l$^{-1}$ h$^{-1}$) exceeded day-time rates (15 pmol H$_2$ l$^{-1}$ h$^{-1}$) by 66%.

In contrast, at Stn 13 the day-time rates of biological $^3$H$_2$ oxidation (42 pmol H$_2$ l$^{-1}$ h$^{-1}$) were 68% higher than night-time (25 pmol H$_2$ l$^{-1}$ h$^{-1}$) (Table 1). In this respect, the temporal variability in biological $^3$H$_2$ oxidation rates reflect the temporal patterns observed in the rate of $^{15}$N$_2$ assimilation and the AR assay. The measured rates of $^3$H$_2$ oxidation were equivalent to 11-63% of $^{15}$N$_2$ assimilation and 1-7% of C$_2$H$_4$ production as measured by the AR assay.

Previous measurements of biological H$_2$ consumption have been reported from other aquatic habitats including coastal seawater (Punshon \textit{et al.}, 2007), shallow lakes (Conrad \textit{et al.}, 1983), and river systems (Paerl 1982). These previous studies have revealed H$_2$ turnover times ranging from $<1$ h in a eutrophic shallow lake (Conrad \textit{et al.}, 1983) to 2–3 days in high-latitude coastal seawater (Punshon \textit{et al.}, 2007). In comparison, the H$_2$ turnover times measured in this study at two sampling stations ranged from 22–40 h (Table 1).

\textit{Estimation of the production and consumption of H$_2$ associated with N$_2$ fixation}
The measured rates of N\textsubscript{2} fixation using the AR assay at Stn 3 and 13 were used to estimate the production of H\textsubscript{2} derived from nitrogenase (Table 2). We use laboratory-derived measurements of net H\textsubscript{2} production by \textit{Trichodesmium} and \textit{Crocosphaera} cultures described in the Introduction to provide upper and lower boundaries for H\textsubscript{2} production. Therefore in contrast to Price \textit{et al.} (2007) who estimated net H\textsubscript{2} production at 55\% of N\textsubscript{2} fixation in the marine environment, we set maximum and minimum net H\textsubscript{2} production rates at 25\% and 1\% of C\textsubscript{2}H\textsubscript{4} production, respectively. The resulting estimates of net H\textsubscript{2} production range from 0.004 to 0.84 nmol H\textsubscript{2} l\textsuperscript{-1} h\textsuperscript{-1} in the upper water column. Furthermore, the calculations indicate that N\textsubscript{2} fixation can replenish the dissolved H\textsubscript{2} pool in as little as 1 h and extending up to 34 hrs, with the exception of 19 September during the night time which has an excessively long upper estimate of 245 h (Table 2).

The estimates of net H\textsubscript{2} production in surface seawater as listed in Table 2 can be compared with the biological $^{3}$H\textsubscript{2} oxidation measurements which were conducted on the same seawater samples (Table 1). The rates of $^{3}$H\textsubscript{2} oxidation were equivalent to 0.8 – 6.6 \% of the AR assay (Table 2) indicating biological consumption was equivalent to the lower end of estimated rates of net H\textsubscript{2} production \textit{i.e.} comparable to rates of net H\textsubscript{2} production by \textit{Crocosphaera}. This suggests that concentrations of dissolved H\textsubscript{2} may increase in the presence of \textit{Trichodesmium} and stimulate the diel cycles of H\textsubscript{2} in surface seawater as observed by Herr \textit{et al.} (1984) in the South Atlantic. However in this study, the increase in \textit{Trichodesmium} abundance was not matched by an increase in net H\textsubscript{2} concentrations (Fig. 1) suggesting that field populations of \textit{Trichodesmium} may re-assimilate more of the H\textsubscript{2} produced via nitrogenase compared to their cultured counter-
parts and are therefore more energetically efficient. Alternatively, other sinks of H\textsubscript{2} in
the upper ocean may contribute to the loss of dissolved H\textsubscript{2} and these are considered in the
next section.

\textit{H\textsubscript{2} cycling in the open ocean}

The oceanic H\textsubscript{2} cycle depends not only on biological production and consumption as
discussed with reference to diazotrophs, but also physical forcing mechanisms. The
physical processes can be considered with respect to the sink terms for H\textsubscript{2}, comparing
estimates of air-sea gas exchange and downwards diffusion with biological oxidation.

The downward diffusion of H\textsubscript{2} can be estimated from the concentration gradient between
depths of 45 and 75 m, using the vertical eddy diffusion coefficient reported by Ledwell
\textit{et al.} (1993) (Table 3). The flux of H\textsubscript{2} to the atmosphere can be estimated according to
Equation 2, where \( S \) is the Bunsen solubility coefficient (Wiesenburg and Guinasso,
1979), \( \Delta p \) is the difference in partial pressure (\( p \)) between the atmosphere and ocean, and
\( k \) is the transfer velocity. An atmospheric H\textsubscript{2} concentration of 0.53 ppmv was used in the
flux calculations (Novelli \textit{et al.}, 1999). The transfer velocity (\( k \)) was calculated
according to Wanninkhof (1992) (Equation 3) where \( U \) is the wind speed (m sec\textsuperscript{-1})
normalized to 10 m above the sea surface and \( Sc \) represents the Schmidt number for H\textsubscript{2} at
\textit{in situ} seawater temperature and salinity (Jähne \textit{et al.}, 1987).

\begin{equation}
F = k \cdot S \cdot \Delta p
\end{equation}

\begin{equation}
k = 0.31 \cdot U^2 \cdot (Sc/660)^{0.5}
\end{equation}

To obtain depth-integrated estimates of H\textsubscript{2} consumption we used historical
measurements of N\textsubscript{2} fixation profiles at Stn ALOHA (HOT cruises #202-213) to calculate
the relationship between N\textsubscript{2} fixation measurements at 25 m and 0-45 m depth integrated values (y=46.12x + 23.8, \textit{r}^2=0.82). The conversion factor was applied to the rates of N\textsubscript{2} fixation (Fig.1) using the percentage of AR assay and \textsuperscript{15}N\textsubscript{2} assimilation (Table 1) to provide a lower and upper estimate of biological H\textsubscript{2} consumption respectively, integrated across the 0-45 m depth horizon. While there is approximately an order of magnitude difference between the upper and lower estimates of biological consumption (Table 3), the median values for turnover times compare favorably with the rates of H\textsubscript{2} consumption calculated from the \textsuperscript{3}H\textsubscript{2} oxidation measurements for discrete seawater samples collected from 25 m (Table 1). It is evident that for this time period, biological consumption and downward diffusion represented the main loss pathways for dissolved H\textsubscript{2} in the upper ocean. The estimated flux of H\textsubscript{2} to the atmosphere ranged from 0.03 - 0.33 \textmu mol m\textsuperscript{-2} h\textsuperscript{-1} (Table 3) and should be considered a low estimate of H\textsubscript{2} loss to the overlying atmosphere due to the predominantly low wind speeds (<5 m sec\textsuperscript{-1}) during the cruise.

**Conclusion**

During a 10 day sampling period in the NPSG, dissolved H\textsubscript{2} concentrations were 147–560\% supersaturated with respect to atmospheric equilibrium. Measured rates of \textsuperscript{15}N\textsubscript{2} assimilation and AR revealed a change in the prevalence of N\textsubscript{2} fixation from night-time to day-time, which was accompanied by a decrease in the abundance in Group B \textit{nifH} gene copies, and an increase in the abundance of \textit{Trichodesmium} \textit{nifH} gene copies. Prior to this study it was hypothesized that varying abundance of larger, day-time N\textsubscript{2} fixing microorganisms \textit{e.g.} \textit{Trichodesmium} might influence the dissolved pool of H\textsubscript{2} in surface seawater due to their relatively high rates of net H\textsubscript{2} production (Wilson \textit{et al.},
However the absence of varying dissolved H$_2$ concentrations indicate that field populations of *Trichodesmium* may be more efficient at recycling H$_2$ compared to laboratory cultures. Biological H$_2$ oxidation measurements in seawater sampled from 25 m depth indicate that H$_2$ production needed to exceed 1-6% of C$_2$H$_4$ production to cause an increase in the ambient pool of dissolved H$_2$ (Table 1). This is considerably lower than in laboratory-maintained *Trichodesmium* cultures where the rate of net H$_2$ production was equivalent to 25% of C$_2$H$_4$ production (Wilson *et al.*, 2012b). Using either the AR assay or the $^{15}$N$_2$ assimilation technique caused approximately 1 order of magnitude variability when calculating the efficiency of H$_2$ cycling. We consider the AR assay to be more representative of nitrogenase activity but recognize that it is an indirect measurement and not widely used in oceanographic studies on non-concentrated seawater samples. Comparison of the loss mechanisms for dissolved H$_2$ in the upper ocean indicated that biological oxidation represented the most prevalent sink compared to downward diffusion and flux to the atmosphere (Table 3).

It should be noted that oceanic H$_2$ cycling is not limited to diazotrophs, and opportunistic H$_2$-oxidizing microorganisms *e.g.* aerobic anoxygenic photosynthetic bacteria and heterotrophic bacteria will also metabolize H$_2$. Furthermore, other sources of H$_2$ such as photochemical degradation of dissolved organic matter (Punshon and Moore 2008) and fermentation (Schropp *et al.*, 1987) should be considered when considering H$_2$ cycling in the upper water column. Nonetheless, this study confirms that wherever diazotrophs occur in the natural environment, the ecosystem becomes enriched in dissolved H$_2$ (Conrad 1988) although the cycling of H$_2$ is more subtle than suggested from laboratory cultures of diazotrophs.
Method

Dissolved H₂ concentrations were measured with a reduced gas analyzer (Peak Laboratories, Mountain View) adapting the method of Moore et al. (2009). The rate of H₂ consumption was quantified by measuring the production of ³H₂O from tracer additions of ³H₂ as previously used in laboratory cultures of diazotrophs (Chan et al., 1980) and environmental microbial assemblages (Paerl, 1983). To determine the rate of N₂ fixation, measurements of ¹⁵N₂ assimilation and AR were carried out as described in Wilson et al. (2012a). The nifH gene abundance was quantified using the methodological protocols previously published by Moisander et al. (2010). Full descriptions of all the analytical methods for measuring H₂ and N₂ fixation and also the accompanying hydrographic datasets are in the Supplementary Information.

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MEGAMER facility grant by the Gordon and Betty Moore Foundation.
References


Table 1. Rates of biological $^3$H$_2$ oxidation conducted on whole seawater samples collected at 25 m (the error bars represent standard deviation of replicate samples, n=3). The rate measurements are compared with the $^{15}$N$_2$ assimilation and C$_2$H$_4$ production values in whole seawater (Fig. 1) to calculate the percentage of N$_2$ fixation accounted for by biological oxidation.

<table>
<thead>
<tr>
<th>Station sampled</th>
<th>Water-column $^3$H$_2$ oxidation (pmol H$_2$L$^{-1}$h$^{-1}$)</th>
<th>% of AR assay accounted for by $^3$H$_2$ oxidation</th>
<th>% of $^{15}$N$_2$ assimilation accounted for by $^3$H$_2$ oxidation</th>
<th>Turnover time of dissolved H$_2$ pool (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stn 3 (Day)</td>
<td>15 ± 1</td>
<td>0.8</td>
<td>18.8</td>
<td>40</td>
</tr>
<tr>
<td>Stn 3 (Night)</td>
<td>25 ± 4</td>
<td>0.9</td>
<td>11.4</td>
<td>23</td>
</tr>
<tr>
<td>Stn 13 (Day)</td>
<td>42 ± 6</td>
<td>1.3</td>
<td>16.2</td>
<td>22</td>
</tr>
<tr>
<td>Stn 13 (Night)</td>
<td>25 ± 2</td>
<td>6.6</td>
<td>62.5</td>
<td>36</td>
</tr>
</tbody>
</table>

Table 2. Estimation of H$_2$ production in the open ocean water-column at a depth of 25 m. The minimum and maximum values are based on 1 and 25 % of C$_2$H$_4$ production.

<table>
<thead>
<tr>
<th>Date sampled</th>
<th>H$_2$ concentration (nmol H$_2$L$^{-1}$m$^{-2}$h$^{-1}$)</th>
<th>AR assay (nmol C$_2$H$_4$L$^{-1}$h$^{-1}$)</th>
<th>Estimated H$_2$ prod. (nmol H$_2$L$^{-1}$m$^{-2}$h$^{-1}$)</th>
<th>Estimated time to replenish H$_2$ stock (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stn 3 (Day)</td>
<td>0.6</td>
<td>1.77</td>
<td>0.018</td>
<td>0.44</td>
</tr>
<tr>
<td>Stn 3 (Night)</td>
<td>0.6</td>
<td>2.87</td>
<td>0.029</td>
<td>0.72</td>
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<tr>
<td>Stn 13 (Day)</td>
<td>0.93</td>
<td>3.34</td>
<td>0.033</td>
<td>0.84</td>
</tr>
<tr>
<td>Stn 13 (Night)</td>
<td>0.93</td>
<td>0.38</td>
<td>0.004</td>
<td>0.10</td>
</tr>
</tbody>
</table>

Table 3. Estimates of sea-air gas flux, downwards diffusion, and biological consumption in comparison with depth-integrated (0-45 m) dissolved H$_2$ concentrations.

<table>
<thead>
<tr>
<th>Date sampled</th>
<th>Depth-integrated (0-45 m) H$_2$ concentrations (µmol m$^{-2}$)</th>
<th>Water-column Sea-air H$_2$ flux (µmol H$_2$L$^{-1}$m$^{-2}$h$^{-1}$)</th>
<th>Downward diffusion H$_2$ flux (µmol H$_2$m$^{-2}$h$^{-1}$)</th>
<th>Biological consumption H$_2$ (µmol H$_2$m$^{-2}$h$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stn 3 (Day)</td>
<td>30.6</td>
<td>0.03 - 0.06</td>
<td>0.42</td>
<td>0.03 - 5.17</td>
</tr>
<tr>
<td>Stn 3 (Night)</td>
<td>30.6</td>
<td>0.04 - 0.08</td>
<td>0.42</td>
<td>0.31 - 3.87</td>
</tr>
<tr>
<td>Stn 13 (Day)</td>
<td>41.0</td>
<td>0.11 - 0.37</td>
<td>0.68</td>
<td>0.47 - 5.80</td>
</tr>
<tr>
<td>Stn 13 (Night)</td>
<td>41.0</td>
<td>0.08 - 0.33</td>
<td>0.68</td>
<td>1.69 - 16.05</td>
</tr>
</tbody>
</table>
Figure 1. Dissolved H$_2$ concentrations (nmol l$^{-1}$) between depths of 5 to 125 m in the North Pacific Ocean. For each sampling occasion, seawater samples were collected at 1300 hrs. The theoretical value of dissolved H$_2$ concentrations in seawater at atmospheric equilibrium (with an atmospheric concentration of 0.5 ppmv) is represented by the dashed line. Error bars where shown represent standard deviation (n=3).
Figure 2. N\textsubscript{2} fixation rates as measured by (A-C) \textsuperscript{15}N\textsubscript{2} tracer assimilation and (D-F) the AR assay for seawater samples collected at 25 m and incubated onboard the ship during either the day or night period. Post-incubation size-fractionation was conducted for replicate \textsuperscript{15}N\textsubscript{2} tracer additions and not for the AR assay. The error bars in A-F represent standard error (n=3). The \textit{nifH} gene abundances collected from the same depth on the same date are shown for UCYN-A, Group B (\textit{Crocosphaera} spp.), (Tricho) \textit{Trichodesmium} and (het) heterocystous cyanobacteria (G-I).
Supplementary Information

Water column structure and biogeochemical properties

Shipboard sampling was conducted in the North Pacific Subtropical Gyre along a cruise track which transited the edge of two anticyclonic mesoscale eddies (Fig S1). A total of 11 sampling stations were occupied during the cruise, spanning a total distance of approximately 170 km. To characterize the upper water column, vertical profiles were conducted using a Conductivity-Temperature-Depth (CTD) system coupled to a rosette consisting of 24 x 12 liter Niskin-like ‘Bullister’ bottles. Oxygen (O\textsubscript{2}) and fluorescence sensors were calibrated against discrete measurements of dissolved O\textsubscript{2} (Carritt and Carpenter, 1966) and chlorophyll \textit{a} (chl \textit{a}) extracted and analyzed by fluorometry (Turner AU-10). Seawater for determination of nutrient concentrations (NO\textsubscript{2}\textsuperscript{-} + NO\textsubscript{3}\textsuperscript{-}, SRP, and Si) was subsampled into acid washed 125 ml polyethylene bottles, capped, and then stored frozen. Sample analysis was performed on land as documented in the online manual for “HOT Laboratory Protocols” (http://hahana.soest.hawaii.edu).

An overview of the water-column biogeochemistry is provided by comparing vertical profiles of nutrients and chl \textit{a} from Stn 3 (24° 43.4’ N, 157° 33.2’ W) during the first part of the transect and from Stn 13 (24° 48’ N, 158° 15.2’ W) during the latter part of the transect (Fig. S1). At Stn 3, the maximum chl \textit{a} concentrations were observed at a depth of 115 m, compared to a depth of 105 m at Stn 13 (Fig. 2). The nutrient profiles revealed a significant difference between concentrations of silicate (Si) (one-tailed t-test, \(P=0.04\)) and soluble reactive phosphorus (SRP) (one-tailed t-test, \(P=0.03\)) in the surface mixed layer (0–45m) between Stn 3 and 13. Furthermore, the vertical profile of SRP concentrations at Stn 3 revealed a distinct subsurface minimum with concentrations
decreasing from 0.09 µM at 25 m to 0.02 µM at 100 m. Beneath 100 m, the
concentrations of nitrate + nitrite (NO$_3^-$ + NO$_2^-$), Si, and SRP increased more rapidly with
depth at Stn 13 where concentrations were 16, 42, and 210% higher than Stn 3 by 175 m,
respectively (Fig. S2).

$H_2$ measurements

Discrete seawater samples for measuring dissolved $H_2$ concentrations were collected into
acid-washed, glass-stoppered 300 ml Wheaton bottles. Samples were analyzed
immediately after collection with a total sample processing time of <2 h. To quantify $H_2$
concentrations, seawater was sub-sampled from the Wheaton bottles into a 50 ml glass
syringe (Perfektum) via 1/8” polyetheretherketone (PEEK) tubing. The syringe was
flushed twice with sample water, ensuring the last flush was free of air bubbles. A
custom-built syringe actuator ensured that a consistent volume of seawater (35 ml) was
always introduced into the syringe. Subsequently 5 ml of $H_2$-free (<10 parts per trillion)
air (Airgas) was introduced into the syringe, $H_2$ was extracted from the seawater using
headspace equilibration, and the headspace was subsequently injected into the gas
analyzer (described below). To prevent accidental addition of seawater following after
the headspace injection, the sampling inlet for the analyzer was fitted with a hydrophobic
syringe filter (13 mm PTFE membrane, 0.2 µm pore size).

We note that the samples are not preserved and therefore $H_2$ concentrations could
potentially change between the time of collection and analysis. The likelihood of this
occurring in oligotrophic seawater samples within < 2 h of sample collection is
considered minimal. The analysis of replicate samples in random order on previous
occasions did not result in any significant increase in the standard deviation of replicate
typically 3) seawater samples. Also whilst this study shows that production of H₂ via N₂
fixation can replenish the dissolved H₂ pool in 1-38 h, we consider the upper estimate of
1 h to be high and the median value of 18 h to be more reasonable which exceeds the 1-2
h required for processing all samples.

H₂ was quantified with a reduced gas analyzer that couples a mercuric oxide (HgO)
bed to a reducing compound photometer (Peak Laboratories, USA). The stoichiometric
reduction of HgO by H₂ gas releases mercury vapor which is quantified using an
ultraviolet absorption photometer located immediately downstream of the HgO bed. For
safety purposes, the gas flow exiting the detector passes through an activated charcoal
mercury vapor scrubber before venting to the atmosphere. Prior to the detector, the
carrier gas (Ultra High Purity air) passes through two analytical columns maintained at
104 °C. The first column is packed with Unibeads 1S (60/80 mesh, 0.32 cm diameter and
41.9 cm length), and the second column with Molecular Sieve 13X (60/80 mesh, 0.32 cm
diameter and 206 cm length). The analytical precision based on the comparison of 4
samples at atmospheric equilibrium (0.3 nmol l⁻¹) was ± 2%. The analyzer was calibrated
using a 1 ppmv H₂ standard (Scott Marrin) that was diluted up to 100-fold using zero-H₂
air. The concentration of dissolved H₂ in equilibrated seawater was calculated according
to the Bunsen solubility coefficients provided by Wiesenbug and Guinasso (1979).

On two separate occasions, the rate of H₂ consumption was quantified by measuring
the production of ³H₂O from tracer additions (0.024–0.046 nM) of ³H₂. This method has
previously been used to measure ³H₂ uptake in laboratory cultures of diazotrophs (Chan
et al., 1980) and environmental microbial assemblages (Paerl, 1983). The seawater
sample was collected in a 40 ml borosilicate glass vial with 2–3 times overflow and sealed with no headspace using Teflon-faced butyl rubber stoppers. Tritium gas (specific activity: 2 TBq/mmol; ViTrax, California) was injected into the vial in tracer quantities (10 to 25 pM) and shaken before quickly venting non-dissolved $^3$H$_2$ in a fume hood. Seawater samples amended with $^3$H$_2$ were incubated for 4 h in the deckboard incubators at repeated intervals during the day and night periods of a diel cycle. Samples were analyzed in triplicate with control samples for abiotic conversion of $^3$H$_2$ consisting of 0.2 µm filtered seawater. No activity was observed in the control samples during the experiments. At the end of the incubation, a 1 ml sub-sample was removed using a syringe and injected into a scintillation vial containing scintillation cocktail (Ultima Gold LLT, Perkin Elmer) and counted immediately in a liquid scintillation analyzer (Tri-Carb 2910 TR, Perkin Elmer) to determine the total activity added to the sample. To quantify the amount of transformed $^3$H$_2$, a separate 2 ml subsample was added to a scintillation vial and purged with N$_2$ (100 ml min$^{-1}$ for 3 min) in a fume hood to remove any remaining $^3$H$_2$. A 1 ml aliquot of the sparged samples was subsequently pipetted into a second scintillation vial containing liquid scintillation cocktail and counted. To account for isotopic discrimination effects when calculating the rate of H$_2$ oxidation, we used the fractionation factor reported in Soffiento et al. (2006). It should be noted that as acknowledged by Soffiento et al. (2006), fractionation effects may vary between the different hydrogenase enzymes e.g. iron(Fe)-only hydrogenase compared to the nickel-iron (NiFe) hydrogenases contained by cyanobacteria (Tamagnini et al., 2007). This should be resolved by analyzing the fractionation factor in phylogenetically distinct
hydrogenase-containing microorganisms before assessing the consequences of measuring $^3$H$_2$ oxidation in mixed microbial assemblages.

**N$_2$ fixation rate measurements**

Rates of N$_2$ fixation were measured using both the $^{15}$N$_2$ tracer technique and the acetylene reduction (AR) assay at three sampling stations: Stn 3, 7, and 13, which were occupied on the 9, 13, and 19 September, respectively. The AR assay was conducted using a reduced gas analyzer, similar to the instrument described in ‘$H_2$ measurements’, for the quantification of C$_2$H$_4$ production (Wilson *et al.*, 2012). The increased sensitivity (5 pmol l$^{-1}$) provided by the reducing compound photometer compared to standard C$_2$H$_4$ quantification using gas chromatography-flame ionization detector (GC-FID) permits the AR assay to be conducted on seawater samples with no preconcentration of the biomass. Control treatments consisted of 0.2 µm filtered surface seawater, analyzed in triplicate alongside the regular seawater samples. The blank to signal ratio, indicative of the biological production relative to the background presence of C$_2$H$_4$ ranged from 75–82%.

Both samples and controls were incubated using deckboard incubators with typical incubation periods of 3–4 h.

Alongside the AR assay the rate of $^{15}$N$_2$ assimilation into particulate biomass was also measured in seawater samples. The $^{15}$N$_2$ tracer was added to seawater samples as $^{15}$N$_2$ enriched seawater’, prepared onboard the ship by filtering seawater collected from 25 m through a 0.2 µm filter, followed by vacuum degasification (250 mbar for 40 min). The $^{15}$N$_2$ gas (98% purity; Isotech Laboratories, Inc.) was dissolved in the sterile, degassed seawater and 50 ml of $^{15}$N$_2$ enriched seawater was added to the seawater samples in 4.3
liter polycarbonate bottles to give a final $^{15}$N$_2$ enrichment of 1.5 atom%. Samples were incubated in the presence of $^{15}$N$_2$ tracer for either 11 h or 13 h corresponding to the day/night-time, respectively. Seawater samples designated for night-time analysis were collected at the same time as the day-time samples and incubated without tracer additions until spiked with $^{15}$N$_2$ enriched seawater at 2000 hrs. Post-incubation, the seawater samples were filtered onto combusted 25 mm glass fiber filters as both unfiltered (whole) seawater and the <10 µm size fraction (representing UCYN-A and Group B). The samples were then stored at -20°C prior to analysis on land to quantify the $^{15}$N$_2$ enrichment of particulate material using an elemental analyzer-isotope ratio mass spectrometer, as described in Montoya et al. (1996).

Molecular analysis of nifH

Discrete seawater samples (2–4 liters) were collected at 1300 hrs using the CTD-rosette from a depth of 25 m, filtered using a peristaltic pump through a 0.22 µm Sterivex filter (Millipore, Billerica, MA, USA) and stored in liquid N$_2$. A full description of methodological protocols including DNA extraction and quantitative PCR analyses has been previously published by Moisander et al. (2010).
References


Figure S1. 14 day composite of satellite derived SSHA 100 km north of the Hawaiian Islands in the Pacific Ocean between 7-21 September 2011 (data from Moderate Resolution Imaging Spectroradiometer). A summary of the cruise transect is indicated by the solid black line and the labeled white circles represent the sampling stations discussed in the text. Station ALOHA, the long term sampling station for the Hawaii Ocean Time-series (HOT) program, located at 22° 45’ N, 158° W is also highlighted.
Figure S2. Representative water column profiles for the two sections of the cruise track, (A-B) Stn 3 and (C-D) Stn 13.