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Summary: The goal of this exercise was to investigate the ease and effectiveness of techniques used to produce $^{15}N_2$ enriched seawater for measurement of rates of nitrogen fixation. Seawater was either used at ambient gas concentration (RTP, herein referred to as ambient seawater) or was degassed using a combination of vacuum, heat and/or helium sparging. $^{15}N_2$ was added to degassed water in serum bottles, exetainers and a gas bag and was added to ambient seawater in exetainers. The atom percent enrichment of the resulting seawater was measured immediately (data presented below) and over a period of months to test storage (data to follow) using a membrane inlet mass spectrometer at Plymouth Marine Laboratory (UK). In summary, the highest percent atom enrichment (~ 43 %) was produced by adding 1ml of $^{15}N_2$ gas to 10 ml of ambient seawater in an exetainer (no overpressure) and vortexing for 5 min.

Degassing seawater

The aim was to reduce the oxygen/dissolved gas content of seawater to less than 20% saturation. We used filtered (0.2 μm) aged low nutrient seawater, a 2 L or 5 L Erlenmeyer flask with side arm, stir plate and magnet, heat plate, vacuum pump and helium line and frit. From each treatment, samples were transferred using silicon tubing via gravity into oxygen bottles (analyzed by Winkler titration), 125 ml serum bottles (x 3, crimped sealed within 1 min) and 10 ml exetainers. The oxygen concentration in seawater samples was determined within 24 hours to monitor the efficiency of degassing.
Degassing was performed under the following conditions:

Table 1. Treatment conditions for degassing seawater and percent oxygen saturation.

Treatment	**Oxygen (% saturation)**
Ambient oxygen content at T_{zero}	100.60 %
Vacuum only (850 mbar) while stirring for at 1-2 hours (5L flask with ~ 1L of seawater)	24.24 ± 0.021 %
Vacuum (800 mbar) and heated to ~ 50°C for 1-2 hours (2L flask with 1L of seawater)	15.65 ± 0.714 %
Sparge with helium for 20 minutes (5 bar) and vacuum (850mbar) for 1 hour (5L flask and ~ 1 L of water)	13.29 ± 1.428 %
Sparge with helium for 20 minutes (5 bar) and vacuum (850mbar) for 1 hour (5L flask and > 2 L of water)	18.51 ± 1.40 %
Sparge with helium for 20 minutes (5 bar)	4.74 ± 3.92 %

Addition of $^{15}N_2$ to seawater

98% $^{15}N_2$ Cambridge was added to degassed and ambient seawater using a gas tight syringe as follows:

(a) 1ml of $^{15}N_2$ gas was injected (using 23G, 1 ¼ inch needles) via a septum (Supelco, PN 27201, teflon faced butyl septa for headspace vial) into the crimp sealed (Superlco, PN 27200) serum bottles (125ml). Assuming a temperature of 22°C, salinity of 36 and volume of 125ml, the theoretical atom % enrichment of $^{15}N_2$ relative to $^{14}N_2$ was ~ 47 % in the serum bottles. Note that the bubble did not dissolve in the serum bottles when shaken vigorously, vortexed or left overnight in the fridge.
(b) Degassed water (He sparged+vacuum) was allowed to gravity flow into an evacuated gas bag via a silicon tube. The weight of the bag was 1.716 kg (including bag with weight 26.6g). We injected 10ml of $^{15}N_2$ into the gas bag and slapped the

bag with a ruler. Within 20 minutes, the large bubble had disappeared. The bag was placed in the fridge overnight. By morning, the gas was still dissolved. The bag was removed from fridge and allowed to come to room temperature to check if the bubble reappeared. It did not. An additional 7 ml of $^{15}N_2$ was added to the bag and the bag was slapped with a ruler although a small bubble remained. The sample was transferred to 60 ml serum bottles and 10 ml exetainers. Assuming a temperature of 22°C, salinity of 36 and volume of seawater 1716 ml and 17ml of $^{15}N_2$, the theoretical atom % enrichment of $^{15}N_2$ relative to $^{14}N_2$ was ~ 52 % in the gas bag.

(c) As recommended by Dr. Gaute Lavik, we filled 10ml exetainers with filtered ambient seawater, capped them and added 1ml of $^{15}N_2$ by (a) using an exit needle to remove 1ml of seawater while adding 1ml of $^{15}N_2$, (b) using an exit needle to add 0.5ml of $^{15}N_2$ but removing the exit needle for the final 0.5ml of gas in order to over pressurize the gas in the container. All exetainers were vortexed for 5 minutes (please note, we did not run a time course for vortexing so it may be possible to vortex for < 5 mins, needs to be investigated). Assuming a temperature of 22°C, salinity of 36 and volume of seawater of 9 or 9.5 ml and 1ml of $^{15}N_2$, the theoretical atom % enrichment of $^{15}N_2$ relative to $^{14}N_2$ was ~ 92 % in the exetainer.

Gas analysis

Masses 28, 29, 30, 32 and 40 were measured in seawater samples using a Hiden HPR20 gas analyser at Plymouth Marine Laboratory. Dr. Vassilis Kitidis at PML performed instrument setup and calculations. Analysis time per sample was 10-15 minutes. Calculations were performed as follows:

(a) m/z 28, 29 and 30 were corrected for drift.
(b) The theoretical N_2 concentration in the seawater 'standard' was calculated.
(c) The blank (pump off) was subtracted
(d) The MIMS output for seawater was calibrated against step (b) above
(e) The concentrations of 28, 29 and 30-N_2 was calculated for all samples
(f) The atom % enrichment and total N_2 was calculated based on step (e) above.

Table 2. Concentration of $^{30}N_2$ and $^{28}N_2$ (μmol/kg), atom percent enrichment and percent of total N_2 relative to seawater at equilibrium in degassed and ambient seawater samples with $^{15}N_2$ added.

Vessel	Treatment	$^{30}N_2$ (μmol/kg)	$^{28}N_2$ (μmol/kg) ± S.D.	^{15}N at-%	% $\sum N_2$ cf to sw at eqm.
Glass bottle	Seawater no treatment (T=22 °C; S=36)	0.005 ± 0.001	398 ± 7	0.32 ± 0.09	99 ± 2
Serum Bottle	Degassed seawater (Vacuum only) + 1 mL $^{15}N_2$ into 125ml serum bottle	149 ± 0	171 ± 13	32 ± 2	80 ± 7
Serum Bottle	Vacuum plus He sparging + 1 mL $^{15}N_2$	177 ± 14	139 ± 9	36 ± 1	78 ± 2
Exetainer	Vacuum only + 1 mL $^{15}N_2$	356 ± 63	149 ± 4	41 ± 1	126 ± 17
Exetainer	Vacuum plus He sparging + 1 mL $^{15}N_2$	335 ± 50	178 ± 28	39 ± 3	127 ± 6
Exetainer	Ambient seawater + 1 mL $^{15}N_2$ (no overpressure) + 5 min vortex	275 ± 6	141 ± 55	40 ± 3	103 ± 15
Exetainer	Ambient seawater + 1 mL $^{15}N_2$ (no overpressure + 5 ml vortex	439 ± 63	139 ± 4	43 ± 1	143 ± 15
Exetainer	Ambient seawater + 1 mL $^{15}N_2$ (overpressure by 0.5 mL) + 5 ml vortex	422 ± 45	143 ± 23	43 ± 2	140 ± 6
Exetainer	Vacuum + He + Gas Bag	323 ± 28	152 ± 5	40 ± 1	118 ± 6

Notes:

(a) The use of exetainers to prepare $^{15}N_2$ enriched water is straightforward and quick, meaning that $^{15}N_2$ enriched seawater could be prepared on the same day or just before collection of samples for rate measurements. However, the atom% enrichment is similar to that prepared by degassing etc and therefore the user would need to prepare and add 50 to 100 ml of $^{15}N_2$ enriched seawater (with 43% enrichment) to achieve an atom% > 0.8-1.3% in the incubation bottle (Figure 1). This is a very low enrichment and could potentially cause overestimation of nitrogen fixation rates (Figure 2). The exetainers technique is also an inefficient use of gas because only a small amount of the $^{15}N_2$ added dissolves into seawater.

Figure 1. Estimated $^{15}N_2$ atom% enrichment of 4.5L seawater with varying volumes of 43% atom enriched seawater.

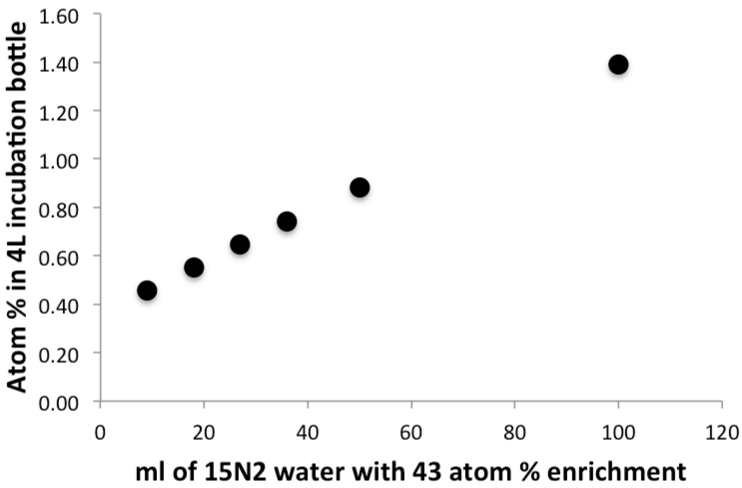

Figure 2. Estimated rates of N2 fixation (nmol h^{-1}) with varying initial $^{15}N_2$ atom % enrichments