Assimilation of Diazotrophic Nitrogen into Pelagic Food Webs

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Abstract

The fate of diazotrophic nitrogen (N\textsubscript{D}) fixed by planktonic cyanobacteria in pelagic food webs remains unresolved, particularly for toxic cyanobacteria that are selectively avoided by most herbivorous zooplankton. Current theory suggests that N\textsubscript{D} fixed during cyanobacterial blooms can enter planktonic food webs contemporaneously with peak bloom biomass via direct grazing of phytoplankton on cyanobacteria or via the uptake of bioavailable N\textsubscript{D} (exuded from viable cyanobacterial cells) by palatable phytoplankton or microbial consortia. Alternatively, N\textsubscript{D} can enter planktonic food webs post-bloom following the remineralization of bloom detritus. Although the relative contribution of these processes to planktonic nutrient cycles is unknown, we hypothesized that assimilation of bioavailable N\textsubscript{D} (e.g., nitrate, ammonium) by palatable phytoplankton and subsequent grazing by zooplankton (either during or after the cyanobacterial bloom) would be the primary pathway by which N\textsubscript{D} was incorporated into the planktonic food web. Instead, in situ stable isotope measurements and grazing experiments clearly documented that the assimilation of N\textsubscript{D} by zooplankton outpaced assimilation by palatable phytoplankton during a bloom of toxic Nodularia spumigena Mertens. We identified two distinct temporal phases in the trophic transfer of N\textsubscript{D} from N. spumigena to the plankton community. The first phase was a highly dynamic transfer of N\textsubscript{D} to zooplankton with rates that covaried with bloom biomass while bypassing other phytoplankton taxa; a trophic transfer that we infer was routed through bloom-associated bacteria. The second phase was a slowly accelerating assimilation of the dissolved-N\textsubscript{D} pool by phytoplankton that was decoupled from contemporaneous variability in N. spumigena concentrations. These findings provide empirical evidence that N\textsubscript{D} can be assimilated and transferred rapidly throughout natural plankton communities and yield insights into the specific processes underlying the propagation of N\textsubscript{D} through pelagic food webs.

Introduction

Eutrophication is recognized as a critical contributing factor to systemic ecosystem shifts in shallow coastal habitats [1,2] and despite a growing understanding of the proximate symptoms of eutrophication (e.g., hypoxia, reduced benthic-pelagic coupling, species loss), the ultimate consequences for biogeochemical processes and ecological function remain relatively unknown [3,4]. Due to their potentially rapid growth rates and direct physiological response to nutrient conditions, noxious blooms of phytoplankton are one of the most common and potentially devastating symptoms of coastal eutrophication in coastal ecosystems [5]. Planktonic cyanobacteria are particularly problematic because they are often toxic and form light-limiting surface scums. Blooms of diazotrophic cyanobacteria are unique in that diazotrophy (the ability to fix dissolved N\textsubscript{2}) circumvents nitrogen limitation [6,7] while generating an input of novel nitrogen to aquatic food webs [6,7,8]. Herbivorous grazing by microzooplankton (c. 20–200 µm body length) and mesozooplankton (c. 200–2000 µm) is the primary process by which phytoplankton are consumed and organic nutrients remineralized; yet, toxic cyanobacteria are often avoided by planktonic herbivores [9,10,11]. Despite this, grazing by zooplankton on cyanobacteria has been hypothesized to explain the rapid assimilation of diazotrophic nitrogen (N\textsubscript{D}) from a toxic cyanobacterium into zooplankton [12]. In the absence of direct grazing, cyanobacterial cells are assumed to accumulate throughout the bloom period until microbial remineralization of detrital bloom material renders the N\textsubscript{D} available to other, more palatable phytoplankton groups [13], and thence to zooplankton. Alternatively, it has been shown that viable cyanobacterial cells are capable of releasing up to 35% of fixed N\textsubscript{D} to surrounding microenvironments as inorganic NH\textsubscript{4}\textsuperscript{+} [14,15] in addition to releasing dissolved organic nitrogen (DON) exudates [16]. This extracellular release of dissolved inorganic nitrogen (DIN) and DON to surrounding waters represents a lateral subsidy of N\textsubscript{D} to
the consortial communities of bacteria that colonize cyanobacterial cells [9,17] and to sympatric phytoplankton. Stimulation of bacterial communities and palatable phytoplankton taxa through extracellular exudation of N_2 represents an indirect trophic pathway by which fixed N_2 in ungrazed cyanobacteria can be assimilated into planktonic food webs.

Our current understanding of plankton trophodynamics and cyanobacterial physiology suggests that N_2 derived from cyanobacteria can enter planktonic food webs contemporaneously with peak bloom biomass via: 1) direct grazing of zooplankton on cyanobacteria [9]; or 2) zooplankton foraging on either palatable phytoplankton or consortial bacteria that have assimilated nitrogenous exudates from viable cyanobacterial cells [12,18]. Alternatively, the transfer of N_2 to planktonic food webs can occur post-bloom following the coupled respiration and remineralization of particulate and dissolved bloom biomass [13]. We tested this conceptual model during a bloom of the cyanobacterium Nodularia spumigena in Lake King, Gippsland Lakes, Australia. The filamentous N. spumigena is a toxic diazotrophic cyanobacterium that produces the hepatotoxin nodularin [9] and there is substantial evidence that herbivorous zooplankton preferentially avoid grazing on this species under natural conditions [9,11,19]. We hypothesised that palatable phytoplankton taxa would be avoided grazing on this species under natural conditions [9,11,19]. We hypothesised that palatable planktonic food taxa would be central to the trophic transfer of N_2, be it via uptake of N_2 exuded by N. spumigena during the bloom or remineralised post bloom. We expected evidence of a bloom-contemporary transfer of N_2 to zooplankton, although we hypothesized that this was likely to be small relative to post-bloom processes. We also hypothesized that direct grazing of zooplankton on N. spumigena would be negligible and we conducted grazing experiments to quantify the role of direct grazing. We used δ¹⁵N stable isotope data to track the fate of bloom-derived N_2 and generate estimates of mass-specific flux rates of N_2 through different functional groups of the plankton community. Patterns in N_2 flux rates and compositional changes in the plankton community were interpreted to infer the role of contemporaneous (i.e., bacterially-mediated lateral trophic transfer) and post-bloom processes (i.e., cellular decomposition) as delivery mechanisms of N_2 to the planktonic food web. Our study verifies the transfer of N_2 from cyanobacteria to multiple elements of the estuarine food web and identifies the specific trophic routing that facilitates the propagation of N_2 to higher trophic levels.

Methods

The Gippsland Lakes are an interconnected chain of three lagoonal estuaries in southeastern Victoria (Australia) that are subject to anthropogenic eutrophication and experience episodic blooms of Nodularia spumigena Mertens [8]. In anticipation of a bloom during the 2011–2012 autumnal summer, plankton sampling was initiated at two open-water sampling sites in Lake King (north site: 37.8757° S, 147.7574° E; south site: 37.9161° S, 147.7792° E). No special permitting was required because sampling did not occur within a protected area and was limited to the collection of ambient water and planktonic organisms. Water and plankton samples were collected from each sampling location on 21 September, 5 October, 28 November 2011, and at weekly–biweekly intervals from 15 December 2011 to 28 March 2012. Hereafter, calendar days will be reported as study days, with study day 1 = 1 September 2011.

Grazing Experiments

Bioassay experiments were conducted using the dilution method [20] to quantify growth rate and grazing pressure on different components of the autotrophic community. For each iteration of the bioassays (N = 9), ambient water samples were collected from the northern sampling site in opaque 3 L containers for transfer back to the laboratory. Here and in later descriptions, all samples were collected from c. 20 to 25 cm subsurface to avoid potential biases associated with the unique composition of matter in surface scums.

Three replicate 100-ml incubations were prepared in 150 ml chambers by diluting ambient estuary water with 0.7 μm GF/F filtered water in ratios of 1:0 (i.e., ambient), 1:4 and 1:19. Incubation chambers were maintained in a water bath at in situ temperatures under natural light conditions: ~100 μmol photons m⁻² s⁻¹ illumination and a 14-hr light:10-hr dark cycle. The composition of the autotrophic community was analysed non-destructively in a Phytopam (Heinz Walz GmbH, Effeltrich, Germany), which quantifies the relative contribution of green (chlorophytes), brown (diatoms and dinoflagellates) and cyan (cyanobacteria) groups to the total measured chlorophyll fluorescence (factory calibration used). Regression of Phytopam output against direct cell counts showed the intensity of the cyan channel to be an excellent predictor of N. spumigena biovolume (n = 16, r² = 0.95, N. spumigena [mm² L⁻¹] = 1.53 x Intensitycyan – 0.36). During the experiments, there was evidence that N. spumigena fluorescence was contributing to the intensity of the green channel; therefore, the green channel was not considered in the data analysis. This was justified on the basis that chlorophytes made a negligible contribution to total phytoplankton biomass throughout this study. Dark-adapted fluorescence measurements were taken at the start of each incubation and every 1–2 days thereafter from each chamber. Incubations were maintained for 7–12 d; however, growth and grazing rate calculations were based on the initial response of each phytoplankton group (i.e., until growth stabilized or reversed) to minimize the influence of bottle-effects on rate measurements.

Daily growth rates of phytoplankton (G, d⁻¹) associated with color channels were calculated for each replicate incubation chamber. A least-squares linear regression was fitted to G (dependent variable) across the three different dilution regimes (independent variable) and the extrapolated intercept interpreted as the maximum predicted daily growth rate (Gmax) in the absence of zooplankton grazing. The difference between Gmax and the mean G observed in the undiluted control samples yields an estimate of grazing rate (Gz, d⁻¹) by zooplankton on each phytoplankton group. Differences in Gz between the cyan and brown groups were tested with a separate variance t-test due to evidence of heteroskedasticity in the data (variance ratio test; F₁,₁₇ = 0.05, p<0.001).

Sampling the Plankton Community

We targeted four unique components of the planktonic food web for stable isotope analysis: phytoplankton (0.7–63 μm), microzooplankton (80–149 μm), mesozooplankton (≥150 μm), and N. spumigena isolates. At each sampling site, an ambient 2 L water sample was collected and immediately preserved with Lugol’s iodine solution to provide an unfiltered sample for later identification and quantification of the plankton community. Identification and direct counts of plankton taxa were conducted by an experienced plankton ecologist using light microscopy and replicate counts with a haemocytometer (small cells only <3 μm) and Sedgewick Rafter chamber (larger taxa). Biovolumes of N. spumigena were estimated using the Sedgewick Rafter chamber on the Lugol’s preserved samples. Additional biovolume estimates were provided by ancillary sampling conducted by the Victorian Environmental Protection Agency (EPA).
The 0.7–63 μm phytoplankton samples were collected by filtering a known volume of 63 μm prefiltered ambient water onto pre-combusted glass fiber filters (Whatman GF/F, 0.7 μm nominal pore dia.). Prefiltering removed most N. spumigena filaments in the sample and subsequent analysis verified that the day-specific δ15N of the 63 μm filtered phytoplankton was significantly enriched relative to ambient water samples during the bloom (one-tailed paired t-test; \( t = 2.08, df = 7, p = 0.04 \)) but not after (one-tailed paired t-test; \( t = 0.00, df = 7, p = 0.47 \)). Larger size classes of plankton were collected by toying an 80 μm plankton net for 3–5 min at ~ 3 km h\(^{-1}\) at each location. High concentrations of N. spumigena filaments in net samples precluded the partitioning of plankton size classes in the field; therefore, whole plankton samples were stored in sample bottles with 0.7 μm filtered ambient water and held on ice for up to several hours until stored at ~20 °C in the laboratory.

In the laboratory, samples were thawed and a pipette used to siphon the positively buoyant N. spumigena filaments from other plankton in the water samples. A 150-μm sieve was used to separate the remaining plankton into the 80–149 μm microzooplankton and ≥150 μm mesozooplankton size classes. After filtering, light microscopy was used to verify that biomass of N. spumigena was a negligible component of the size-fractioned plankton samples. If substantial N. spumigena filaments were apparent (i.e., more than ~ 10% numerical abundance), size-fractioned samples were diluted with deionized water and centrifuged at 2000–4000 rpm in a refrigerated swinging bucket centrifuge for 5–10 min. Non-N. spumigena plankton typically settled out first and formed the apex of the sediment; whereas, N. spumigena remained suspended in solution or formed a recognizable green upper layer on the sediment. The supernatant and any filaments in the sample and subsequent analysis verified that the day-specific δ15N of the 63 μm filtered phytoplankton was significantly enriched relative to ambient water samples during the bloom (one-tailed paired t-test; \( t = 2.08, df = 7, p = 0.04 \)) but not after (one-tailed paired t-test; \( t = 0.00, df = 7, p = 0.47 \)). Larger size classes of plankton were collected by toying an 80 μm plankton net for 3–5 min at ~ 3 km h\(^{-1}\) at each location. High concentrations of N. spumigena filaments in net samples precluded the partitioning of plankton size classes in the field; therefore, whole plankton samples were stored in sample bottles with 0.7 μm filtered ambient water and held on ice for up to several hours until stored at ~20 °C in the laboratory.

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The size classes in this study generally correspond to taxonomically and functionally different plankton components [12,21] and visual examination of a subset of our size-fractionated samples supported our broad classifications. Biomass in the phytoplankton samples was primarily autotrophic phytoplankton (e.g., diatoms: Aulacoseira sp., Pseudo-nitzschia sp., Skeletonema costatum), with scattered mixotrophs (e.g., dinoflagellates: Ceratium furca, Prorocentrum minimum) and heterotrophs (e.g., ciliates: Facella sp.) also present at low levels. Microzooplankton biomass was primarily heterotrophic microzooplankton (e.g., copepod nauplii, ciliates) with mixotrophic dinoflagellates and Coscinodiscus sp. contributing a small fraction to the total organic biomass. Biomass in the mesozooplankton was almost exclusively heterotrophic zooplankton (e.g., copepods: Ocnus media, Salinus conflictus; bivalve veligers).

Samples were held in an oven at 60°C until completely dried (min 48 hrs), after which they were ground to a fine homogenous powder and packed into sterile tin capsules for N-isotope analysis. Isotope samples were analysed on a Hydra 20–22 isotope ratio mass-spectrometer and coupled ANCA-GSL2 elemental analyser (Sercon Ltd., UK) at Monash University. Isotope values are reported in the δ notation relative to ambient air with precision ±0.1%.

Least-squares linear regression was used to assess the magnitude of observed declines in δ15N of plankton groups at each site from initial conditions (pre-bloom) to the termination of the bloom (22 September 2011–7 February 2012).

### Flux Rate Calculations

Daily uptake rates of diazotrophic N (ND) into the non-N. spumigena plankton groups were calculated using isotope mass balances. Mass-specific rate calculations were carried out using the mass-balance approach described by Montoya et al. [22], modified to include a trophic fractionation term [23,24]. Trophic fractionation of δ15N was included in estimates of ND flux into the micro- and mesozooplankton groups, but not the phytoplankton group. The proportion of ND present in the biomass of plankton group i at day \( t \) was estimated by calculating the proportion of ND in each plankton sample, \( p \), using a simple two-endmember mixing model:

\[
p_{i,t} = \frac{\delta N_{i,t} - \delta N_{i,0} - A}{\delta N_{nod} - \delta N_{i,0}}
\]

where \( \delta N_{i,t} \) is the nitrogen isotope value of plankton group i at time \( t \), \( \delta N_{i,0} \) is the nitrogen isotope value of the plankton group prior to the N. spumigena bloom, \( \delta N_{nod} \) is the average nitrogen isotope value of N. spumigena, and \( A \) is the trophic fractionation value assigned to that particular plankton group.

The specific rate of nitrogen uptake, \( V(d^{-1}) \), was calculated as:

\[
V_{i,t} = \left( \frac{1}{\rho_{i,t}} \right) \times p_{i,t}
\]

where \( \rho_{i,t} \) is the proportion of the total nitrogen derived from ND in plankton group i at day = \( t \). Mass-specific uptake of ND, \( \rho_{i,t} \) (nmol N mg\(^{-1}\) d\(^{-1}\)), was then calculated as:

\[
\rho_{i,t} = V_{i,t} \times TN_{i,t}
\]

where TN\(_{i,t}\) is the concentration of total N in plankton group i at day = \( t \) given as nmol N per mg dry weight.

We used a nominal one-trophic level enrichment of \( A = 3.4 \% [24,25] \) for the zooplankton size classes relative to their primary putative N-source (phytoplankton). During initial model construction, sensitivity analysis showed that \( \rho \) estimates increased by 14.9±8.2 (SD) nmol N mg\(^{-1}\) d\(^{-1}\) (microzooplankton) and 27.8±13.0 nmol N mg\(^{-1}\) d\(^{-1}\) (mesozooplankton) for each 1% increase in the value of the \( A \) parameter. These patterns were consistent: estimates of ND flux increased (decreased) for a plankton group if the group was assigned to a higher (lower) estimated trophic position or if the value of the fractionation term was increased (decreased). Although the absolute value of our flux estimates are contingent on the value of \( A \), the relative changes and the overall patterns among plankton groups were unaffected by the presence or absence of the fractionation term. In the absence of empirical trophic position estimates for zooplankton in this study and based on the flexible feeding ecologies of these taxa, we opted to include \( A \) while using a conservative estimate of 1 trophic exchange between zooplankton groups and their putative food source.

Fractionation of δ15N\(_{D}\) can also occur during assimilation of dissolved ND by phytoplankton [26,27] or nitratification of diazotrophic NH\(_4\)\(^{+}\) in the water column [28,29]. Isotopic fractionation during these processes was not considered here due to persistently low DIN concentrations in the environment and evidence of N-limitation among phytoplankton. Total DIN concentrations in the upper water column were consistently low throughout the bloom (north = 0.89±0.53 [SD] μM; south = 1.19±0.95 μM) suggesting N-limiting conditions among
non-diazotrophic phytoplankton. Indeed, ancillary nutrient additions conducted in tandem with grazing experiments indicated marked N-limitation among diatoms and dinoflagellates throughout the study period (See Appendix S1). In addition, phytoplankton will assimilate both NH$_4^+$ and NO$_3^-$, particularly when N-limited; thus, the mass-balance of the combined uptake of total DIN by phytoplankton would not reflect the kinetic discrimination of $^{15}$N during nitrification. Taken together, these conditions suggest that declines in $^{15}$N values across plankton groups were primarily driven by assimilation of N$_2$ into their tissues rather than a reduction in the concentration of $^{15}$N in the DIN pool.

The focus of this study is on the flux of N$_2$ through planktonic food webs; yet ultimately, the planktonic web is only one of several interacting components of the larger aquatic food web. We investigated the transfer of bloom-associated N$_2$ to the demersal food web by analyzing the white muscle and liver tissue $^{15}$N composition of a commercially caught finfish, black bream Acanthopagrus butcheri, collected over the course of the study. The $^{15}$N composition of fish muscle and liver tissue equilibrate to the isotopic composition of diet (with fractionation) at different rates [30]; therefore, differential shifts in the $^{15}$N value of these tissues during a time-series can provide an indicator of the timing and relative magnitude of N$_2$ flux from the pelagic food web to the demersal food web. All fish were collected with gill nets from areas affected by the *N. spumigena* bloom and immediately frozen. White muscle from the dorsal musculature and liver tissue were dried at 60°C, then pulverized and analysed for $^{15}$N on the isotope ratio mass-spectrometer as previously described.

**Results**

There was a large bloom of *N. spumigena* throughout Lake King during the summer months of November 2011 to February 2012. *N. spumigena* was present in low concentrations (c. 0.5 mm$^3$ L$^{-1}$) at both sites in Lake King by late September (study day = 21) but substantial biovolumes did not develop until mid-November (study day = 89; Fig. 1). The bloom had two distinct peaks in biomass at both sites, with maximum initial and secondary biovolumes of 9.9 mm$^3$ L$^{-1}$ and 3.8 mm$^3$ L$^{-1}$ at the northern site and 7.0 mm$^3$ L$^{-1}$ and 12.2 mm$^3$ L$^{-1}$ at the southern site. By early February (study day = 155), the secondary bloom of *N. spumigena* had collapsed at both sites and biovolumes declined to concentrations of ~0.2 mm$^3$ L$^{-1}$; by May *N. spumigena* was no longer detectable in the water column.

Prior to the *N. spumigena* bloom, the autotrophic plankton community was dominated by the cyanobacterium *Eucaiposi* sp., the diatom *Coscinodiscus* sp. and a chlorophyte *Kirchnerella* sp. Copepods, primarily adult *Sulcanus conflictus* (Calanoida) and a mixed assemblage of unidentified adult-stage calanoids and nauplii, were the most abundant zooplankton taxa during the same pre-bloom period. There was a shift in the plankton community coincident with the *N. spumigena* bloom that was marked by declines in most of the dominant pre-bloom taxa and the appearance of previously unobserved taxa. The autotrophic plankton community of the mid-summer peak bloom period was numerically dominated by *N. spumigena* with the remainder composed of a suite of diatoms (e.g., *Aulacoseira* sp., *Coscinodiscus* sp., *Melosira* sp., *Pseudo-nitzschia* sp., *Skeletonema costatum*), dinoflagellates (e.g., *Ceratium furca*, *Dinophysis acuminata*) and a small number (i.e., 3–6% of recorded spp.) of unidentified chlorophytes. Non-*N. spumigena* phytoplankton typically contributed an average of <5% to total autotrophic cell counts during the bloom. The cyclopid copepod *Oncella media* replaced *S. costatus* as the most abundant copepod species with copepod nauplii, bivalve veligers, and ciliates (e.g., *Facella* sp.) also present. Following the final collapse of the *N. spumigena* bloom, the diatoms *Pseudo-nitzschia* cf. *pungens* and *S. costatum*, dinoflagellates *Prorocentrum minimum*, *Prorocentrum lima*, and *C. furca*, and unidentified chlorophytes dominated the phytoplankton. Abundant post-bloom zooplankton included the ciliates *Facella* sp. and *Mesodinium rubrum*, several cyclopid copepods including *O. media*, and a mixed assemblage of copepod nauplii.

Results from the grazing experiments indicated significant differences in the timing and magnitude of grazing rates, $r_G$ (d$^{-1}$), between *N. spumigena* and a combined diatom and dinoflagellate group. For *N. spumigena*, growth rate peaked at $G = 0.34 \pm 0.12$ (SD) d$^{-1}$ on study day = 106, coincident with the first peak in bloom biovolume. Among the diatoms and dinoflagellates, $G$ peaked later in the time series at 0.46 ± 0.15 on study day = 161 following the collapse of the *N. spumigena* bloom. Estimates of specific grazing rates (Fig. 2) indicated that grazers avoided *N. spumigena* ($r_G = -0.25 \pm 0.23$) and preferentially selected (separate variance $t$-test; $t = -4.20, df = 26, p < 0.001$) the diatoms and dinoflagellates ($r_G = 0.62 \pm 0.97$). These taxon-specific patterns in grazing rate were consistent over the course of the bloom cycle (Fig. 2). The negative $r_G$ observed for *N. spumigena* was due to increased growth rates occurring at higher grazer concentrations, suggesting that increased grazing pressure on sympatric diatoms and
Figure 2. Plankton grazing and growth experiments. Zooplankton grazing rates ($G_r$ day$^{-1}$; upper left panel) and phytoplankton specific growth rates under different dilution conditions ($G_p$1-0.1 [filtered/ambient dilution], $G_p$1-19:1 [filtered/ambient]; upper right panel) calculated from mesocosm experiments with subsurface water from Lake King, Australia. Abscissae of upper plots coded by fluorescence color channels: cyan (cyanobacteria), brown (diatoms/dinoflagellates). Box plot components: measure of central tendency = median, outer edges of box = 25th and 75th interquartile range, whiskers = quartiles ± (1.5 x interquartile range), circles = outliers. Time-series of average $G_r$ (error bars show ±1 SD) for the cyan and brown fluorescence channels (lower panel). The x-axis shows the number of days elapsed since 1 September 2011 (study day = 1). doi:10.1371/journal.pone.0067588.g002

Figure 3. Time series of plankton diazotrophic nitrogen flux rates. Time series of size-fractioned plankton $N_\text{D}$ flux rates (mmol $N_\text{D}$ mg dry wt$^{-1}$ day$^{-1}$) collected at a north (upper panel; $N_{\text{isotope}} = 52$) and south (lower panel; $N_{\text{isotope}} = 48$) sampling site in Lake King, Australia. The x-axis shows the number of days elapsed since 1 September 2011 (study day = 1). doi:10.1371/journal.pone.0067588.g003
13.9±1.1 % by study day 202. Conversely, δ15N values of liver tissue underwent rapid depletion approximately 2–4 weeks post-bloom, declining from an average value of 11.3±0.9 ‰ during the bloom period (study days: 106–159) to 9.9±0.1 ‰ after the post-bloom depletion (study days: 181–202). Post-hoc tests of differences between bloom and post-bloom periods for each tissue showed no difference for muscle tissue (n = 31, t = 0.49, p = 0.63); whereas, the decline in liver δ15N was significant between bloom periods (n = 31, t = 4.94, p<0.001; see Fig. 4).

Discussion

Using a naturally occurring stable isotope tracer, we documented the in situ propagation of N2 through multiple components of an estuarine food web. These findings unequivocally demonstrate the propagation of N2 from N. spumigena into a pelagic food web and a demersal fish species under natural conditions. These results shed light on the specific trophic mechanisms underlying the transfer of N2 to consumer zooplankton as well as the temporal dynamics of these mechanisms relative to the trajectory of the cyanobacterial bloom.

Within the phytoplankton, the net result of disparate dissolved nitrogen-generating pathways dominating at different bloom phases appears to be a continuous increase in the pool of bioavailable N2 that peaks several weeks post-bloom. During N. spumigena blooms, proliferation of filamentous cells leads to the formation of tangled colonies that remain buoyant, even after senescence, due to intercellular gas-filled vesicles [9]. This buoyancy effectively nullifies sedimentation by the majority of the bloom biomass and compartmentalizes initial nutrient exchange from N. spumigena within the pelagic food web [32]. Both free-floating filaments and aggregate colonies of N. spumigena support diverse consortia of bacteria [33] that are capable of remineralizing DON exudates from growing and senescing cyanobacterial cells [18,34]. During exponential and stationary phases of N. spumigena growth, the availability of N2 to other phytoplankton is limited to assimilation of DIN [14,16] and DON [35,36,37] exudates from viable N. spumigena, or assimilation of remineralized organic N2 [16,38]. Direct measurement of total DIN indicated that concentrations were low at both sampling sites (See Methods: Flux rate calculations) and nutrient addition experiments showed N-limitation among diatoms and dinoflagellates (See Appendix S1). With the onset of senescence (c. study day 155), concentrations of DIN increased at both sites at a rate of 0.45 μM day⁻¹ to an average of 11.5±4.4 μM by study day 182. This increase corresponded with peak flux rates of N2 into the phytoplankton, presumably concurrent with maximum respiration and remineralization rates of detrital N. spumigena biomass [38].

Our results show that the integrative effect of these disparate pathways is to dampen the temporal variability in bioavailable N2 and decouple N2 uptake rates by phytoplankton from the short-term stochasticity of N. spumigena bloom dynamics.

Among the heterotrophic zooplankton, potential pathways for the transfer of N2 from N. spumigena are either via direct ingestion of N. spumigena cellular material or the indirect transfer of labile N2 through one or more intermediary functional groups. Results from our grazing experiments verified the absence of measurable grazing of N. spumigena by the natural zooplankton community throughout the bloom period and agree with previous work demonstrating preferential avoidance of N. spumigena by zooplankton grazers in the Gippsland Lakes [11] and other coastal ecosystems [9].

In the absence of direct grazing, we infer that N2 is being routed through bloom-associated bacteria as the primary, initial trophic intermediary between N. spumigena and heterotrophic zooplankton. This inference is consistent with recent experiments using 15N labelling and nanoscale secondary ion mass spectrometry (nanoSIMS) that indicate a rapid transfer of N2 from filamentous cyanobacteria to epiphytic bacteria (M. Kuyper, personal communication). Bacterivory is very common among the protozoan and certain metazoan (e.g., copepod nauplii) taxa that constitute the microzooplankton [21,39,40]. Foraging of microzooplankton upon N. spumigena-associated bacteria has been inferred from experimental mesocosms [38] and field collections [39] and represents the most plausible initial mechanism for the trophic transfer of N2 to the microzooplankton. In turn, mesozooplankton are effective predators of microzooplankton [38,41] and scavengers of detrital material (e.g., Oncorhynchus spp.; [40]) in addition to functioning as grazers of phytoplankton. The tight temporal covariation in p that we observed between the micro- and mesozooplankton suggests a close coupling between these two functional groups. Thus, nutrient transfer from N. spumigena to consortial bacteria followed by heterotrophic transfers from bacteria to microzooplankton to mesozooplankton represents the most likely trophic pathway supporting the flux of N2 into heterotrophic zooplankton taxa.

Although unexpected, the higher concentration of N2 in the zooplankton functional groups versus the autotrophic producers throughout the bloom and immediate post-bloom phases (Fig. 5) makes sense in light of the dynamic foraging capabilities of the zooplankton taxa and compositional shifts in the dominant taxa. Mixotrophy and omnivory are common trophic strategies among micro- and mesozooplankton [40,42] and these flexible feeding behaviours could serve to accelerate the radiation of N2 in these groups relative to primary producers. For example, mixotrophic dinoflagellates would have access to remineralized DINp in the water column as well as to organic N2 bound in the tissue of bacterial prey. Similarly, omnivorous copepod species such as Sagitta sp. would assimilate N2 during opportunistic grazing on diatoms or dinoflagellates, heterotrophic predation of microzooplankton [42,43], or scavenging of biofilm-covered detritus. In particular, detrital feeding on senescent N. spumigena colonies could be an important mechanism by which N2-laden consortial bacteria and microzooplankton are consumed by mesozooplankton scavengers. Changes in the identity of dominant zooplankton

Figure 4. Time series of black bream muscle and liver δ15N isotope composition. Time series of mean δ15N isotope (‰) error bars show ±1 SD values of black bream Acanthopagrus butcheri liver (empty circles) and white muscle tissue (filled circles) collected in Lake King, Australia. Area bounded by vertical dotted lines indicate the intervening period between N. spumigena bloom collapse (study day — 155) and depletion of liver δ15N values. The x-axis shows the number of days elapsed since 1 September 2011 (study day = 1). doi:10.1371/journal.pone.0067588.g004

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taxa at different phases of the bloom could have also contributed to the rapid assimilation of $\text{N}_\text{D}$. During the bloom, $\text{S. conflictus}$ was replaced by the cyclopoid copepod $\text{O. media}$ as the dominant mesozooplankter and several studies have documented the close association between $\text{Oncaea}$ spp. and suspended detrital aggregates (i.e., marine snow; [44,45,46]). This association is apparently trophic – the modified maxillae of $\text{Oncaea}$ spp. [45] can be used to scrape particulate-laden (<2–5 μm) biofilms from aggregate surfaces [46,47]. Such a feeding strategy would permit direct consumption of microbial consortia associated with $\text{N. spumigena}$ aggregates.

Results from the tissue analysis of black bream provide initial evidence that the post-bloom uptake of remineralized $\text{N}_\text{D}$ by non-$\text{N. spumigena}$ phytoplankton represents a larger fraction of the total $\text{N}_\text{D}$ flux to the demersal food web and higher trophic levels. The rapid decline in black bream liver $\delta^{15}$N that occurred 2–4 weeks post-bloom indicates a large pulse of isotopically light nitrogen entered the demersal food web during that time; this corresponds closely with the accelerating flux of remineralized $\text{N}_\text{D}$ into the phytoplankton. Black bream are omnivores, suggesting that opportunistic foraging on available prey could explain the tight temporal coupling between $\text{N}_\text{D}$ flux dynamics and this particular fish species. Together with the plankton data, these results support the hypothesis that $\text{N}_\text{D}$ radiation is dominated by post-bloom processes in estuarine food webs. The observed depletion in the black bream liver $\delta^{15}$N further supports the contention that remineralized bloom detritus is capable of contributing to measurable production among higher trophic levels. Interestingly, the temporal lag between the $\text{N. spumigena}$ bloom cycle and the post-bloom assimilation of most $\text{N}_\text{D}$ by palatable phytoplankton may interrupt the phenology of important predator-prey dynamics in the estuary. For example, the timing, magnitude and composition of spring–summer plankton cycles can be critical to the growth and survival of young-of-the-year fish and invertebrate species [48,49]. This interval is also an important foraging period for adult-stage organisms that require sufficient energetic stores to support the metabolic demands associated with overwintering, migration or gonad development. Thus, despite the potential fertilization effect of an extended $\text{N. spumigena}$ bloom, it is not clear if such a bloom represents a net positive or negative influence on productivity and food web dynamics in this (or another) estuary. Further work is needed to quantify the total flux of $\text{N}_\text{D}$ during different bloom phases as well as the allocation of assimilated $\text{N}_\text{D}$ to the somatic and metabolic demands of higher trophic level consumers within the estuarine food web.

There is a growing consensus that $\text{N}_\text{D}$ constitutes a potentially important subsidy for production among higher trophic levels in coastal and oceanic pelagic food webs [34,50]. In this study, 30–95% of the phytoplankton and 55–95% of the zooplankton nitrogenous biomass was composed of $\text{N}_\text{D}$ during the bloom and the post-bloom weeks (Fig. 5). In conjunction with the black bream data, this information suggests that $\text{N}_\text{D}$ can rival the contribution of alternative sources of N-loading to the nutrient budget and trophic structure of estuaries during certain times of the year. Studies using a myriad of methods [34,41,50,51,52] have traced the transfer of $\text{N. spumigena}$ $\text{N}_\text{D}$ or biomass into different compartments of the planktonic food web; yet, translating these findings into a holistic understanding of the processes underlying the transfer of $\text{N}_\text{D}$ up the food web has been hampered by the artificial conditions imposed by bench-scale experiments and (or) the short-term ‘snapshot’ characteristics of the sampling regimes. Other studies have noted the transfer of $\text{N}_\text{D}$-fixation products from diazotrophic cyanobacteria to consortial bacteria [18], and have thus set the stage for our study by suggesting microbial trophic intermediaries could play an important role in the transfer of $\text{N}_\text{D}$ from cyanobacteria to zooplankton. Our results provide strong support for this consensus and emphasize the trophic linkages between $\text{N. spumigena}$-associated bacterial communities and the eukaryotic elements of the planktonic food web (Fig. 6). These linkages serve to accelerate the transfer of $\text{N}_\text{D}$ from $\text{N. spumigena}$ into the heterotrophic components of the plankton community, leading to a tight coupling of zooplankton assimilation rates with cyanobacterial bloom dynamics. The reliance of non-$\text{N. spumigena}$ phytoplankton on the dissolved pool of $\text{N}_\text{D}$ and the various pathways contributing to that pool over the bloom-cycle serves to

Figure 5. The proportional diazotrophic nitrogen content of zooplankton versus phytoplankton over time. Phase plot of the proportional contributions of diazotrophic nitrogen ($\text{N}_\text{D}$) to the total nitrogen ($\text{N}_\text{T}$) of microzooplankton (empty circles, final date = triangle) and mesozooplankton (filled circles, final date = triangle) versus $\text{N}_\text{D}/\text{N}_\text{T}$ of phytoplankton at the north (upper panel; $N_{\text{microzooplankton}} = 9$, $N_{\text{mesozooplankton}} = 7$) and south (lower panel; $N_{\text{microzooplankton}} = 9$, $N_{\text{mesozooplankton}} = 8$) sampling sites over the course of a $\text{Nodularia spumigena}$ bloom in Lake King, Australia. Data points are daily means connected in temporal sequence by dashed (microzooplankton) or solid (mesozooplankton) lines with study day = 35 and 155 (2 February 2012; date of $\text{N. spumigena}$ bloom collapse) provided for reference. The 1:1 ratio is given by the dotted line. doi:10.1371/journal.pone.0067588.g005
decouple phytoplankton uptake from the *N. spumigena* bloom dynamics.

**Supporting Information**

**Appendix S1 Nutrient addition bioassays.** Details on methods and results from nutrient addition bioassays can be found in Appendix S1.

**Acknowledgments**

The authors thank Vera Eate, Peter Faber, Yafei Zhu, Dave Collins, Chris Garland, Guillaume Martinez and Emilie Laurent for their help in the field and laboratory. Victor Escard and Vera Eate at the Water Studies Centre (Monash University, Clayton, Victoria AUS) analysed samples and provided the stable isotope data. The authors would like to thank Sam Lake, Gilbert Cabana, John Raven and Jeff Shimeta for providing comments on an earlier draft of this manuscript.

**Author Contributions**

Conceived and designed the experiments: RJW PLMC. Performed the experiments: RJW PLMC DPH JB JS TS. Analyzed the data: RJW PLMC. Contributed reagents/materials/analysis tools: PLMC JB JS. Wrote the paper: RJW PLMC DPH JB JS TS. Performed field work: RJW DPH TS. Performed plankton identification: JS.

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