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Limited importance of primary production in the deep chlorophyll layer for macro-zooplankton in an oligotrophic karst lake: A whole-lake ^{15}N experiment

Wayne A. Wurtsbaugh[1,*], María Rosa Miracle[2,†], Antonio Camacho[2], Javier Armengol[2] and Eduardo Vicente[2]

[1] Watershed Sciences Department/Ecology Center, Utah State University, Logan, UT 84322-5210, U.S.A.
[2] Cavanilles Institute for Biodiversity and Evolutionary Biology, University of Valencia, E-46980 Paterna, Spain.

* Corresponding author: wayne.wurtsbaugh@usu.edu

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ABSTRACT

Limited importance of primary production in the deep chlorophyll layer for macro-zooplankton in an oligotrophic karst lake: A whole lake ^{15}N experiment

Deep-chlorophyll layers (DCL) in oligotrophic lakes contribute significantly to primary productivity, but the importance of this production for the rest of the food web and for other strata is unknown. In Laguna El Tejo, a sheltered 1.7-ha Spanish karst lake, chlorophyll levels were < 2 µg/L in the epilimnion but reached 10 µg/L in the metalimnion and upper hypolimnion where cyanobacterial picoplankton dominated. Particulate nitrogen levels were 2-14 times higher in the metalimnion than in the epilimnion and 42 % of the primary productivity occurred in the deeper strata where the DCL was located. To address the trophic importance of the high biomass and production in the deep chlorophyll layer, we injected $^{15}NH_4^+$ and rhodamine into a 0.5-m strata (15-16 m) in the metalimnion of the lake. The $^{15}NH_4^+$ taken up by the nitrogen-limited phytoplankton allowed us to measure the importance of biologically mediated transport whereas the rhodamine traced physical eddy diffusion. After 28 days 55 % of the ^{15}N could be accounted for: 71 % remained in the metalimnetic seston (11-18 m), 10 % was in the hypolimnetic seston (18-20 m), 11 % was found above in the epilimnetic seston, and only 8 % had sedimented into the anoxic layer below 20 m. Only negligible amounts of rhodamine (corrected for degradation) moved beyond the 14-18 m strata in the lake, but bio-diffusivity of ^{15}N was 3 times greater than the physically induced diffusivity of rhodamine. A mixing model indicated that the deep chlorophyll layer contributed only 1-2 % of the diet of epilimnetic macrozooplankton but 14-33 % of the diet of the meta-hypolimnetic zooplankton. The data indicate the overall importance for primary production and the sequestration of nutrients in the DCL, but relatively limited importance for the macrozooplankton in the lake.

Key words: deep chlorophyll maxima, DCM, diffusivity, tracer

RESUMEN

Relevancia limitada de la capa profunda de clorofila para la producción del del macrozooplancton en un lago kárstico oligotrófico: un experimento de adición de ^{15}N en todo el lago

En lagos oligotróficos los máximos profundos de clorofila (Deep-chlorophyll layers, DCL) contribuyen significativamente a la producción primaria, pero se desconoce la importancia relativa que tiene esta producción para el conjunto de la red trófica y para otros estratos lacustres. En la laguna del Tejo, una laguna cárstica española de 1,7 ha de superficie, encajada en una dolina que la protege del viento; los valores de clorofila fueron < 2 µg/L en el epilimnion alcanzando valores de 10 µg/L en el metalimnion y en la parte superior del hipolimnion, donde el picoplancton, formado por picocianobacterias, era dominante. Los valores de nitrógeno particulado fueron entre 2 y 14 veces superiores en el metalimnion que en el epilimnion, dándose el 56 % de la producción primaria en los estratos profundos donde se localizaba el DCL. Para investigar la importancia trófica de la elevada biomasa y producción primaria de este máximo profundo de clorofila, inyectamos $^{15}NH_4^+$ y rodamina en un estrato de 0.5-m en el metalimnion (entre 15-16 m). El $^{15}NH_4^+$ tomado por el fitoplancton (que estaba

limitado por N), nos permitió medir la relevancia del transporte biológico; mientras que la rodamina nos permitió trazar la difusión pasiva por causas físicas. Tras 28 días, el destino del 55 % del ^{15}N fue: el 71 % permaneció en el seston metalimnético (11-18 m), el 10 % fue localizado en el hipolimnético (18-20 m), el 11 % más arriba, en el seston del epilimnion, y un 8 % había sedimentado en la capa anóxica, por debajo de 20 m. Únicamente cantidades insignificantes de rodamina (corregida por su degradación) se desplazaron más allá de los 14-18 m en el lago, pero la biodifusividad del ^{15}N fue 3 veces mayor que la difusividad física de la rodamina. Un modelo mixto indicó que el máximo profundo de clorofila contribuyó tan sólo al 1-2 % de la dieta del zooplancton epilimnético, sin embargo, contribuyó al 14-33 % de la del zooplancton meta-hipolimético. Los datos indican la importancia general que para la producción primaria y la captación de nutrientes tiene el DCL, pero también su relativamente limitada importancia para el macrozooplancton en este lago en su conjunto.

Palabras clave: *máximos profundos de clorofila, zooplancton, difusividad, trazador*

INTRODUCTION

Oligotrophic lakes and oceans usually have zones enriched in phytoplankton, or deep chlorophyll layers (DCL) below the mixed layer (Fee, 1976; Camacho, 2006; Cullen, 2015; Silsbe & Malkin, 2016; Leach *et al.*, 2018). The peak in chlorophyll is frequently referred to as the deep chlorophyll maximum (DCM). DCLs can account for over 50 % of primary production in oligotrophic systems and contain much of the particulate organic matter (seston) in the water column (Silsbe & Malkin, 2016; Giling *et al.*, 2017; Scofield *et al.*, 2020). The high production in the DCL is a consequence of physiological adaptions of the phytoplankton taxa living there (Camacho & Vicente, 1998; Camacho *et al.*, 2001; Camacho, 2006; Cullen, 2015). Much of the research on DCLs has focused on the mechanisms that drive their formation (e.g. Pilati & Wurtsbaugh, 2003; Cullen, 2015; Scofield *et al.*, 2017; Lofton *et al.*, 2020), transport of nutrients between strata (e.g. Pilati & Wurtsbaugh, 2003; Letelier *et al.*, 2004), and their importance as a food source for grazing zooplankton which often migrate between the surface and deep layers (Williamson *et al.*, 1996; Winder *et al.*, 2003). The relative influence of top-down vs. bottom-up control on algal composition and dominance in the DCL varies across phytoplankton groups (Lofton *et al.*, 2020). Additionally, the importance of DCLs for zooplankton feeding in lakes has been debated, with some studies showing them to be very important (Matthews & Mazumder, 2005; Francis *et al.*, 2011; Twiss *et al.*, 2012) whereas others have found a limited role (Wilkinson *et al.*, 2014). Some of this research has focused on diel vertical migration (DVM) of zooplankton that often inhabit the darker, colder water during the day to avoid fish predation, and move into the mixed layer at night, presumably to feed on higher quality foods (Lampert *et al.*, 2003). The underlying assumptions of these migration studies have, however, been questioned (Wurtsbaugh & Neverman, 1988; Williamson *et al.*, 1996; Armengol *et al.*, 2012).

To address some of these questions, we injected a stable isotope of nitrogen ($^{15}NH_4^+$) along with inert rhodamine into the deep chlorophyll layer of a small doline lake in central Spain. The plankton in the lake, Laguna El Tejo, are nitrogen limited (Camacho *et al.*, 2003a), so the tracer was quickly incorporated into the food web. The ensuing isotopic analyses and routine limnological sampling allowed us to address the following questions: (1) How important is the DCL for production processes? (2) What taxa dominate in the DCL? (3) How important is production in the DCL for grazing zooplankton? (4) How fast is biological movement of the ^{15}N in relationship to physical processes that move the rhodamine? (5) What is the half-life of nitrogen in Laguna El Tejo?

MATERIAL AND METHODS

Study site

The experiment was conducted in Laguna El Tejo, located at 39.987° N, 1.878° W, 20 km east of the city of Cuenca, Spain. The circular lake lies at an elevation of 1012 m in a karst depression with an area of only 1.7 ha (Fig. 1A). Steep walls rise 22-50 m above the entire lake so that it is protected from winds and the surface watershed is very

small. At the time of the study, the maximum and mean depths of the lake were 26 and 12 m with a volume of 158 000 m^3. No surface inflows enter the lake, and sublacustrine springs and precipitation to the lake surface provide water and nutrients. A capped spring located on the south wall of the depression approximately 30 m above the lake level was sampled occasionally to provide an idea of the nutrient supply in the groundwater. The lake level fluctuates considerably: a morphometric analysis done in 1987 (personal communication, C. Lentisco) indicated that the lake was 5 m deeper than in 1997 when our experiment was conducted and the lake's depth declined approximately 0.4 m during the 231 days we monitored it. The limnology of lakes in this region has been studied extensively (Miracle & Vicente, 1983; Miracle *et al.*, 1992; Armengol & Miracle, 1999; Miracle *et al.*, 2000; Camacho, 2006).

The carbonate rocks in the watershed provide the lake with a pH varying between 8.5-9.1 in the surface waters, and alkalinities near 5.5 meq/L. Lake whitings from $CaCO_3$ precipitation sometimes occur during summer as in the adjacent and better-studied Laguna La Cruz (Rodrigo *et al.*, 1993; Miracle *et al.*, 2000). Laguna El Tejo is oligo-mesotrophic, with summer epilimnetic chlorophyll *a* concentrations varying from 1.5-2 µg/L and Secchi depths from 6-7 m. Dissolved inorganic nitrogen and phosphorus concentrations are near levels of detection in most cases (P < 0.9 µg/L; N < 0.3 µg/L). Both epilimnetic and metalimnetic phytoplankton change seasonally between nitrogen and phosphorus limitation (Camacho *et al.*, 2003a). Dissolved organic carbon in the epilimnion of the lake usually ranges from 3-4 mg/L. Submerged macrophytes (*Myriophyllum spicatum* and *Chara aspera*) are intermittently present around the steep littoral zone but are not abundant. Introduced minnows (*Luciobarbus guiraonis* and *Achondrostoma arcasii*) are abundant in the lake. The young of both species and adult *A. arcasii* are omnivorous and feed on zooplankton.

^{15}N Tracer Injection and processing

Field collections

We injected $^{15}NH_4Cl$ and rhodamine WT dye into the lake's metalimnion on 17 September 1997 (Fig.1). Prior to the injection 145 g of 10-atom % $^{15}NH_4Cl$ and 652 g of a rhodamine WT impregnated wax cake were mixed together. To inject the tracers, we pumped water up from a depth of 15.5 m with an electric pump and mixed it with the $^{15}NH_4Cl$ and rhodamine. The mixture was then immediately injected at 4-5 L/min through 9 holes in a vertical steel pipe into a 0.5-m thick strata at a depth of 16 m. The tracers

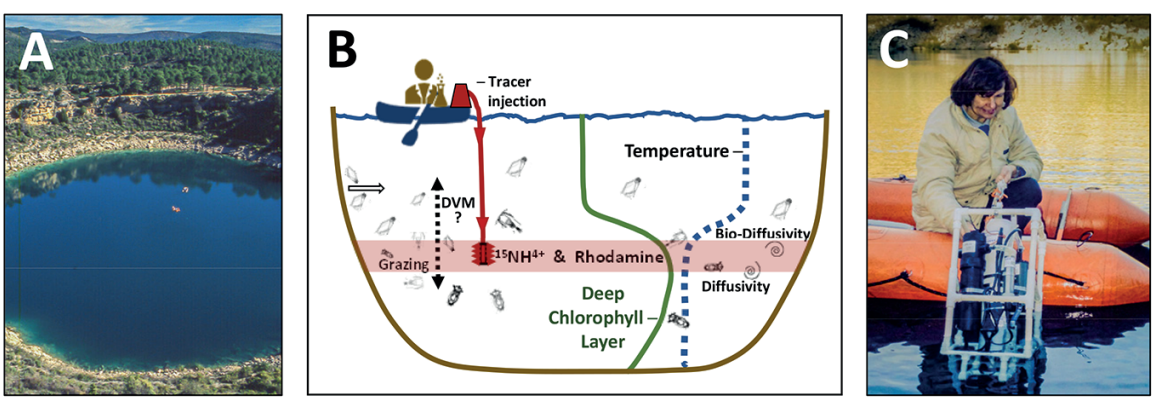

Figure 1. A. Laguna El Tejo with two sampling rafts in upper center. B. Schematic showing tracer injection methods and processes studied. C. Dr. Maria Rosa Miracle sampling with a Seabird salinity-temperature-depth profiler in El Tejo. *A. Laguna del Tejo, con las barcas desde las que se hacia el muestreo. B. Esquema que muestra los métodos de inyección del trazador y el ^{15}N, y los procesos estudiados. C. La Profesora María Rosa Miracle, midiendo la salinidad y temperatura con una sonda multiparamétrica en la Laguna del Tejo.*

were injected for ca. 30 min at each of 9 stations located roughly equal-distant around the lake over depths > 20 m. Some limited tracer contamination of the epilimnion likely occurred when the hose and pipe were pulled to the surface without first flushing them with epilimnetic water.

The main experiment was run for 28 days. Samples were obtained from the injection date, September 17th, to October 15th, though measurements of certain variables where taken up to one year later. Seston samples were collected at 7-9 depths between 12:00-19:00 h with a 2.7 L, 50-cm long Ruttner bottle at four stations placed equidistant around the lake over maximum water depths of ca. 23 m. Lake water samples were stored at 4 °C and processed the next day. We filtered 700-1500 ml of each sample on pre-combusted (550 °C, 12 h) 47-mm Whatman™ GF/F filters, until clogging occurred. The filters were dried for 24 h at 45 °C and frozen. To encapsulate the seston material in tin capsules, a 36-mm diameter punch was used to subsample 81 % of the seston. The encapsulated samples were then frozen at -20 °C for up to 3 months before further processing.

Zooplankton were normally sampled between 12:00-20:00 hours with a 25-L Schindler-Patalas plankton trap fitted with a 100-µm net. Repetitive casts were made until between 25-200 L of water were collected from each depth. Higher volumes were collected in the epilimnion because zooplankton were sparse there during the day. The 100-µm net may have retained a small amount of filamentous phytoplankton in addition to the zooplankton, though this type of phytoplankton is scarce in Laguna El Tejo (Morata *et al.*, 2003; our data). The zooplankton samples were filtered on pre-combusted, 25-mm GF/F filters, placed in Eppendorf capsules, dried 24-48 hours at 45 °C and then encapsulated for isotopic processing.

Sedimenting material was collected in four traps placed at each station. The traps were 12-cm diameter, and 50-cm long plastic cylinders. Prior to deployment, these were filled with deionized water containing 5 g/L reagent grade NaCl in order to create a density gradient and harsh conditions to prevent lake water and non-sedimenting plankton, respectively, from entering the trap during deployment. We then injected 120 ml of 2 % formaldehyde and 1 % of sodium tetraborate into the bottom of each trap to preserve sedimenting material. Formaldehyde has minimal effects on measurements of isotopic enrichment (Bicknell *et al.*, 2011). The traps were then suspended in the anoxic layer with the trap mouth at 21-m for 1-4 weeks. When a trap was brought to the surface, we homogenized the contents, measured the volume, and a 60-100 ml subsample was filtered on pre-combusted 47-mm GF/F filters. The filters were then dried and processed in the same manner as the seston samples.

^{15}N analyses and calculations

Nitrogen mass and isotopic composition were determined with a Europa Scientific ANCA 2020 mass spectrometer. To estimate the flux of ^{15}N into different compartments we calculated the increment in the total amount of ^{15}N above those at the beginning of the experiment, under the assumption of stable conditions over the 28-day period. We did not collect base-line conditions for the δ^{15}N of sedimenting material. Consequently, we used the depth-weighted mean δ^{15}N of seston in the water column prior to the injection ($^{+}\delta$ 4.8) as the background level for sedimenting material. If epilimnetic seston, with lower δ^{15}N levels ($^{+}\delta$1.4 - 1.9), sedimented more than that in the metalimnion, our calculation would have underestimated the amount of material transported to the sediments.

The proportions of epilimnetic (f_1) and metalimnetic seston (f_2) consumed by zooplankton was estimated with a simple mixing model (Phillips, 2012):

$$f_1 = \frac{(\delta^{15}N_{mix} - \delta^{15}N_{tr}) - \delta^{15}N_2}{\delta^{15}N_1 - \delta^{15}N_2}$$

$$f_2 = 1 - f_1$$

Where:

$\delta^{15}N_{mix}$ = Mean incremental isotopic enrichment above background in zooplankton from a given strata on day 28 of the experiment

$\delta^{15}N_{tr}$ = Mean trophic enrichment factor of

zooplankton above seston prior to tracer addition (δ 8.2)

$\delta^{15}N_1$ = Mean incremental enrichment in seston from epilimnion (3 and 8 m)

$\delta^{15}N_2$ = Mean incremental enrichment in seston from metalimnion (14 -18 m)

Rhodamine fluorescence was measured on water samples collected with the Ruttner bottle used for ^{15}N-seston samples that were frozen and subsequently filtered (GF/F) and analyzed with a Hitachi F4500 fluorescence spectrophotometer set with excitation and emission wavelengths set at 540 and 576 nm, respectively, and with 5 nm slit widths. Because rhodamine degrades when exposed to light, we conducted an experiment that allowed us to correct for this degradation in the lake. Rhodamine WT was dissolved in deionized water and placed in Pyrex bottles and then either exposed directly to the sun for 120 hours or held in the dark. In the dark bottles there was no significant degradation during the experiment ($p = 0.19$). Light measurements were measured concurrently and the relationship between accumulated light (PAR, in Einsteins = mol Photon m^{-2} s^{-1}) and rhodamine fluorescence in the bottles was established:

% of initial = 4.554 + -0.00256 ln (Einsteins), $r^2 = 0.91$

A model of rhodamine degradation in the lake was established using a mean vertical extinction coefficient for PAR of 0.201/m, and assuming 14 h of full sunlight each day:

$$\% \text{ of initial rhodamine} = e^{(4.55 - 0.00256 * E)}$$

where E = cumulative Einsteins over a given number of days

At depths of 10, 14 and 16 m this resulted in a correction factors of 0.54, 0.80 and 0.82, respectively, after the 28-day main experiment in the lake. The model, however, did not correct for differential rhodamine degradation with shifting wavelengths at different depths, so it is only approximate.

Eddy diffusivity of the rhodamine (K_z) and "biodiffusivity" (K_b) of the ^{15}N were calculated as follows (Quay *et al.*, 1980):

$$K_{(z \text{ or } b)} = \frac{\sigma_t^2 - \sigma_o^2}{2 (t - t_0)}$$

Where:

t = initial (t_0) and time since injection (σ_t; seconds)

σ_t = mean square distance (m)
= ½ distance between depth at which the rhodamine or ^{15}N was 0.1 x the maximum concentration

σ_o = 0.5 m, tracer injection thickness

Initial mean square distances were calculated with data from the mean rhodamine fluorescence profiles recorded ~5 hours after the injection on a Wetstar® (Wetlabs, Inc.) in situ fluorometer mounted to a Seabird 19 CTD. Fluorescence recorded 7-hours previously was subtracted from the rhodamine signal because phycoerythrin in cyanobacteria fluoresced slightly in the rhodamine spectra. Because rhodamine and ^{15}N were injected concurrently, this fluorescence measurement should provide an accurate picture of the relative dispersion of the tracers at the injection depth. Subsequent measurements of the tracers were made from the seston or rhodamine samples collected from the Ruttner bottle. Because the Ruttner bottle was 0.5-m long, and because we sampled only at 1.5-3 m intervals, our estimates of K_z and K_b are not precise. However, the *relative magnitudes* of the two diffusivity estimates should not have been influenced by the sampling design.

Physical and chemical measurements

In addition to the Seabird CTD profiles, we collected additional measurements at one station in the lake on each sample date. *In situ* temperature, conductivity and oxygen profiles were made with WTW meters (LF 191 and WTW Oxi91). Light penetration (photosynthetically active radiation, 400-700 nm) in the lake was measured by a 4π scalar irradiance sensor (Li-Cor). Chemical analyses were performed according to standardized methods (Golterman, 1978; APHA, 1992) on three dates (17 Sep, 30 Sep, 15 Oct). Ammonia (detection limit (DL) = 2.8 µg N/L) was measured

by the indophenol blue method. Nitrate plus nitrite (DL = 0.3 µg N/L) was measured as nitrite after reduction of nitrate by a cadmium-copper couple in an alkaline solution, and colorimetric determination with sulfanilamide and N-(1-naphtyl) ethylenediamine dihydrochloride. Particulate nitrogen (PN) from samples retained on GF/F filters was analyzed with the mass spectrometer as described above. Soluble reactive phosphorus (SRP; DL = 0.9 µg P/L) was measured by the phosphomolybdic acid method with ascorbic acid. Total phosphorus (DL = 0.9 µg P/L) was determined as orthophosphate after persulfuric acid digestion of the sample for 2 h at 135 ºC.

Plankton measurements

Phytoplankton were identified and counted from each of the sampling depths for samples collected on 17 Sept., 2 Oct., and 15 Oct. Phytoplankton composition was relatively stable on these three dates, and we have consequently presented data from only the midpoint of the experiment (2 Oct.) when the biovolume of each taxon were also estimated. Eukaryotic phytoplankton counts were made using the Utermöhl sedimentation method (Uthermöhl, 1958) in an Olympus inverted microscope at 200-1000 magnification. Species were identified following the taxonomic keys described in Sendra *et al.* (2019). Biovolume estimates were made by measuring several cells of each phytoplanktonic species, then geometric shapes were used for calculations (Rott, 1981). Picocyanobacterial counts were performed by epifluorescence microscopy on a Zeiss III epifluorescence microscope as described by Camacho *et al.* (2003c). Chlorophyll *a* was measured on each sampling date after filtration of the samples

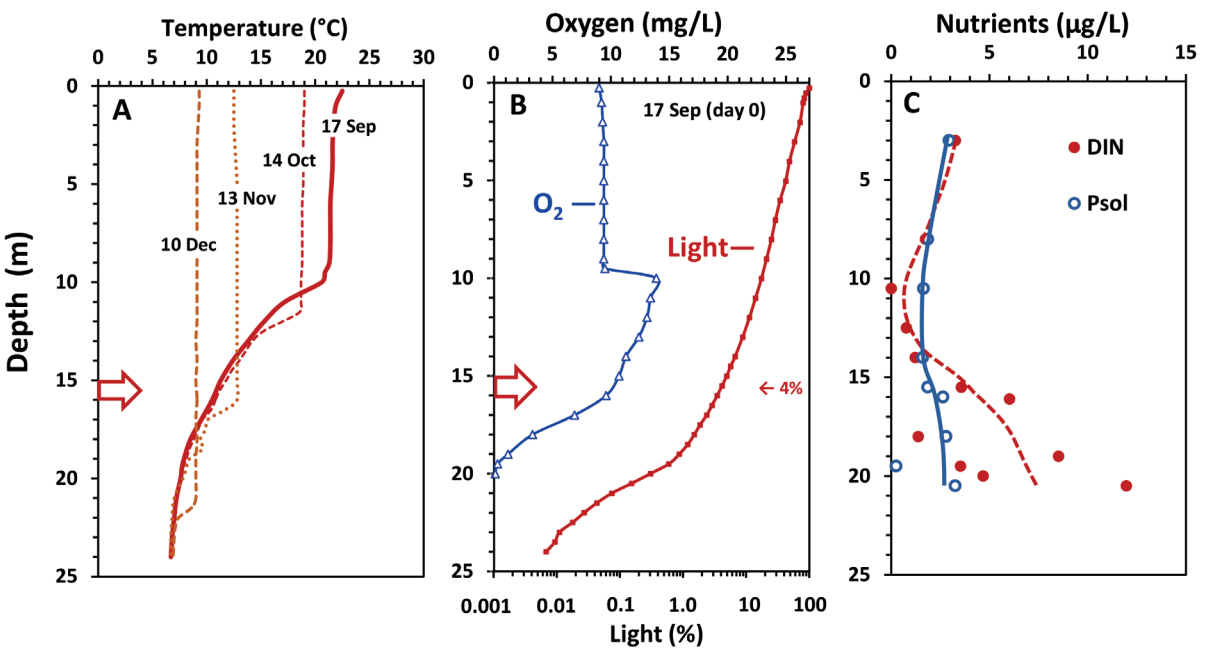

Figure 2. A. Profiles of temperature on five dates during the experiment in Laguna El Tejo. B. Profiles of oxygen concentration and light intensity at the start of the experiment on 17 September. The arrows on the y axes show the depth that ^{15}N and rhodamine were injected into the deep chlorophyll layer. C. Mean dissolved inorganic nitrogen (DIN) and soluble reactive phosphorus (SRP) measured on three dates during the 28-day experiment. *A. Perfiles verticales de temperatura medidos en cinco fechas diferentes durante el experimento realizado en la Laguna del Tejo. B. Perfiles verticales de la concentración de oxígeno y la radiación luminosa medidos al principio del experimento, el 17 de septiembre. Las flechas muestran la profundidad en la que se inyectó el ^{15}N y la rodamina en la profundidad donde estaba localizado el máximo profundo de clorofila. C. Concentraciones promedio de nitrógeno orgánico disuelto (DIN) y fósforo reactivo soluble (SRP) en tres fechas a lo largo de los 28 primeros días del experimento.*

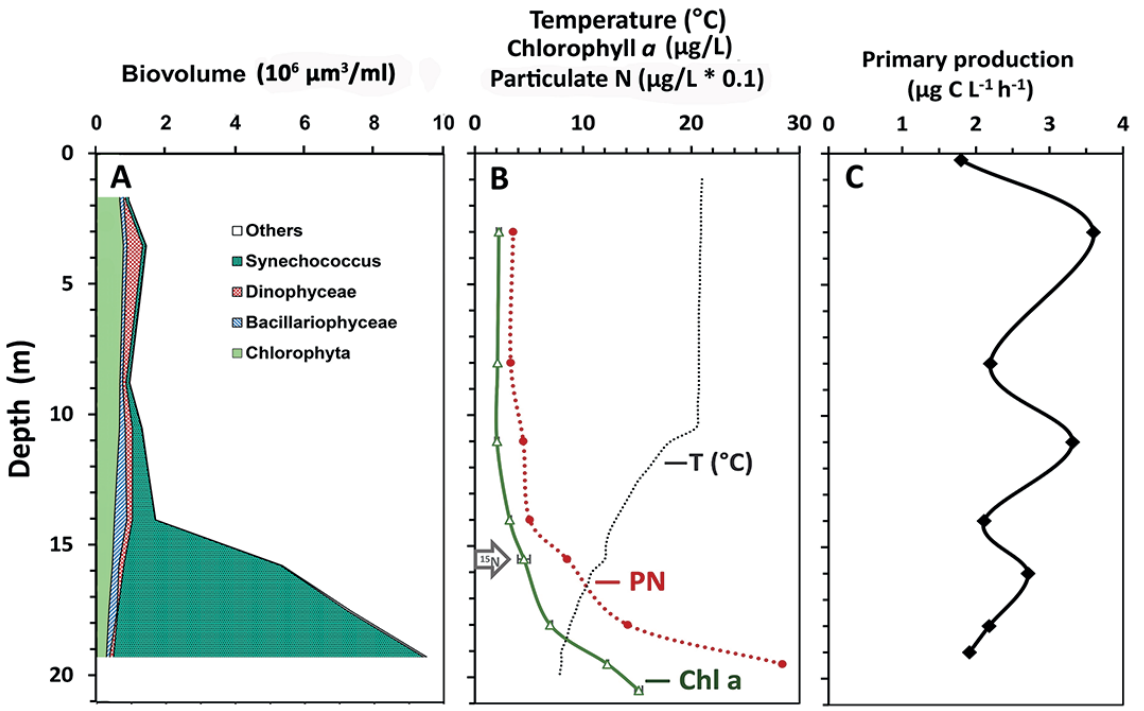

Figure 3. A. Biomass composition of phytoplankton groups in the water column of Laguna El Tejo on Day 15 (2nd-October) of the experiment. B. Chlorophyll *a*, particulate nitrogen concentrations (PN) and temperature in the water column on the Day 13 (30th Sept.). C. Plankton primary production profile on Day 13. *A. Composición (en biomasa) de los grupos principales de fitoplancton a lo largo de la columna de agua de la Laguna del Tejo en el día 15 del experimento (2 de octubre). B. Concentraciones de clorofila-*a *y de nitrógeno particulado (PN) y temperatura en el día 13 del experimento (30 de septiembre). C. Perfil vertical de la producción primaria en el día 13 del experimento.*

through a GF/F glass fiber filter, and subsequent extraction with acetone: DMSO, following the procedures described in Picazo *et al.* (2013). Carbon photosynthetic assimilation was measured *in situ* on 30 Sept. using the ^{14}C technique (Steeman-Nielsen, 1952) following the procedure described by Camacho & Vicente (1998). Samples were directly placed in 60-ml tissue culture bottles (Nunclon, Intermed) and kept in the dark during manipulation. Two clear and a dark bottle were used for each depth. 4 µCi of $NaH^{14}CO_3$ was added to each bottle and they were then incubated at depth for 4 hours. Inorganic carbon estimates were made using measures of alkalinity, pH, temperature, and ionic strength.

Midway through the study (30 Sept. - 1 Oct.) we measured diel vertical migration of the zooplankton at one station. At midday and midnight vertical profiles were made with the Schindler-Patalas trap and the samples preserved in 4 % formalin for subsequent identification and biomass estimates. Entire samples were counted on an inverted microscope at 100 X. For crustacean species, > 100 individuals were measured to estimate their biomass utilizing length–weight regressions (Lawrence *et al.*, 1987). To characterize the fraction of smaller zooplankton (30-100 µm, mainly rotifers) we re-filtered the 1L 100-µm filtrate with a 30-µm Nitex net. These samples were preserved and counted using an inverted microscope at 100–400 magnification. Estimates of the biomass of rotifers were made following Ruttner-Kolisko (1997).

Four-five minnows were sampled with a seine at the start of the experiment and after 15, 28 and 57 d. These were dried, ground and subsamples were analyzed for ^{15}N enrichment as described for zooplankton.

RESULTS

Limnological characteristics

The experiment was conducted during the fall, and consequently the epilimnion cooled and deepened from mid-Sep until December. However, the change was relatively minor during the main experiment, from 17 Sep. to 14 Oct. (Fig. 2A). Oxygen concentration peaked at the top of the metalimnion, where the density gradient minimized upwards diffusion of the photosynthetically-produced oxygen, and declined to 0 mg/L by 20 m. Secchi disk visibilities were 7.2 m and 7.4 m, respectively, at the start and end of the 28-day experiment. Light levels declined nearly exponentially through the epilimnion and upper metalimnion but dropped rapidly in the hypolimnion where the deep chlorophyll layer was well developed (Fig. 2B). Light levels were 4 % of surface intensities at the ^{15}N injection point. The depth of 1 % light intensity changed from 18.7 m at the start of the experiment to 17.9 m on day 28. The 1 % light level was near the peak of the Synechococcus layer (see below). Dissolved inorganic nutrients were low in the epilimnion, dropped lower at the top of the metalimnion, and increased below 15 m in the deep chlorophyll layer (Fig. 2C). On most dates total P in the epilimnion was near the level of detection (0.9 µg/L), increased to 3 µg/L in the upper DCL and reach 16 µg/L at 20 m. The spring water entering El Tejo that was sampled on three dates had mean concentrations of 3.6 ± 3.2 µg N/L NH_4^+, 760 ± 526 µg N/L $NO_3^+NO_2$ and 1.0 ± 0.7 µg/L soluble P.

Algal composition in Laguna El Tejo varied markedly with depth (Fig. 3A). In the epilimnion, green algae (*Cosmarium laeve*, *C. abbreviatum*, and others) were co-dominants with a large dinoflagellate (*Peridinium* sp.), a small population of diatoms (*Fragilaria ulna*, *Cyclotella radiosa*) and *Synechococcus*. In the upper part of the deep chlorophyll layer (11-14 m), green algae (*C. abbreviatum*, *C. laeve* and others) and diatoms (*C. radiosa*) were abundant, along with dinoflagellates and increasing populations of *Synechococcus* sp.. A very small population of the cyanobacterium *Pseudanabaena* sp. was found in the epilimnion and metalimnion on one date. At 15-16 m in the deep chlorophyll layer, green algae (*Chlorella vulgaris*; *Cosmarium* spp.), diatoms (*C. radiosa*) and dinoflagellates remained abundant, but were overshadowed by increasing densities of *Synechococcus* which represented 85 % of the phytoplankton biovolume. At the anoxic interface at 19.5 m, the biomass of green algae, diatoms and dinoflagellates decreased and cryptophytes (*Cryptomonas erosa*, *C. phaseolus*) and euglenophytes (*Euglena agilis*) increased moderately. At 19.5 m, however, *Synechococcus* densities reached 4-5 x 10^6 cells/ml, and they represented 95 % of the autotrophic plankton biovolume. At 19.5 m estimated phytoplankton biovolume was 10-times higher than in the epilimnion.

All three biomass metrics (algal biovolume, seston N and Chl. *a*) had similar depth profiles, suggesting that most of the seston was comprised of phytoplankton. However, the seston N metric also would have included detrital material. The changes in phytoplankton biovolume with depth were paralleled by increases in particulate nitrogen, which increased from 34 µg/L in the epilimnion, to 85 µg/L at the tracer injection depth, to 284 µg/L at 19.5 m (Fig. 2B). Extracted chlorophyll *a* concentrations also followed the same pattern as seston N, increasing some in the upper metalimnion and then markedly in the strata where *Synechococcus* predominated (Fig. 3B). The chlorophyll profile changed little throughout the experiment, with the exception that the low epilimnetic concentrations extended to 12.5 m by day 28, coincident with the increased mixing depth (Fig. 2A).

Photosynthesis, on the one day it was measured, had an irregular pattern with one peak in the epilimnion, a second one at the top of the deep chlorophyll layer, and moderate production throughout the *Synechococcus* layer (Fig. 3C). At 19 m, where the light intensity was only 12 µE m^{-2} s^{-1}, primary production equaled that of phytoplankton at the surface. The production at the top of the deep chlorophyll layer was accompanied by a peak in oxygen concentrations (Fig. 2B).

The deep layers of the lake dominated algal biomass metrics, and to a lesser degree, primary production. Only 21-38 % of the algal biovolume, chlorophyll and particulate nitrogen

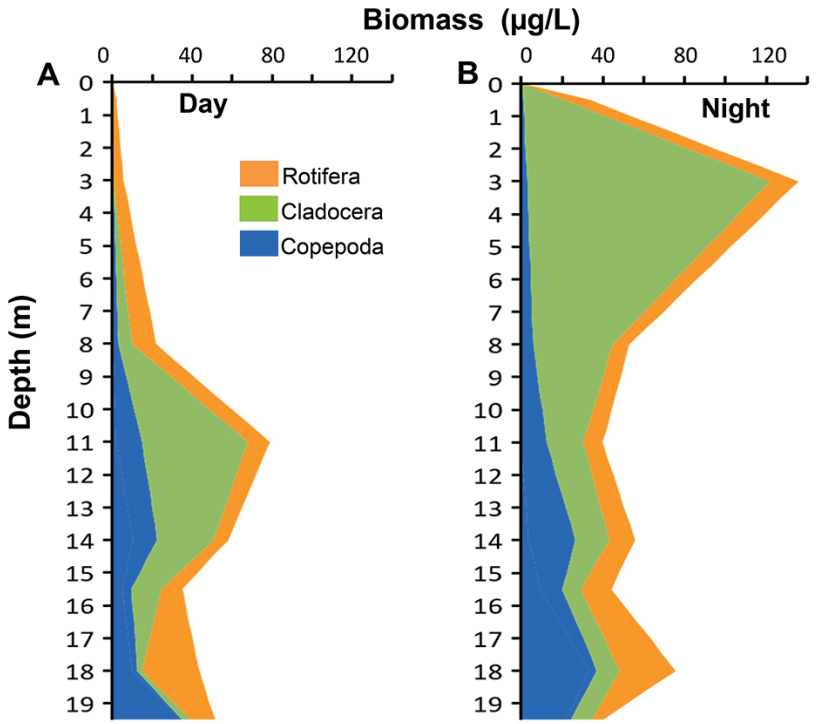

Figure 4. Dry biomass of different zooplankton groups at different depths of Laguna El Tejo (A) during the day and (B) night on 1 Oct. *A. Biomasa (peso seco) de los diferentes grupos de zooplancton en el perfil vertical de la Laguna del Tejo (A) durante el día y (B) por la noche, del día 1 de octubre.*

biomass occurred in the epilimnion (0-10.5 m), whereas 58 % of the primary production occurred there (Fig. 3). An additional 10-31 % of biomass metrics occurred in the 10.5-14 m strata above the *Synechococcus*-dominated zone, and 19 % of primary production occurred there. The Synechococcus zone (> 14 m), accounted for 48-70 % of the biomass metrics, and 23 % of the primary production.

Zooplankton biomass in the lake was dominated by the herbivores *Diaphanosoma brachyurum* and *Tropocylops prasinus* and by the predaceous copepod, *Cyclops abyssorum* (Fig. 4). For both copepods, larval stages (which are mostly herbivores) accounted for a 60 % of their biomass. During the day, the grazers (*D. brachyurum* and *T. prasinus*) occurred primarily in the upper part of the deep chlorophyll layer (11-14 m) at temperatures between 13° and 19 °C. Their densities declined at 15.5 and particularly at 18 and 19.5 m where *Synechococcus* increased markedly. At night, the *Diaphanosoma* increased in the epilimnion, primarily as a result of diel horizontal migration (Armengol *et al.*, 2012). Rotifers represented around 25 % of the biomass in the water column and *Anuraeopsis fissa*, a species that frequently peaks in the hypolimnion of stratified lakes (Miracle & Armengol, 1995), were particularly abundant in the *Synechococcus* zone. Nitrogen concentrations of the zooplankton followed the same profile as the zooplankton biovolume estimates, but concentrations were less than 2 % of that in the seston (data not shown).

^{15}N enrichment of the community

Prior to the addition of tracers, the natural ^{15}N enrichments of phytoplankton and zooplankton community increased with depth (Fig. 5). $\delta^{15}N$ levels for seston increased from $^{+}1$ in the epilimnion to $^{+}6$ in the hypolimnion. Zooplankton $\delta^{15}N$ levels increased in a similar manner,

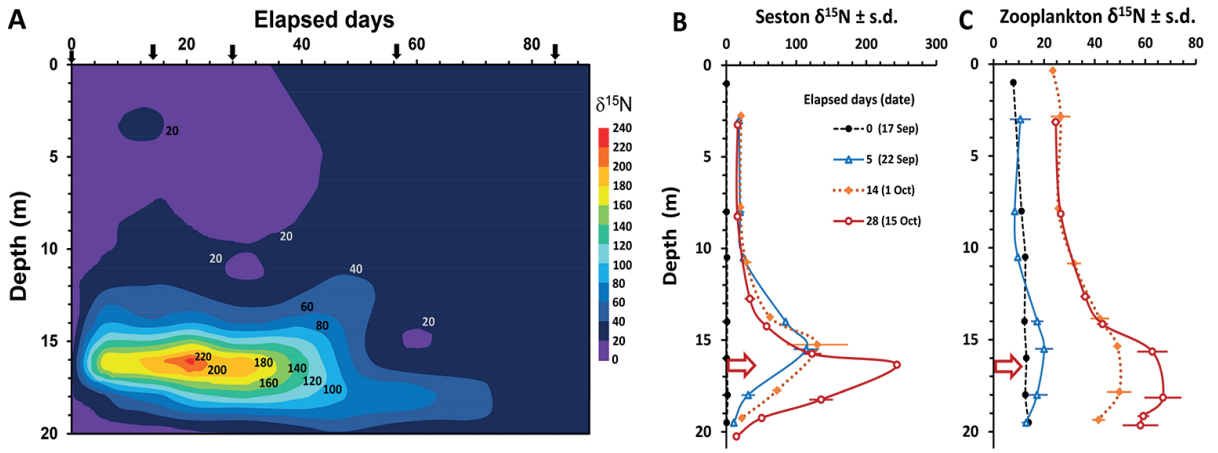

Figure 5. A. Isopleths showing ^{15}N enrichment in the seston for the first 85 days of the experiment. Arrows show the dates where isotopic enrichment was measured. B. Depth profiles of ^{15}N enrichment of the seston on four dates. C. ^{15}N enrichment of the zooplankton on four dates. *A. Isolíneas del enriquecimiento en ^{15}N del seston en los primeros 85 días del experimento. Las flechas muestran la fecha y profundidad en la que se inyectó el trazador. B. Perfiles verticales del enriquecimiento en ^{15}N del seston en cuatro fechas durante el estudio. C. Enriquecimiento en ^{15}N del zooplancton en cuatro fechas durante el estudio.*

from +8 near the surface to +14 at 19.5 m. Zooplankton, on average, had a trophic enrichment factor (+$\delta^{15}N$) 8.2 units above the seston.

After the addition of $^{15}NH_4^+$, the seston pool became labeled with the tracer, particularly near the depth of the injection (Fig. 5A-B). Within 5 days $\delta^{15}N$ levels at 15.5 m increased to +110 and continued to increase until day 30. On day 30 we sampled one additional depth (16.1 m) and found even higher labeling there. Surprisingly, by day 5 ^{15}N labeling also increased to near +20 in the epilimnion, likely the result of contamination when our injection tube with pulled up from 15.5 m without first flushing it. Epilimnetic seston labeling did not increase subsequently during the main 28-day experiment. By 13 Nov. when winter mixing had begun, seston enrichment was near +30 throughout the mixed layer that extended to 16 m, and by 10 Dec. the lake was nearly isothermal (Fig. 2A) and the isotope had mixed throughout the water column, enriching the seston to +33 (Fig. 5A).

Zooplankton also became enriched with ^{15}N, particularly in the deep chlorophyll layer (Fig. 5C). After 5 days, enrichment was limited to zooplankton in the deep chlorophyll layer, but by days 14 and 30, zooplankton became enriched throughout the water column. In the epilimnion, enrichment averaged +26, higher than the seston in that layer, but consistent with the trophic enrichment factor noted prior to the tracer addition, indicating that the epilimnetic zooplankton were feeding little in the deep chlorophyll layer. In the deep chlorophyll layer, zooplankton enrichments peaked at +50 (day 14) and +67 (day 30) at 18 m.

The mixing model analysis of zooplankton diets also indicated that zooplankton collected in the epilimnion (3-8 m) were consuming almost entirely seston from that layer (Fig. 6; 3-8 m). At the top of the metalimnion (12.5-14 m), the model estimated that 14 % of the diet was from the deep chlorophyll layer, and in the *Synechococcus* layer (15.5-19 m), 33 % of the zooplankton diets were from that layer, with 64 % estimated to have been from epilimnetic seston.

^{15}N injected into the deep chlorophyll layer and incorporated in the seston and zooplankton moved in the water column 10 times faster than the rhodamine (Fig. 7). Vertical bio-diffusivity for the ^{15}N seston was 1.24 * 10^{-6} m^2/s and the diffusivity of rhodamine was only 0.12 * 10^{-6} m^2/s.

$\delta^{15}N$ increased slightly in fish from +10.6 ± 0.2 at the start, to +12.0 ± 1.7 on day 28 (p =

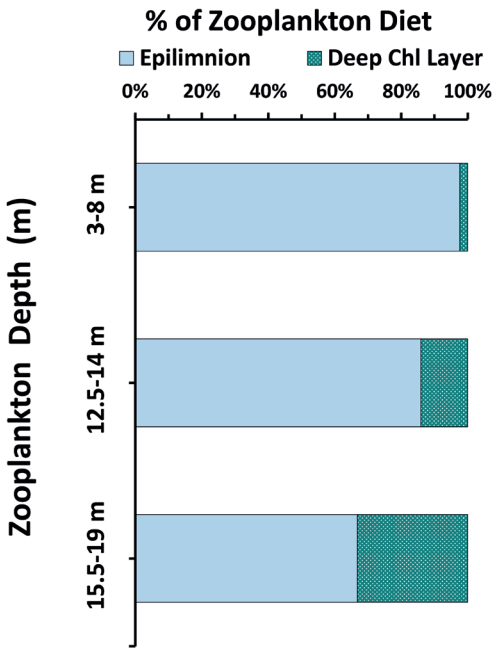

Figure 6. Mixing-model estimates of the diet proportions of macrozooplankton from different depths consumed from the epilimnion, and from the deep chlorophyll layer. Because the model assumes that the zooplankton had reached isotopic equilibrium, the diet proportion from the deep chlorophyll layer may be underestimate. Data are for day 28 of the experiment. *Estimaciones, mediante un modelo mixto, de la proporción de la dieta del macrozooplancton de diferentes profundidades consumida en el epilimnion y en el máximo profundo de clorofila. Dado que el modelo asume que el zooplancton ha alcanzado su equilibrio isotópico, es posible que la proporción de la dieta ingerida en el máximo profundo haya sido subestimada. Los datos corresponden al día 28 del experimento.*

0.09), but then decreased to +9.9 ± 1.3 in mid-November (p = 0.63), indicating that fish benefited little from the deep chlorophyll layer.

An analysis of the ^{15}N distribution on day 28 indicated that 55 % of the injected tracer was accounted for. The seston in the metalimnion (11.5-18m) accounted for over 70 % of this, indicating relatively little movement into the epilimnion and hypolimnion (Fig. 8). Zooplankton incorporated only 1 % of the ^{15}N that was added and 8 % was found in the sediment traps. Mean sedimentation rates for the first and second two-week periods were 26.7 and 21.8 mg N m^2/d, but these were not significantly different (2-tailed t-test, p = 0.16). Average particulate nitrogen levels in the 20-m water column above the traps was 1400 mg N/m^2, yielding an average daily loss rate of 1.6 %/d. In contrast, the sedimentation loss rate for ^{15}N was eight times lower: only 0.2 %/d.

The year-long analysis of ^{15}N in the epilimnion of El Tejo showed that the tracer declined exponentially after the lake mixed (Fig. 9). After 335 days post-injection the epilimnetic seston remained +δ 5.6 above background enrichment. The decline was slow, with an estimated tracer half-life of 170 d.

DISCUSSION

The sampling demonstrated that much of the algal production (42 %) and biovolume (78 %) in this karst lake occurred in the metalimnion and hypolimnion. This has routinely been found in oligotrophic lakes and oceans (Giling *et al.*, 2017) and even in many mesotrophic systems

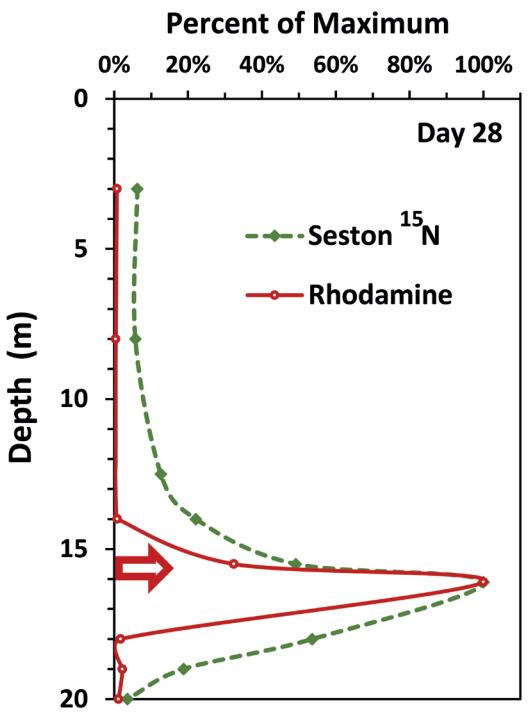

Figure 7. Distribution of rhodamine and ^{15}N-labeled seston on day 28 of the experiment. The arrow shows the injection depth of the tracers. *Distribución de la rodamina y del seston marcado con ^{15}N en el día 28 del experimento. La flecha muestra la profundidad de inyección de los trazadores.*

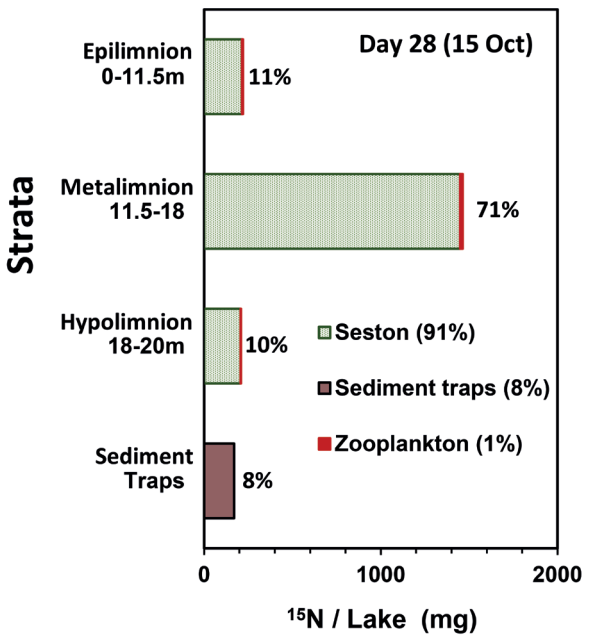

Figure 8. Estimated distribution of the ^{15}N recovered at the end of the experiment (day 28). *Distribución estimada del ^{15}N recuperado al final del experimento (día 28).*

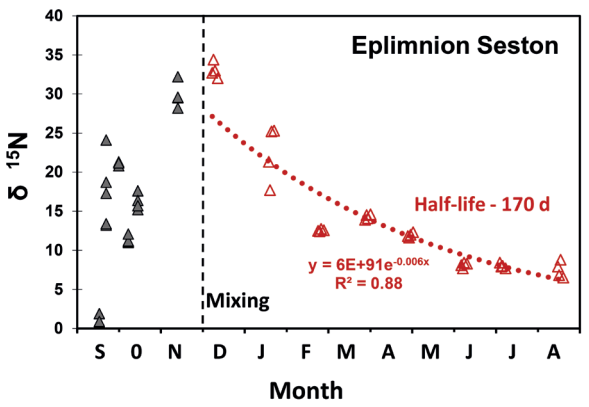

Figure 9. Enrichments of ^{15}N in the seston of Laguna El Tejo over one year. The solid grey triangles show isotopic enrichment of seston prior to lake mixing. The red open triangles show the decline in enrichments after the lake had mixed nearly to the bottom. *Enriquecimiento en ^{15}N del seston en la Laguna del Tejo un año después de la inyección. Los triángulos grises muestran el enriquecimiento del seston antes de la mezcla del lago. Los triángulos rojos vacíos muestran la caída del enriquecimiento tras la mezcla vertical del lago.*

(Williamson *et al.*, 1996). In Lake El Tejo, phytoplankton biomass and chlorophyll levels began to increase in the top of the metalimnion, but the large increase was deep in the water column where a cyanobacterial *Synechococcus* population developed at a light intensity near 4 % of surface radiation. Although DCLs frequently development near the top of the metalimnion, Leach *et al.* (2018) found that 33 % of lakes they analyzed with DCM had chlorophyll peaks below the metalimnion. They also found that the median light level for development of the DCM was 4.8 %, similar to that found in El Tejo, whereas DCL are common at up to 1 % PAR depths (Camacho, 2006; Scofield *et al.*, 2020).

Deep chlorophyll layers are typically composed of motile algal species and/or neutrally buoyant cyanobacteria with gas vacuoles or ballast substances (Richardson & Cullen, 1995; Camacho *et al.*, 2000; Leach *et al.*, 2018). The upper part of the DCL in El Tejo was due primarily to a diatom (*Cyclotella radiosa*), but the large increase in chlorophyll and algal biomass was due to *Synechococcus*, which has a diameter near 2 µm and a low cellular density, thus having negligible sinking rates (Camacho *et al.*, 2003b). The large peak in *Synechococcus* is similar to that of the nearby Lake La Cruz (Camacho *et al.*, 2003c) where active growth occurs in the deeper part of the metalimnion, and cell densities reach nearly 10^7 cells/ml. Light-harvesting and growth of these picocyanobacterial is possible in the deep layers because they contain phycoerythrin (Camacho *et al.*, 1996; Camacho *et al.*, 2001; Camacho *et al.*, 2003b). Consequently, they can thrive at depths where nutrient availability is higher than in the surface layers (Camacho, 2006). Moreover, our primary production determinations showed a peak of carbon assimilation coinciding with the deeper DCL formed almost exclusively by *Synechococcus*, which evidenced *in situ* photosynthetic activity even near the oxic-anoxic interface. However, its activity was much lower per biomass unit than the upper (subsurface and metalimnion) primary production peaks, which yield higher specific productivity (production/biomass). Earlier experiments demonstrated that even though they have phycoerythrin, the *Synechococcus* are nevertheless light

limited, but they take advantage of the higher nutrient availability in these deeper layers to support *in situ* growth (Camacho *et al.*, 2003b). Negligible sinking rates and low grazing pressure in the cold hypolimnion minimize population losses of *Synechococcus* in the DCL, allowing high biomass accumulation, despite low growth rates (Camacho *et al.*, 2003a, 2003b).

The seston in El Tejo became heavily labeled with ^{15}N within five days of the addition, primarily in the DCL at 15.5 m. On day 28 of the experiment we sampled ^{15}N slightly deeper (16.1 m) because rhodamine profiles measured with the WetStar fluorometer mounted on the Seabird CTD indicated the peak was there, and we found that isotopic labeling was nearly double that of the seston at 15.5 m. The higher enrichment at 16.1 m was likely due to either a slight measurement error with our injection line, or to a higher density of the injected tracers that could have carried them below their injection point. The seston in the epilimnion was also enriched to $\delta^{+}18$ within five days but showed no additional enrichment during the main 28-day experiment. Wilkinson *et al.* (2014) injected ^{13}C tracer into the metalimnion of a similar-sized lake and found no enrichment of seston in the epilimnion until after 20 days. Consequently, we believe the epilimnetic enrichment in El Tejo was primarily due to some spillage from our raft, or from accidental addition when the injection tube was raised from depth without first flushing it. Rhodamine was not measured on the first sampling date following the injection, but on day 16 of the experiment average epilimnetic rhodamine was 2.6 % of the concentration at 15.5 m, indicating that some tracer contamination of the epilimnion occurred during the injection.

After 28 days we were only able to account for 55 % of the ^{15}N tracer. Other pools that we did not measure that could account for this missing tracer are: (1) ^{15}N uptake by periphyton or benthic bacteria, which can be significant in oligotrophic lakes (Brothers *et al.*, 2016; Vadeboncoeur & Power, 2017); (2) denitrification, which could have been appreciable since the $^{15}NH_4^+$ injected could have easily been nitrified and then denitrified at the interface of the oxic-anoxic zone at 19-20 m (e.g. Brezonik, 2013; Castellano-Hinojosa *et al.*, 2017); (3) $^{15}NH_4^+$ in the injection strata which was not taken up by the plankton and tracer transformed into dissolved inorganic nitrogen, neither of which were analyzed for; (4) tracer leaving the lake via groundwater, and; (5) errors in our measurements of the other pools. Because we didn't have a lake-wide estimate of fish abundance or biomass we could not calculate the amount of ^{15}N moving into this pool, but the insignificant ^{15}N enrichment in their tissue, and the normally small contribution of fish to the biomass of the biota (Wurtsbaugh, 2007), suggests they were not an important pool for the tracer.

Only 8 % of the recovered tracer had fallen into the sediment traps. This low amount was not surprising given that: (1) much of the tracer was likely incorporated into *Synechococcus*, with very low sedimentation rates (Camacho *et al.*, 2003b; Camacho, 2006) and; (2) macrozooplankton grazing in the DCL was likely very low, with consequent low export of fecal material that can account for significant losses of particulate matter from the water column of lakes and oceans (Pilati & Wurtsbaugh, 2003; Turner, 2015; Maszczyk & Wurtsbaugh, 2017). Additionally, grazing on the abundant *Synechococcus* was likely done by the abundant rotifer, *Anuraeopsis fissa*, that graze bacteria and picoplankton efficiently (Ooms-Wilms, 1997; Twiss *et al.*, 2012). This, however, would not cause vertical movements of the ^{15}N, since rotifers have limited vertical migrations (Armengol & Miracle, 2000) and they do not produce dense (settling) excreta such as the fecal pellets of copepods. The sedimentation rate of the ^{15}N was eight times lower than the sedimentation rate of unlabeled N, suggesting that much of the settling material originated above the heavily labeled *Synechococcus* layer.

The comparison of the final rhodamine and ^{15}N profiles in the DCL indicated that biotic processes were 10X more important for tracer movement than the physical process of vertical eddy diffusion. One obvious reason for the faster movement of ^{15}N was sedimentation of the phytoplankton and excreta of zooplankton that fed in this layer. Although ^{15}N sedimentation rates were low, they likely contributed to the downward spread of the tracer. The greater

upward movement of the tracer could have been due to mobile flagellates, or to ^{15}N in the excreta of zooplankton that had some vertical movement in the water column (Fig. 4; Armengol *et al.*, 2012). The greater dispersion of the ^{15}N must be viewed in the context of the low diffusivity of the rhodamine tracer, $0.12 * 10^{-6}$ m^2/s, which is near that for molecular diffusion (Kalff, 2002). This low diffusivity is consistent with the very small fetch of El Tejo (140 m) and the surrounding 20-50 m cliffs that protect the lake from winds. Based on the surface area of El Tejo, the equation of Maiss *et al.* (1994) predicts a rhodamine diffusivity of 0.435×10^{-6} m^2/s, somewhat higher than what we observed, but consistent with the wind-protected lake surface. The higher diffusivity for ^{15}N via biotic processes (biodiffusivity) is important because researchers often address the upward vertical movement of nutrients as a strict consequence of the physical mixing processes, and thus they are likely underestimating the true movement of nutrients. Although biodiffusivity has been studied extensively to address solute transport from the sediments (Matisoff, 1996; Kristensen *et al.*, 2012), its importance in the water column has focused primarily on how zooplankton influence physical mixing (Simoncelli *et al.*, 2017; but see Pilati and Wurtsbaugh (2012); Houghton *et al.*, 2018).

After fall mixing dispersed ^{15}N throughout the water column, we continued to sample the epilimnetic seston until the following August, allowing us to estimate a half-life for this tracer of 170 d. Given that there are no surface inflows to El Tejo, and that the lake lost depth during our study, we expect that groundwater exchange with the lake was low during the study thus providing minimal flushing. However, we do not have a water residence time to compare with the ^{15}N tracer, because we could not use the long-term loss rate of rhodamine due to unknown spectrally-controlled photo-bleaching. Nevertheless, the data demonstrate that denitrification and sedimentation loss of nitrogen was low in this lake.

Our data indicates that the production in the DCL was only moderately important as a food source for crustacean zooplankton, despite the amount of primary production and particularly algal biomass in this layer. After 28 days, only 1 % of the recovered ^{15}N was found in the zooplankton. This was somewhat expected, given that only 1.8 % of N in the plankton was in the crustacean zooplankton. The mixing model also indicated that the DCL was relatively unimportant as a food resource for the zooplankton: only 2 % of the food consumed by epilimnetic zooplankton and 14-33 % by macrozooplankton captured in the DCL was from the deep layer. Many crustacean zooplankton can feed on *Synechococcus* (Lampert *et al.*, 1986), so the low values were unexpected. We did not, however, measure isotopic enrichment of rotifers, as nearly all of them would have passed through the 100-µm mesh we used to separate seston and zooplankton. Rotifers can feed heavily on picoplankton such as the *Synechococcus* in the DCL of El Tejo (Twiss *et al.*, 2012), so it is likely we underestimated the transfer to the zooplankton. Additionally, the zooplankton sampled on day 28 may not have come into full equilibrium with ^{15}N tracer in the seston and this also could have caused us to underestimate the importance of the DCL. However, in a ^{13}C metalimnetic enrichment experiment similar to ours (Wilkinson *et al.*, 2014) zooplankton isotopic labeling was complete within 30 days, suggesting that the plankton in El Tejo may have been close to equilibrium.

Based on their ^{13}C experiment Wilkinson *et al.* (2014) inferred that the DCL contributed only minimally to the crustacean zooplankton diet, and Sanful *et al.* (2017) studying natural populations, reached a similar conclusion. However, Francis *et al.* (2011), using natural-abundance isotopic analyses, concluded that the DCL was a very important food source in oligotrophic lakes. Others have found conflicting evidence of the food value of seston in the DCL (Williamson *et al.*, 1996; Cole *et al.*, 2002). Cold temperatures at the depth of the DCL in El Tejo (8-13 °C) and other lakes should also reduce zooplankton grazing in this stratum (Dawidowicz & Loose, 1992; Lampert *et al.*, 2003). Much of the literature on the importance of the DCL for zooplankton has focused on diel vertical migration (DVM), and the consumption of deep-chlorophyll phytoplankton during the day when zooplankton migrate into this layer. However, in El Tejo there was minimal DVM of the zooplankton: instead, the increase of

plankton in the epilimnion at night was due to migration from the epilimnetic sediments or from macrophytes in the littoral zone (Armengol *et al.*, 2012). The importance of the DCL for zooplankton is thus likely dependent on the degree of DVM, which can vary seasonally as the relative amounts and qualities of the seston in the epilimnion and metalimnion change (Brindza, 2002; Matthews & Mazumder, 2005). Additional work will be necessary to determine the importance of these temporal shifts, and the significance of the DCL for the upper trophic levels in a variety of lakes.

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