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Denitrification in a meromictic lake and its relevance to nitrogen flows within a moderately impacted forested catchment

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## **Biogeochemistry**

# Denitrification in a meromictic lake and its relevance to nitrogen flows within a moderately impacted forested catchment --Manuscript Draft--

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Abstract:	We analysed the spatial and temporal variability of benthic nitrogen fluxes and denitrification rates in a sub-alpine meromictic lake (Lake Idro, Italy), and compared in- lake nitrogen retention and loss with the net anthropogenic nitrogen inputs to the watershed. We hypothesized a low nitrogen retention and denitrification capacity due to meromixis. This results from nitrate supply from the epilimnion slowing down during stratification and oxygen deficiency inhibiting nitrification and promoting ammonium recycling and its accumulation. We also hypothesized a steep vertical gradient of sedimentary denitrification capacity, decreasing with depth and oxygen deficiency. These are important and understudied issues in inland waters, as climate change and direct anthropic pressures may increase the extent of meromixis. Nearshore sediments had high denitrification was negligible. The littoral zone, covering 10% of the lake surface, contributed 50% of total denitrification, while the monimolimnion, which covered 70% of the sediment surface, contributed to < 13% of total denitrification. The persistent and expanding meromixis of Lake Idro is expected to further decrease its nitrogen removal capacity (31% of the incoming nitrogen load) compared to what has been measured in other temperate lakes. Values up to 60% are generally reported for other such lakes. Results of this study are relevant as the combination of anthropogenic pressures, climate change and meromixis may threaten the nitrogen processing capacity of lakes.		
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2	Denitrification in a meromictic lake and its relevance to nitrogen
3	flows within a moderately impacted forested catchment
4	
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#### 21 Abstract

22 We analysed the spatial and temporal variability of benthic nitrogen fluxes and denitrification 23 rates in a sub-alpine meromictic lake (Lake Idro, Italy), and compared in-lake nitrogen retention and 24 loss with the net anthropogenic nitrogen inputs to the watershed. We hypothesized a low nitrogen 25 retention and denitrification capacity due to meromixis. This results from nitrate supply from the 26 epilimnion slowing down during stratification and oxygen deficiency inhibiting nitrification and 27 promoting ammonium recycling and its accumulation. We also hypothesized a steep vertical 28 gradient of sedimentary denitrification capacity, decreasing with depth and oxygen deficiency. 29 These are important and understudied issues in inland waters, as climate change and direct 30 anthropic pressures may increase the extent of meromixis. Nearshore sediments had high denitrification rates (87 mg m<sup>-2</sup> d<sup>-1</sup>) and efficiency (~100%), while 31 32 in the monimolimnion denitrification was negligible. The littoral zone, covering 10% of the lake 33 surface, contributed ~50% of total denitrification, while the monimolimnion, which covered 70% of 34 the sediment surface, contributed to < 13% of total denitrification. The persistent and expanding 35 meromixis of Lake Idro is expected to further decrease its nitrogen removal capacity (31% of the incoming nitrogen load) compared to what has been measured in other temperate lakes. Values up 36 37 to 60% are generally reported for other such lakes. Results of this study are relevant as the 38 combination of anthropogenic pressures, climate change and meromixis may threaten the nitrogen 39 processing capacity of lakes.

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#### 43 Introduction

44 Reactive nitrogen (Nr) inputs to watersheds have increased many fold in the last century, exceeding their uptake and storage capacity and causing large Nr exports with detrimental effects 45 on receiving aquatic ecosystems (Galloway et al. 2003; Howarth et al. 2006). Several assessments 46 47 have been made of Nr pathways and fate along the terrestrial to aquatic continuum, highlighting 48 knowledge gaps (Howarth et al. 1996; Seitzinger et al. 2006; Bartoli et al. 2012). Namely, the Nr 49 exported by rivers to the coastal zone worldwide averages 25% of the Nr loading generated by 50 human activities in their watersheds, although large differences exist among catchments (Howarth 51 et al. 1996; Howarth et al. 2012). Further effort continues to be needed to elucidate the fate of Nr not reaching coastal waters, the roles played by different landscape and ecosystem components, and 52 53 the effects of anthropic activities on Nr processing (Seitzinger et al. 2006). 54 Lakes and reservoirs are biogeochemical reactors where the excess Nr is either retained or 55 permanently removed as N<sub>2</sub> (David et al. 2006; Harrison et al. 2009; Lassaletta et al. 2012). The 56 ratio of Nr retention to Nr removal differs among lakes, depending on a suite of environmental, 57 hydrological, morphological and geographical conditions (Seitzinger et al. 2006; Bruesewitz et al. 2011; Finlay et al. 2013; Rissanen et al. 2013). Ultimately, a wide variety of interlinked 58 59 biogeochemical processes control Nr (Burgin & Hamilton 2007), with lakes potentially acting as Nr 60 filters in the watershed. In a lake, Nr is assimilated and retained by primary producers, which in turn 61 fuel the food web and, ultimately, deliver organic N (Norg) to sediments. Microbial ammonification 62 recycles a fraction of the sedimentary Norg as ammonium (NH4<sup>+</sup>). NH4<sup>+</sup> is in turn assimilated by primary producers or transformed through a sequence of microbial processes comprised of 63 64 nitrification, anaerobic NH<sub>4</sub><sup>+</sup> oxidation (anammox), denitrification and dissimilative nitrate (NO<sub>3</sub><sup>-</sup>) reduction to NH<sub>4</sub><sup>+</sup> (DNRA). These processes have different ecological effects as only denitrification 65

and anammox permanently remove Nr, whereas DNRA recycles the Nr within the ecosystem. 66 Benthic Nr transformations are intense in both shallow littoral or deep sediments (Nizzoli et al. 67 68 2010; 2014), where microbial processes are stimulated by the availability of organic carbon and 69 electron acceptors (David et al. 2006; Revsbech et al. 2006; Wenk et al. 2014). 70 Although denitrification is recognized as a major Nr sink in lakes, to date only a few studies 71 have assessed the contribution of in-lake denitrification with respect to Nr loadings from the 72 watershed (Mengis et al. 1997; David et al. 2006; Bruesewitz et al. 2011; Rissanen et al. 2013; 73 McCarthy et al. 2016). Factors controlling Nr transformations are mainly inferred from mass 74 balance or simulation models (Harrison et al. 2009; Finlay et al. 2013). Further, processes responsible for Nr removal have not yet been adequately measured. Studies of in-lake 75 76 denitrification are often biased by the use of the acetylene-inhibition techniques to measure 77 denitrification. This method does not allow for the assessment of the coupling between nitrification 78 and denitrification, because acetylene is a strong inhibitor of nitrification (Groffman et al. 2006). 79 Measurements performed with sediment slurries are also unsuitable, since they may overestimate 80 denitrification rates (Groffman et al. 2006). 81 The occurrence and persistence of thermal stratification and mixing control in-lake 82 biogeochemical processes. Oxygen and nutrient availability especially limit biological activity. Thermal conditions and availability of oxygen, NO<sub>3</sub><sup>-</sup> and organic carbon control benthic 83 84 denitrification (Piña-Ochoa & Álvarez-Cobelas 2006). Under eutrophic conditions, the stable 85 thermal stratification induces dissolved oxygen depletion in the hypolimnion, which in turn shifts 86 the benthic microbial metabolism from aerobic to anaerobic (Matthews et al., 2008). In the hypolimnion, under anoxic conditions, denitrification depletes NO<sub>3</sub><sup>-</sup> in association with two 87 concurrent factors. The NO<sub>3</sub><sup>-</sup> supply from the epilimnion slows down during stratification, while 88

89 persistent anoxia hampers microbial nitrification, which requires oxygen. Furthermore, the end-90 products of anaerobic metabolism such as sulfides inhibit denitrification and foster DNRA (Burgin 91 & Hamilton 2007; Nizzoli et al. 2010; Azzoni et al. 2013). Persistent stratification in meromictic 92 lakes deeply affects biogeochemical processes, where deep-water anoxia may last over decades, 93 thus altering redox conditions (Lehmann et al. 2015). Furthermore, deep temperate lakes are 94 undergoing less frequent water turnover, alarmingly shifting toward holo-oligomictic conditions, 95 with the possible onset of meromixis due to the climate changes (Salmaso et al., 2014; Jeppesen et 96 al. 2015; Kraemer et al., 2015; Ficker et al. 2017). For these reasons, meromictic lakes are 97 especially suited for studying Nr pathways and fate. The coupled assessment of their watershed 98 processes could help to evaluate and predict the lake ecosystem responses to altered mixing regime 99 and to increasing anthropogenic pressures in the watershed.

100 This study has two objectives: 1) to investigate benthic Nr processing in a meromictic lake and 101 2) to put such Nr processing in the framework of whole basin N-budgets. We hypothesized that 102 meromictic conditions can depress Nr removal, as the occurrence of anoxia may stimulate  $NH_4^+$ 103 recycling and limit denitrification. Specifically, the aims of this work were: 1) to assess the 104 contribution of denitrification to the lake Nr budget, 2) to evaluate and compare denitrification rates 105 in deep and littoral lake sediments, and 3) to integrate the lake Nr budget into a detailed watershed 106 Nr budget.

We considered Lake Idro, a sub-alpine meromictic lake, as a model system to assess spatial and temporal variability of microbial N-transformations and to elucidate the role of benthic denitrification as a Nr sink. To put the in-lake Nr removal capacity into a context, we also quantified the total Nr inputs delivered to the lake by its tributaries, the Nr export by the lake outflow and the Net Anthropogenic Nitrogen Input (NANI) to the lake watershed. The NANI

approach is widely used to quantify the net anthropogenic Nr load to catchments and to evaluate the
spatial and temporal variability of Nr retention within the watershed (Han & Allan 2008; Lassaletta
et al. 2012; Hong et al. 2012).

115

#### 116 Material and methods

117 Study site

Lake Idro is located on the southern slopes of the Alps at 368 m a.s.l. (Table 1 and Figure 1). 118 119 The watershed has an area of 609 km<sup>2</sup>, an average elevation of 1610 m a.s.l. and is mostly forested 120 (>70%). Only one fourth of the lake catchment area is exploited for agriculture, mainly by pastures 121 and rough grazing (17.4%), while cropland agriculture (7.4%) is located only in the lowland (Figure 1 and Table 1). Population density is 40 ind km<sup>-2</sup>, while livestock units (1 LSU = 1 adult dairy cow) 122 account for 6 LSU km<sup>-2</sup>. Poultry (96000 ind), rabbits (16000 ind) and dairy cows (2300 ind) are the 123 124 main species farmed (ISTAT 2010). Additionally, 7 trout aquaculture activities operate in the 125 catchment with an annual average production of 1700 t. 126 Since 1930s, Lake Idro has been regulated by a top releasing dam and used for water supply to 127 lowland irrigation and hydroelectric power generation. The Chiese and Caffaro rivers contribute ~45% and ~41% of the total water inflow to the lake (2.1 x  $10^6$  m<sup>3</sup> d<sup>-1</sup>), respectively. The lake has a 128 129 total volume of 0.85 km<sup>3</sup>, a maximum depth of 124 m and is considered meromictic (Garibaldi et al. 130 1996). The average residence time of water is about 1 year (Garibaldi et al. 1996). A steep chemocline is present between 40-50 m depth, and the monimolimnion accounts for ~50% of the 131 132 total water volume. The littoral zone (<10 m depth) accounts for less than ~10% of the total lake

133 surface; the shore is gently sloping on the northern and southern sides, and rather steep on the

134 western and eastern sides. The lake is meso-eutrophic with whole lake total P and N concentration 135 averages of 110 and 960  $\mu$ g L<sup>-1</sup>, respectively. In the last 40 years, eutrophication and hypolimnetic 136 anoxia has been exacerbated. Total P concentrations in surficial waters increased from an average 137 of 9  $\mu$ g L<sup>-1</sup> in the early 1970s to 21  $\mu$ g L<sup>-1</sup> today. NO<sub>3</sub><sup>-</sup> concentrations increased steadily from an 138 average of 196  $\mu$ g N L<sup>-1</sup> to 868  $\mu$ g N L<sup>-1</sup> in the same period (S1 Supplementary material).

139

#### 140 Sediment and water features, nutrient fluxes and benthic denitrification

141 Sediment cores were collected on 24 January, 16 May, 8 August and 21 November 2011 at four 142 sites at 3, 6, 20 and 120 m depth (Figure 1). On each occasion, 3 replicate sediment cores (30 cm length and 4 cm internal diameter) were collected at each site to analyze sediment characteristics, 143 144 while 4 replicate sediment cores (30 cm length and 8 cm internal diameter) were collected for denitrification rates and dissolved inorganic nitrogen (DIN =  $NH_4^+ + NO_2^- + NO_3^-$ ) flux 145 146 measurements. In parallel, temperature, oxygen, dissolved sulphides,  $NH_4^+$  and  $NO_x$  ( $NO_2^- + NO_3^-$ ) 147 were measured at each site to provide context for flux measurements. Temperature and oxygen 148 were measured directly *in situ* with a multi parameter probe (Idronaut Ocean 316) at 1 m depth 149 intervals. Water column samples for DIN and dissolved sulphides were collected with a Ruttner 150 bottle at surface, 2.5, 10, 20, 30, 40, 50, 60, 90, and 120 m depths at the site of maximum depth and 50 cm above the sediment at the other sites. The samples were immediately filtered (0.45 µm) and 151 152 stored frozen until analysis. Additionally, 25 L of water were collected at each site for core 153 maintenance and incubation.

154 Immediately after collection all sediment cores were placed in a cool box and returned to the 155 laboratory within 6 hours. Cores collected from the maximum depth station, were immediately 156 submerged in anoxic water retrieved at the same depth and closed with a rubber stopper in order to

157 minimize the exposure to atmospheric oxygen. Once in the lab the sediment cores were transferred to separate tanks, submerged in lake water collected at the corresponding sampling site and 158 159 maintained overnight in the dark in constant temperature rooms at the average temperature 160 measured in the field. Oxic conditions in the tank water collected from the mixolimnion were 161 assured by bubbling air with airstones. Anoxia in the tank water collected from the monimolimnion 162 was assured by covering the incubation tank with a plastic bag and bubbling the water with N<sub>2</sub>. The water inside cores was gently stirred avoiding sediment resuspension during the pre-incubation and 163 164 incubation period with magnetic stirrers driven by a large magnet rotated by an external motor at 40 165 rpm. Measurements began the day after the sampling.

The cores for sediment characterization were processed as follows. The upper 0-5 cm section was sliced and rapidly homogenised; 5 cm<sup>3</sup> of the homogenate were subsampled with a cut-off 10 cm<sup>3</sup> syringe, transferred into pre-weighed aluminium dishes and weighed to determine sediment density. Sediment porosity (cm<sup>3</sup> H<sub>2</sub>O cm<sup>-3</sup> of sediment) was determined as weight loss from wet sediments after 24 hours at 70 °C. Sedimentary organic matter (OM) was determined from 0.5 g dry sediments as loss on ignition at 450 °C over 3 hours.

Fluxes of dissolved inorganic N across the water-sediment interface were quantified via short-172 173 term batch incubations in the dark following the protocol described by Dalsgaard (2000; see S2 174 Supplementary material). The water inside the tanks was replaced with new water from the same 175 site prior to initiating the incubation to maintain near *in situ* dissolved nutrient concentrations. To 176 initiate incubations, the water level in the tank was lowered to below the core tops and the cores 177 sealed with floating Plexiglass lids. At the beginning of the incubation period before closing the cores and at the end, water samples were taken from each core for NH<sub>4</sub><sup>+</sup> and NO<sub>x</sub> analyses. Water 178 179 samples were immediately filtered (0.45 µm) and stored frozen until analysis. The incubation time

(from 1 to 3 hours, depending on season) was chosen based on test incubations for oxygen fluxes, to maintain the mean change of water column oxygen concentration within the 20% of the initial saturation (Dalsgaard et al., 2000). Anoxic cores collected from the monimolimnion were incubated for 5 hours at all sampling dates.

184 In situ denitrification ( $D_T$ ) rates were measured on the same set of cores used for flux 185 measurements following the Isotope Pairing Technique - IPT - (Nielsen 1992). The IPT is based on the addition of  ${}^{15}NO_3$  to the water phase of each core followed by measurements of the produced 186 187 labelled N<sub>2</sub>. After flux measurements the cores were left submerged in the tanks for one hour 188 without lids. To initiate measurements, the water level in the tank was lowered to below the core tops and  ${}^{15}NO_3$  was added to the overlying water of each core at a final concentration of 420 µg L<sup>-</sup> 189 <sup>1</sup>. Before and 5 min after the addition of  ${}^{15}NO_3^{-}$ , water samples were collected from each core to 190 calculate the <sup>15</sup>NO<sub>3</sub><sup>-</sup> enrichment. The overlying water was then allowed to equilibrate with sediment 191 192 pore water for 30 min. After this equilibration time the cores were closed with floating Plexiglass lids to avoid gas exchange with the atmosphere. In May 2011, <sup>15</sup>NO<sub>3</sub><sup>-</sup> was added to the water phase 193 of each core to reach 4 concentration levels (420, 980, 1400 and 2100 µg N L<sup>-1</sup>). Different <sup>15</sup>NO<sub>3</sub><sup>-1</sup> 194 195 additions were used to evaluate the dependency of potential denitrification rates (denitrification of the *in situ*  ${}^{14}NO_3^{-}$  + denitrification of the  ${}^{15}NO_3^{-}$ ) on  $NO_3^{-}$  availability at the 4 different depths. At 196 197 the end of all incubations 5 ml of 7 M ZnCl<sub>2</sub> were added to the water of each core to inhibit further 198 bacterial activity. Just after the addition of ZnCl<sub>2</sub>, the sediment was slurried and sub-samples were carefully collected with a glass syringe equipped with a 10 cm long gas-tight Tygon® tube. The 199 200 samples were immediately transferred into 12 ml glass vials (Exetainer, Labco) ensuring that no 201 bubbles formed during sampling. Overflow of at least three Exetainer volumes was assured before

202	sealing and poisoning with additional ZnCl <sub>2</sub> (200 $\mu L$ 50% w/v) for subsequent analysis of the $^{29}N_2$
203	and $^{30}\text{N}_2$ composition of the dissolved $N_2$ pool. All of these cores were incubated for the same
204	incubation time as for flux measurements.
205	
206	Nitrogen mass balance at watershed scale and Nr loading to the lake
207	The Nr budget of the whole catchment was computed with the Net Anthropogenic Nitrogen
208	Input model (NANI, Howarth et al., 1996):
209	
210	$NANI = N_{Dep} + N_{Fert} + N_{Fix} + N_{Trade}$
211	
212	Where
213	$N_{Dep} = atmospheric Nr deposition$
214	N <sub>Fert</sub> = synthetic Nr fertilizer applied to agricultural soils
215	N <sub>Fix</sub> =agricultural N <sub>2</sub> fixation
216	$N_{Trade}$ = net exchange of Nr as food and feed.
217	NANI was first calculated at the municipal scale, which is the smallest administrative unit at
218	which most of the national statistics are available. To calculate the contribution of each
219	municipality to the Nr budget, municipality-level data were then aggregated at the catchment scale
220	by weighting each municipality based on the spatial distribution of land use areas in the watershed
221	(Han & Allan 2008).
222	$N_{Dep}$ was estimated using wet and dry deposition of both oxidized and reduced Nr. By
223	convention only oxidized Nr deposition is used in the NANI estimate. $NH_3$ is short lived in the
224	atmosphere, and deposition of reduced Nr likely reflects local recycling (Hong et al. 2012).

225 However, NH<sub>3</sub> deposition may be from sources outside the Lake Idro watershed, and thus not locally recycled. This is because of the small size of the watershed and the relatively small apparent 226 227 contribution of agriculture, a major source of local reduced Nr. To account for local recycling, we 228 subtracted NH<sub>3</sub> volatilization from atmospheric deposition, assuming that all the NH<sub>3</sub> volatilization 229 was redeposited locally. Nr deposition measured at three gauging stations of the CONECOFOR network in the investigated area averages  $750\pm130$  and  $875\pm140$  mg N m<sup>-2</sup> v<sup>-1</sup> for oxidized and 230 reduced Nr respectively (Rogora et al. 2006). These values are in agreement with those estimated 231 232 with the GAINS-Italy model at a 20x20 km resolution (De Marco personal communication). 233 However, they are lower than estimates extrapolated from the 50×50 km resolution grid of the Co-234 operative Programme for Monitoring and Evaluation of the Long-range Transmission of Air Pollutants in Europe (EMEP 2010). The latter are equal to 950 and 1900 mg N m<sup>-2</sup> v<sup>-1</sup> for oxidized 235 and reduced Nr, respectively. We therefore considered an average value of  $N_{Dep} = 1900 \pm 760 \text{ mg N}$ 236  $m^{-2} y^{-1}$ , we assumed constant over the catchment, and multiplied this estimate by the watershed 237 238 area. Ammonia volatilization was estimated as the percentage loss of the supplied Nr using 239 published emission values. Nr potentially lost via volatilization in the Lake Idro watershed is 240 primarily related to cattle and chicken manure (30%) and urea fertilizers (15%) (Bussink and 241 Oenema 1998; Misselbrook et al. 2004).

Fertilizer Nr application, agricultural Nr fixation and net Nr food and feed imports were calculated using agricultural and demographic census data from the Italian National Institute of Statistics, year 2010 (ISTAT 2010). Fertilizer Nr application was estimated using available data on fertilizer sales and Nr content for each fertilizer type (ISTAT 2010). The fertilizer sales data were available at the province level divided by form: urea,  $NH_4^+$ ,  $NO_3^-$ , Nr solutions and miscellaneous forms. We assumed that fertilizers were applied in the same province in which they were sold.

However, because the Lake Idro watershed lays within provinces that include intensive agricultural 248 lowland areas outside the investigated catchment, a simple downscaling based on the proportion of 249 250 watershed area to province area could overestimate the fertilizer input. Therefore, we calculated an average province-level fertilizer application rate by dividing fertilizer sales by the potentially 251 252 fertilized land area in the province. We then multiplied this average value for the potentially 253 fertilized land area in each municipality included in the watershed. The potentially fertilized area 254 was estimated as the sum of temporary and permanent agricultural crops; excluding N-fixing crops, 255 permanent meadows and pastures.

The amount of fixed N<sub>2</sub> associated with pastures, rough grazing and permanent meadows which collectively represent more than the 95% of the N-fixing crops in the watershed - was estimated by multiplying the area of the specific N-fixing crop by the areal N-fixation coefficients of each crop type. N-fixation coefficients were estimated as the product of yield and Nr content corrected for the ratio of total biomass produced to harvested biomass (Lassaletta et al. 2012). Average values were 2400 (pastures), 800 (rough grazing) and 11800 (meadows) kg N km<sup>-2</sup> y<sup>-1</sup>, comparable to literature values (Hong et al. 2012).

263 The net Nr imports with food and feed trade were calculated as the difference between Nr production, i.e. sum of crop and livestock Nr production in the catchment, and Nr consumption, 264 calculated as the sum of humans and livestock Nr intake. Human Nr consumption was estimated by 265 266 multiplying human population by Nr consumption per capita (6.8 kg N capita<sup>-1</sup> y<sup>-1</sup>). The latter was calculated assuming a protein consumption of 111 g capita<sup>-1</sup> d<sup>-1</sup> (FAOSTAT 2010), with protein = 267 268 N x 6.25 (Jones, 1941). The inhabitant number in each municipality was obtained from the 2010 269 Demographic Census (ISTAT 2010). This number was integrated with data on tourist presence 270 (Regional Agency for Environmental Protection).

Livestock Nr consumption and excretion were calculated from livestock numbers, multiplied by the intake and excretion parameters for each livestock type. Per animal Nr intake and excretion parameters of the different livestock classes were obtained from the regional plans for water protection (Lombardy Region 2003). When data were not available, we referred to published values (S3 supplementary material). Livestock Nr production was calculated as the difference between animal Nr intake and excretion (Hong et al. 2012).

The amount of Nr produced by crops was calculated by multiplying the average harvested yield of each crop (ISTAT 2010) by the average Nr content of each harvested crop type (Lombardy Region 2003). Harvested Nr with crops was distributed between humans and livestock, corrected for fractions lost during food and feed production according to the FAO Food Balance Sheet of Italy (FAOSTAT 2010). When data were not available, we referred to published values (Hong et al. 2012). The coefficients used are summarized in the S4 supplementary material.

283 This estimate of the NANI is based on all the best available information. The accuracy could be 284 limited by the quality of the input data, the accuracy of the coefficients used for converting input data 285 into Nr units and the validity of the assumptions made. The uncertainty associated to the NANI calculation was incorporated into the budget output by performing a Monte Carlo simulation (Han & 286 287 Allan 2012), which utilized random sampling for all coefficients, atmospheric deposition and 288 fertilizer data. Census data of livestock numbers, human population and the area of each crop type 289 collected from National Statistics were considered well constrained and kept fixed. All the other terms 290 were allowed to vary stochastically and independently around the mean value and were assumed to 291 follow a normal distribution. A total of 1000 models were run as mean and standard deviation settled 292 to constant values after 500 iterations.

293 Standard deviation for N<sub>Dep</sub> was directly obtained from the variability of data collected for the 294 Lake Idro watershed (coefficient of variation = 40%). Standard deviations for crop yield, Nr content, 295 and livestock Nr excretion were estimated from the average coefficient of variation reported in Soana 296 et al. (2011) which equal to 26%, 25%, and 14%, respectively (S5 supplementary material). Standard 297 deviations for animal Nr intake were estimated assuming a coefficient of variation equal to Nr 298 excretion. Standard deviations for the parameters for which information about variability were not 299 available (crop fraction distributed to humans, Nr fraction lost in food and feed processing, human 300 Nr intake and fertilizer Nr application), were estimated using an intermediate coefficient of variation 301 (20%) between 14 and 26%, the range of uncertainty of the agricultural coefficients (S5 302 supplementary material).

303

#### 304 *Riverine Nr flux estimations*

305 Chemical characteristics of inflowing and outflowing waters were measured monthly from June 306 2010 to March 2012. At each sampling date water samples were collected for NH<sub>4</sub><sup>+</sup>, NO<sub>x</sub>, total 307 dissolved (TDN) and total (TN) nitrogen determinations. Samplings and analyses were performed 308 following standard methods (APHA 1998). The daily Nr loadings to and exports from the lake were 309 computed as the product of average daily flow by concentration of the target chemical species. 310 Annual loadings were calculated as the product of the discharge weighted mean concentration by 311 the mean annual discharge of the two years (Quilbè et al. 2006). The Chiese Consortium, the 312 Management Authority of lake Idro, and the Autonomous Province of Trento provided the daily 313 average water inflows and outflows. Discharge of minor tributaries was calculated from direct 314 measurements of water velocity, with a water velocity meter (Scubla mod. 2030), and cross 315 sectional area in each sampling day. Total Nr input to the lake was computed as the sum of the river 14 316 Nr loadings plus atmospheric Nr deposition (wet and dry deposition of both oxidized and reduced317 Nr) to the lake surface.

318

#### 319 Analytical determinations and calculations

320  $NH_4^+$  (Bower & Holm Hansen 1980) and  $NO_x$  (APHA 1998) were determined by

321 spectrophotometry. TN and TDN were determined as NO<sub>x</sub> after alkaline peroxydisulfate oxidation

322 (Valderrama 1981).

The abundances of  ${}^{29}N_2$  and  ${}^{30}N_2$  in the dissolved N<sub>2</sub> pool were determined with a gas 323 324 chromatograph in line with a mass spectrometer at the National Environmental Research Agency, Silkeborg (DK) following Risgaard-Petersen & Rysgaard (1995). In brief the N2 of each sample 325 326 was extracted from the water in the Exetainer by introducing a helium headspace and shaking 327 vigorously the vial for 5 min. The entire headspace (4 ml) was then carried through the gas 328 chromatograph columns (Roboprep-G-Plus GC) and to a triple-collector mass spectrometer (Europa 329 Scientifica TracerMass) to obtain the isotopic composition of the N<sub>2</sub> by a flow of helium (99.9995% 330 purity). Prior to reaching the mass spectrometer, water vapour, CO<sub>2</sub> and O<sub>2</sub> were removed by 331 passing the sample through a drying tube (10 mm x 200 mm packed with Mg(ClO<sub>4</sub>)<sub>2</sub>), a Carbosorb 332 (10-20 mesh) column and a reduction column (15 mm x 300 mm packed with Cu wires at 650 °C) respectively. The increased abundance of  ${}^{29}N_2$  and  ${}^{30}N_2$  in the samples was obtained by subtracting 333 334 the natural  ${}^{29}N_2$  and  ${}^{30}N_2$  abundance from the signals. Fluxes of NH<sub>4</sub><sup>+</sup> and NO<sub>x</sub> across the sediment-water interface were calculated from the change 335 336 of solutes concentration over time (Nizzoli et al., 2014) using the following formula:

337

338 Fx = ((Cf - Ci) \* V) / A\* t

- 340 Fx = flux of the x species (mg m<sup>-2</sup> h<sup>-1</sup>)
- 341 Cf = final concentration of x (mg  $L^{-1}$ )
- 342 Ci = initial concentration of x (mg  $L^{-1}$ )
- 343 V = volume of the water column (L)
- 344 t = incubation time (hours)
- 345 A= sediment surface area inside the core  $(m^2)$
- 346 Daily rates were calculated by multiplying hourly rates by 24.
- $D_T$ , direct denitrification of <sup>14</sup>NO<sub>3</sub><sup>-</sup> diffusing to the sediment from the water column (D<sub>W</sub>) and
- 348 denitrification of  ${}^{14}NO_{3}$  produced by nitrification within the sediment (D<sub>N</sub>) were calculated
- following Nielsen et al. (1992) as follows:
- $350 D_{15} = p29 + 2p30$
- 351  $D_T = D_{15} \times (p29/2p30)$
- 352  $D_W = ({}^{14}NO_3^- / {}^{15}NO_3^-) * D_{15}$
- $353 D_N = D_{14} D_W$
- 354 where  $D_{15}$  represents denitrification of the added <sup>15</sup>NO<sub>3</sub><sup>-</sup>, <sup>14</sup>NO<sub>3</sub><sup>-</sup> the ambient unlabelled NO<sub>3</sub><sup>-</sup>
- 355 concentration and  ${}^{15}NO_3^-$  the isotopically-labelled  $NO_3^-$  concentration at the start of the incubation,
- 356 p29 and p30 represent the production rates of  ${}^{29}N_2$  and  ${}^{30}N_2$ , respectively. The presence of
- 357 anammox interferes with IPT calculations resulting in an overestimation of  $D_T$  because the  $N_2$
- 358 produced by anammox cannot be discriminated from the N<sub>2</sub> produced by denitrification. Therefore,
- 359 independence of  $D_T$  from added <sup>15</sup>NO<sub>3</sub><sup>-</sup> was checked to validate IPT and exclude significant
- 360 overestimation due to anammox (Risgaard-Petersen et al. 2003).

Benthic denitrification efficiency (DE) was calculated as the percentage of denitrification to
 ammonification according to Eyre & Ferguson (2009):

 $363 \quad DE = D_T / (DIN + D_T) * 100$ 

364 The mass transfer coefficient (m  $y^{-1}$ ) was calculated as the ratio of D<sub>w</sub> to the ambient NO<sub>x</sub> 365 concentration (David et al. 2006).

The contribution of sediments at different depths to the whole lake denitrification was 366 calculated according to David et al. (2006). The lake sediment was first partitioned into three 367 368 bathymetric layers on the basis of similarities in the features of the water overlying the sediment 369 and the sediment itself. Then, annual D<sub>T</sub> rates were calculated in each of the three layers with a 370 linear integration of the measured daily rates over the sampling year multiplied by the 371 corresponding sediment surface area. Finally, the areal D<sub>T</sub> values were summed to estimate the 372 whole lake denitrification rate. This calculation assumes that the change of denitrification rates between two subsequent sampling periods was linear and that denitrification rates were 373 374 homogeneous within each of the three layers.

375 Nr retention within the whole basin (terrestrial + lake) was calculated as the difference between

NANI and Nr export from the lake outlet. Nr retention within the terrestrial part of the watershed was estimated as the difference between NANI and Nr loading exported by rivers and streams to the lake. The lake Nr retention was estimated as the difference between the Nr loading to the lake and the Nr quantity exported through the lake outlet. Nr retention efficiency (%) relative to Nr input was calculated as 100\*(Nr inputs – Nr outputs)/ Nr inputs.

381 Data were analysed with nonparametric tests because of violations of the assumptions of
 382 normality and homogeneity of variance even after data transformation. The non-parametric
 383 Kruskall Wallis H test was used to test differences of denitrification rates and Nr fluxes among

depths  $(n_{120}=n_{20}=n_6=n_3=16)$  and among sampling dates within each depth

 $(n_{spring}=n_{summer}=n_{autumn}=n_{winter}=4)$ . When differences among depth or among seasons were

386 significant, *a posteriori* comparison of the means was performed using a post hoc Tukey and

387 Kremer test (Quinn & Keough 2002). Differences between daily Nr loads measured at lake inlet and

388 outlet were compared using the non-parametric Mann-Whitney U test. All statistical analyses were

389 performed using the statistical computing software R (R Core Team, 2014). Descriptive statistics

390 are presented within the text as mean  $\pm$  standard deviation.

391

#### 392 **Results**

#### 393 Water and sediment characteristics

394 Temperature, dissolved oxygen and Nr underwent steep variations along the water column 395 (Figure 2 and Table 2). Water temperatures displayed a typical seasonal trend in the shallower sites, changing from 5 °C in winter to 23 °C in summer, and was nearly constant in the deepest waters. 396 Dissolved oxygen concentrations decreased with depth, ranging from 8.6 to 14.1 mg  $L^{-1}$  in the surface 397 398 layer to anoxia below 40-50 m depth. NOx concentrations followed a similar pattern, decreasing from 868  $\mu$ g N L<sup>-1</sup> in the surface to <100  $\mu$ g N L<sup>-1</sup> from 40 m to the bottom. NH<sub>4</sub><sup>+</sup> concentrations followed 399 an opposite trend, increasing from  $<20 \ \mu g \ N \ L^{-1}$  in the mixolimnion up to  $\sim 1000 \ \mu g \ N \ L^{-1}$  in the 400 deepest layers. A concurrent accumulation of dissolved sulphides up to 4.3 mg S L<sup>-1</sup> also occurred 401 402 from 60 m to the bottom.

The sediment collected at 3 and 6 m depths was composed of fine sands with a high silt content. The average OM content (5.4±1.6 %) in the upper sediment horizon was similar for the two sites. By contrast, sediment collected at 20 and 120 m depth was composed of fine textured mud. At 120 m depth the sediment appeared dark black up to the sediment-water interface, with a strong

407 sulphide smell, whereas at 20 m depth a thick layer of yellowish oxidised sediment was visible at 408 the sediment-water interface at all the sampling dates. The average OM content  $(9.5\pm1.7 \%)$  in the 409 upper sediment horizon was higher at 20 m depth compared to the deepest layer  $(7.2\pm1.4\%)$ .

410

411 *Benthic Nr fluxes and denitrification rates* 

412 Differences between depths 3 and 6 m were not statistically different (K-W, p>0.05) for

413 benthic fluxes of  $NH_4^+$ ,  $NO_x$  or  $D_T$  rates. Therefore, in the following sections we will refer to these

414 observations collectively as littoral sites (0-10 m bathymetric layer).

415 D<sub>T</sub> rates were significantly different among all sites (K-W, p<0.001; Figure 3a). Average D<sub>T</sub>

416 rates decreased with depth, being from 2.3 to 24 times greater in the littoral sediment (average

417  $87\pm85 \text{ mg m}^{-2} \text{ d}^{-1}$ ) than in the deeper layers. D<sub>T</sub> was especially low to undetectable in the 120 m

418 depth sediment  $(3.5\pm2.9 \text{ mg m}^{-2} \text{ d}^{-1})$ . In the 0-10 m layers, benthic D<sub>T</sub> underwent a clear seasonal

419 pattern, with spring and summer rates 6 times higher than those measured in autumn and winter (K420 W, p<0.001).</li>

421 Nitrification was only a minor source of  $NO_x$  for denitrification and  $D_N$  rates were not 422 statistically different among the four sites (Figure 3a).  $D_W$  accounted on average for more than 90% 423 of  $D_T$  and drove the seasonal patterns and spatial distribution of  $D_T$ . We also demonstrated that 424 denitrification rates were stimulated by increasing levels of  $NO_x$  at all the depths, although the 425 degree of stimulation was one order of magnitude lower in sediments from 20 m to the bottom than 426 in littoral sediments (Figure 4).

427 Overall, the annual denitrification in the benthic system accounted for a N<sub>2</sub> production of 428  $83\pm10$  t N y<sup>-1</sup> (7.2\pm0.4 t N km<sup>-2</sup> y<sup>-1</sup>). An annual rate of 42±10 t N y<sup>-1</sup> (69±8.7 t N km<sup>-2</sup> y<sup>-1</sup>) occurred

429 in the littoral area, while only  $11\pm 2$  t N y<sup>-1</sup> ( $1.3\pm 0.1$  t km<sup>-2</sup> y<sup>-1</sup>) were produced in the 430 monimolimnion sediments (Table 3).

Concurrently, high rates of NO<sub>x</sub> consumption were measured in the littoral sediment (-431  $75.6\pm77.0 \text{ mg m}^{-2} \text{ d}^{-1}$ ) particularly under spring and summer conditions. Much lower NO<sub>x</sub> fluxes 432 were detected in the deeper sediments (-7.0 $\pm$ 2.8 mg m<sup>-2</sup> d<sup>-1</sup>; Figure 3b). On average, the benthic 433 system was a source of NH<sub>4</sub><sup>+</sup> to the water column, with significant differences among depths (K-W, 434 p < 0.01; Figure 3c). A clear seasonal pattern was observed in the littoral zone where NH<sub>4</sub><sup>+</sup> release 435 from the sediment peaked in spring and summer ( $101.1\pm67.2 \text{ mg m}^{-2} \text{ d}^{-1}$ ). At 120 m depth NH<sub>4</sub><sup>+</sup> 436 release was always high (99.4 $\pm$ 58.1 mg m<sup>-2</sup> d<sup>-1</sup>) with lowest efflux measured in autumn (42.9 $\pm$ 4.2 437 mg m<sup>-2</sup> d<sup>-1</sup>) and highest in winter (173.8±23.8 mg m<sup>-2</sup> d<sup>-1</sup>). NH<sub>4</sub><sup>+</sup> fluxes were almost undetectable at 438 20 m depth. 439

In the littoral benthic system, the net DIN flux was on average slightly into the sediments (-16.8 $\pm$ 48.0 mg m<sup>-2</sup> d<sup>-1</sup>) due to the high NO<sub>x</sub> consumption, while the monimolimnion sediment was always a net source of DIN (14-224 mg m<sup>-2</sup> d<sup>-1</sup>), mainly due to NH<sub>4</sub><sup>+</sup> recycling. Denitrification efficiency was elevated in sediments down to a depth of 50 m depth, while Nr recycling dominated the monimolimnion sediments (Table 3).

445

#### 446 *Nitrogen fluxes at watershed scale*

The estimated NANI was  $1785\pm467$  t N y<sup>-1</sup>, corresponding to an areal input of  $2929\pm767$  kg N km<sup>-2</sup> y<sup>-1</sup> (Figure 5). Atmospheric Nr deposition accounted for 61% of NANI, equivalent to  $1781\pm763$  kg N km<sup>-2</sup> y<sup>-1</sup> while the Nr input associated with trade of food and feed was 20% of NANI, equivalent to  $574\pm80$  kg N km<sup>-2</sup> y<sup>-1</sup>. The autotrophic organic Nr production within the catchment ( $484\pm85$  kg N km<sup>-2</sup> y<sup>-1</sup>) was not sufficient to meet the Nr needs of the human and

452 livestock population (1057±67 kg N km<sup>-2</sup> y<sup>-1</sup>); organic Nr was thus imported to the watershed 453 mainly as animal feed (606±78 kg N km<sup>-2</sup> y<sup>-1</sup>). N<sub>2</sub> fixation associated with crops and pastures 454 (524±112 kg N km<sup>-2</sup> y<sup>-1</sup>) accounted for an additional 18% Nr input, while fertilizers (51±7 kg N km<sup>-2</sup> 455  $^{2}$  y<sup>-1</sup>) were < 2% of NANI.

456 TN concentrations measured in the water at the outlet of the main rivers and small streams that 457 drain into the lake showed large variability, among both sampling dates and sites (499-3469 µg N L<sup>-</sup> <sup>1</sup>). The TN pool consisted mainly of  $NO_x$  (up to the 80%), with the highest concentrations generally 458 459 detected in minor creeks (data not shown). The average daily TN load was variable, ranging from a minimum of 860 kg N d<sup>-1</sup> to a maximum of 6021 kg N d<sup>-1</sup> (Figure 6). More than 90% of the total Nr 460 load was delivered by the Caffaro and Chiese rivers, mainly as dissolved Nr forms (> 98%). The 461 annual Nr export by rivers to the lake was 846±586 t N y<sup>-1</sup>, which is equivalent to 47% of NANI. 462 TN concentration measured in river water at the lake outlet (485-1020 µg N L<sup>-1</sup>) was lower 463 464 than in the inflowing waters (Figure 6). The daily Nr exported from the lake ranged from 622 kg N d<sup>-1</sup> to 4065 kg N d<sup>-1</sup> and was significantly lower than the daily input (Wilcoxon, p=0.013). The 465 annual Nr export from the lake was  $598\pm395$  t y<sup>-1</sup>, equivalent to nearly 70% of the annual inputs. 466 Therefore, the lake either retained or lost nearly 30% of the Nr load (270±706 t N y<sup>-1</sup>) prior to 467 riverine export. 468

469

#### 470 **Discussion**

471 Measurements of benthic Nr fluxes and denitrification rates along with Nr mass balances were 472 combined to evaluate the contribution of benthic denitrification to Nr removal under meromictic 473 conditions. These are understudied but important issues in inland waters, as they can provide new 474 insight on the capacity of lake ecosystems to dissipate the Nr load under infrequent to no mixing

475 frequencies. A synopsis of the budgets and fluxes resulting from the study highlights that in-lake
476 denitrification was not efficient in removing Nr in most of the lake's sediment. Further, the
477 watershed had a low Nr retention capacity resulting in a high percentage of Nr (47%) exported to
478 the lake system from tributary rivers (Figure 7).

479

## 480 Spatial and temporal variability of benthic Nr processes

Different benthic nitrogen processes dominated in deep and littoral sediments of Lake Idro.
 Nearshore sediments had higher denitrification rates and denitrification efficiency, compared to the
 monimolimnion sediments where denitrification was almost negligible. Recycling of NH<sub>4</sub><sup>+</sup> from
 sediments to the water column dominated in the monimolimnion.

485 Spatial heterogeneity of denitrification rates was previously observed in dimictic lakes with rates generally higher in the littoral than in the deeper sediments (Saunders & Kalff 2001; 486 487 Bruesewitz et al. 2012), but with exceptions due to local conditions (Rissanen et al. 2013; Small et al. 2014). The average  $D_T$  of 2.8 mg N m<sup>-2</sup> d<sup>-1</sup> measured in the meromictic Lake Idro falls within the 488 lower range of values (1.9 to 84 mg m<sup>-2</sup> d<sup>-1</sup>) detected in other deep sediments (Table 4) suggesting a 489 490 reduced capacity to dissipate Nr. Water column NO<sub>x</sub>, as opposed to nitrification, was by far the 491 dominant source of NO<sub>x</sub> for denitrification. The incubations of sediment cores with increasing NO<sub>3</sub><sup>-</sup> 492 concentrations revealed that in all the investigated sediments denitrification was NO<sub>3</sub><sup>-</sup> limited. Low 493 NO<sub>x</sub> concentrations in the water column can prevent denitrification by limiting the diffusion of NO<sub>x</sub> 494 into the sediment denitrification zone. Furthermore, anoxia precludes nitrification and NO<sub>x</sub> 495 production within the sediment. The comparison of denitrification in littoral and deep sediments 496 also evidenced that NO<sub>x</sub> addition was less effective in the latter sediments (Figure 4). We argue that 497 anoxia and chemically reducing conditions in the monimolimnion can further inhibit denitrification

498 processes. The mass transfer coefficient, which can be regarded as an index of the efficiency of N<sub>2</sub> 499 production relative to NO<sub>x</sub> availability (David et al. 2006), was lower in sediments collected from 500 the monimolimnion compared to the other depths (Table 3). Lower values suggest that environmental conditions in the monimolimnion were less suitable to denitrification compared to 501 502 littoral oxygenated sediments. Denitrification efficiency was less than 5% and anaerobic 503 ammonification was the dominant process of sediment Nr release under these conditions (Figure 8). In contrast, D<sub>T</sub> rates of 87 mg N m<sup>-2</sup> d<sup>-1</sup> measured in the littoral habitat were within the upper 504 range of values (0 to 238 mg N m<sup>-2</sup> d<sup>-1</sup>) measured in other lake ecosystems (Table 4). Here, benthic 505 506 Nr metabolism followed a typical seasonal pattern with peaks of D<sub>T</sub> rates and DIN fluxes in spring 507 and summer. Such high rates likely reflected temperature changes and the consistently high NO<sub>x</sub> 508 availability in the water column throughout the year; low D<sub>N</sub> indicated that sediment nitrification 509 was a minor source of NO<sub>x</sub>. Mineralized Nr was released to the water column as NH<sub>4</sub><sup>+</sup> in littoral 510 sediment due to low nitrification. However, in contrast to the deeper sediments, DIN fluxes across 511 the sediment water interface in the littoral zone were largely driven by microbial NO<sub>x</sub> reduction. 512 Here, denitrification of the NO<sub>x</sub> bulk diffusing from the water column exceeded the NH<sub>4</sub><sup>+</sup> efflux, 513 and the superficial sediment acted as a net DIN sink (Figure 8).

514

#### 515 Benthic denitrification and in lake Nr processing

We scaled up denitrification rates to the whole lake to provide context for the contribution of benthic denitrification in the different bathymetric layers to the in lake Nr metabolism (Table 3). To our knowledge, there are only few examples of the concurrent use of mass balances and direct measurements of in-lake processes to evaluate pathways and fate of the Nr loadings from the watershed (Mengis et al., 1997; David et al. 2006; Bruesewitz et al. 2011; McCarthy et al. 2016). 521 Upscaling from core measurements to the whole lake requires addressing both methodological 522 problems and spatial and temporal variability of rates (Groffman et al. 2006). We addressed both 523 kinds of uncertainty. First, we used a well-established technique for measuring denitrification 524 (Groffman et al. 2006). Second, we sampled sediments in three bathymetric layers, from the 525 shallow littoral zone to the deepest monimolimnion, over four seasons to incorporate both the 526 spatial and temporal variability of fluxes and rates (David et al. 2006; Bruesewitz et al. 2012; 527 Nizzoli et al., 2014).

528 The contribution of the three different bathymetric zones to the whole benthic N<sub>2</sub> production 529 was not proportional to the respective sediment area, contrary to what was previously observed in 530 Lake Shelbyville, a low land polimictic reservoir (David et al. 2006) or in the dimictic Gull Lake 531 (Bruesewitz et al. 2012). In Lake Idro 50% of benthic denitrification occurred in the littoral area. 532 This area accounted for only 10% of the lake surface; whereas the monimolimnion sediments, 533 which extended over 70% of the lake surface, contributed only 13% of D<sub>T</sub> (Table 3). 534 These data demonstrate that shallow aquatic environments characterized by high NO<sub>x</sub> loads can 535 sustain high denitrification rates, and thereby the littoral area has an enormous impact relative to its 536 surface in the control of the external NO<sub>x</sub> loads. The disproportionate contribution of the littoral 537 area can be found not only in meromictic lakes but also in eutrophic temperate lakes where water 538 stratification is followed by oxygen and  $NO_x$  depletion (Bruesewitz et al. 2011). Under these 539 conditions, the deep benthic system progressively loses the capacity to denitrify (Nizzoli et al. 2010; 540 Bruesewitz et al. 2011), which is preserved instead in the more oxidized littoral sediments (Nizzoli 541 et al. 2014). Yet several anthropic pressures affect littoral zones of lakes (Francis et al. 2007). 542 Among others, human exploitation of water resources and extreme hydrological events affect the

magnitude and timing of water-level fluctuations, which in turn may influence benthic metabolism(Hofmann et al. 2008) with possible implications for Nr biogeochemistry.

The in-lake  $D_T (83 \pm 5 \text{ t y}^{-1})$  accounted for only 30% of the Nr retained by the lake, much lower than values of 62-100% found in the alpine meromictic Lake Zug, the dimictic eutrophic lake Baldegg and a lowland reservoir (Mengis et al. 1997; David et al. 2006). This discrepancy could be due to the mass balance and acetylene inhibition methods with sediment slurries, which are known to overestimate the *in situ* denitrification rates (Mengis et al. 1997; Groffman et al. 2006). Furthermore,  $D_T$  accounted for the removal of only 10% of the Nr load to the lake providing a

551 missing quota of 165 t  $y^{-1}$ .

552 Nr retention is the combined result of Norg storage in sediment, living biomass, DIN 553 accumulation in the water column and unmeasured N<sub>2</sub> production. In this work we incubated 554 sediment cores under dark conditions. This experimental set up is appropriate for the deeper zones 555 where light does not reach the sediment surface. By contrast light can penetrate to shallow littoral 556 sediments, and part of the Nr is assimilated by benthic primary producers (Nizzoli et al., 2014). 557 Therefore, our approach could have underestimated Nr retention in the littoral zone, especially in 558 the southern part of the lake, colonized by dense submerged meadows of Lagarosiphon maior (Bolpagni 2013). Assuming assimilation rates of 0.28-1.26 mg N g d<sup>-1</sup> (Nizzoli et al. 2014 and 559 references therein) and an average dry biomass of 250 g m<sup>-2</sup> (Longhi upublished data) Nr 560 561 assimilation would be in the range of 9-38 t N v<sup>-1</sup>. This rate is comparable to littoral denitrification. 562 In meromictic lakes, persistent water column stratification prevents the upward flux of 563 accumulated nutrients from the monimolimnion. Indeed, in Lake Idro, the monimolimnion is storing  $\sim 300$  t of NH<sub>4</sub><sup>+</sup>. However, the long-term fate of this Nr is unclear. Partial turnover of the 564 565 upper monimolimnion can potentially occur due to heavy storms or very cold winters. Such events 25 566 occurred in the nearby meromictic lakes Lugano and Iseo (Salmaso et al. 2014; Lehmann et al.

567 2015). Under these circumstances, the accumulated  $NH_4^+$  can migrate upwards into the mixed layer 568 undergoing oxidation to  $NO_x$  with denitrification to  $N_2$  (Lehmann et al. 2015).

569 The water-sediment interface is commonly considered the most reactive zone of aquatic 570 ecosystems, where biogeochemical processes are amplified. However, recent studies suggest that in 571 meromictic lakes the chemocline could also host intense Nr transformations due to the sharp redox 572 transition (Schubert et al. 2006; Hamersley et al. 2009). For example, in Lake Lugano, in summer 573 ~60% of total denitrification was measured in the water column (Wenk et al. 2014). During this 574 study, the chemocline of Lake Idro occurred from approximately 40 to 50 m depth and accounted for about 0.15 km<sup>3</sup>. Therefore, even low denitrification rates in this bathymetric layer could greatly 575 contribute to the Nr budget. This needs future investigation given the inability to balance lake 576 577 imports with exports and denitrification estimates.

578

### 579 The lake as a metabolic regulator of the NANI from the watershed

580 The Lake Idro watershed is characterized by low anthropogenic pressures, resulting in a NANI far below the average values found for Europe (4000 kg km<sup>-2</sup> y<sup>-1</sup>) and worldwide (Boyer et al. 2002; 581 Hong et al. 2012; Gao et al. 2014). Nutrient budgeting is affected by uncertainties due to difficulties 582 583 in estimating the different budget terms and the assumptions made in model formulation (Oenema 584 et al. 2003; Soana et al. 2011; Hong et al., 2013). Indeed, the NANI of Lake Idro watershed has a 585 relatively high coefficient of variation (CV = 26% of the mean). This CV falls in the upper range of 586 those previously calculated in other watersheds (4 - 20%) with a comparable approach (Yan et al., 2011; Han & Allan 2012; Chen et al. 2016). The variability of the different NANI terms and 587 588 coefficients can explain this high CV. For example, An et al. (2012) used a uniform 5% coefficient 26 of variation. In contrast we applied, when possible, a specific range for each coefficient, from 14 to

590 40% of the mean. In the Lake Idro basin, as in most catchments with relatively low population

591 density and human activities, atmospheric deposition is the largest NANI component. The

592 variability of NANI is thus due to the large uncertainty associated to this term.

593 We compared NANI and the Nr flux from the watershed throughout the lake inlet. The lake

594 catchment exhibited a low Nr retention efficiency and an average of 47% of the NANI was not

retained by the watershed system (Figure 7). Previous studies demonstrated that the Nr flux across

596 watersheds is within 20-25% of the NANI, although large differences have been observed among

597 catchments ranging from < 10% to > 50% (Howarth et al. 2012).

The presence of the lake enhances the Nr retention capacity of the whole watershed. Our inputoutput mass balance indicates that 53% of the NANI is retained by the terrestrial part of the watershed, while the lake retains 13% (Figure 7). This is a high percentage relative to the lake surface which is only 2% of the total watershed area, further confirming that the in-lake biogeochemical processes are quantitatively relevant (Seitzinger et al. 2006; Harrison et al. 2009; Lassaletta et al. 2012). However, the Nr removal efficiency of Lake Idro appears low, when compared to other aquatic ecosystems (Figure 9).

Nr removal efficiency of lakes is extremely variable and influenced by latitude, water residence time and trophic status (Seitzinger et al. 2006; Rissanen et al. 2013; Finlay et al. 2013). Water residence time in particular is widely used as an explanatory factor. It represents the time scale for processes that influence Nr removal (Nr uptake, settling of particulate Nr and diffusion of NO<sub>x</sub> to anoxic denitrification zones) to occur before flushing downstream (Seitzinger et al. 2006). Lake Idro retained 31% of the external Nr load with an efficiency falling in the lower range of values measured in other temperate lakes with similar water residence time or predicted by means of

612 empirical equations from literature. The latter estimates are much greater and are in the range 40-60% (Figure 9). This low retention efficiency can be explained by the fact that 70% of the benthic 613 614 system in Lake Idro is affected by anoxia. NO<sub>x</sub> depletion and extremely chemically reducing 615 conditions favor Nr recycling rather than denitrification. We added increasing amounts of  $NO_x$  to 616 monimolimnic waters above sediments to evaluate denitrification capacity following  $NO_x$  supply due to water overturn. At NO<sub>x</sub> concentrations between 700-840  $\mu$ g N L<sup>-1</sup>, equivalent to those 617 measured at 30 m depth, the theoretical denitrification rates fell to the range of 16.8-21.0 mg m<sup>-2</sup> d<sup>-1</sup> 618 619 corresponding to a Nr dissipation from five to six times higher than that measured *in situ* in this 620 study. Therefore, we can argue that in case of complete overturn, Nr retention efficiency would 621 increase up to ~35% of the external Nr load.

In the temperate zone, anoxia and chemically reducing conditions can develop not only in 622 623 meromictic lakes, but also in the hypolimnion of dimictic eutrophic lakes (Matthews et al., 2008; 624 Nizzoli et al. 2010; Foley et al., 2012). Our results suggest that when the complete turnover becomes less frequent, the denitrification efficiency decreases, especially during the summer 625 626 stratification period. Eutrophication can accelerate this trend, through organic enrichment and 627 oxygen consumption rates. These impacts are likely to become more severe in the future exacerbated by the concurrence of eutrophication and climate changes (Foley et al., 2012; Ficker et 628 629 al. 2017). Numerical thermal lake models (Danis et al. 2004) and direct observations (Salmaso et al. 630 2014; Ficker et al. 2017) suggest that complete turnover could become less frequent with the 631 occurrence of holo-oligomixis and in extreme conditions meromixis. The increased stability of 632 water stratification has been ascribed to altered thermal conditions which are the result of different climate related drivers. Namely the differential increase of water temperature is faster in the surface 633 634 than deep layers, especially in deep lakes (Rempfer et al. 2010; Kraemer et al., 2015; Ficker et al.

2017). Climate change can also cause salinization of many lakes in Mediterranean climate zones
(Jeppesen et al. 2015) and hyper-eutrophication of eutrophic lakes with the accumulation and
settling of the ungrazed biomass (Moss et al. 2011). Organic matter enrichment coupled with less
frequent mixing can induce a positive feedback, since mineralization increases dissolved ions
concentration and consequently water density. These processes can amplify the density differences
with epilimnic waters, enhancing the stratification stability.

In the short term, changes in water circulation along with organic enrichment stimulates denitrification by inducing redox gradients at the water-sediment interface and fueling denitrifiers with an electron source (Seitzinger et al. 2006; Finlay et al. 2013). However, prolonged water stratification could represent a threat for the Nr removal capacity of lakes and their associated ecosystem services. Therefore, factors that impair water mixing and promote the onset of meromictic conditions would indirectly jeopardize the capacity of lake sediments to denitrify and thereby increase Nr delivery to downstream ecosystems.

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649

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- 659

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- 845

847	Figure captions
848	
849	Figure 1 Location of Lake Idro and land use in its watershed (A). Detailed map of the lake showing
850	the sampling sites for sediment and riverine total nitrogen flux estimation (B)
851	
852	Figure 2 Water column profiles of $NH_4^+$ and $NO_x$ concentrations (left), dissolved oxygen (O <sub>2</sub> ) and
853	dissolved sulphides $(H_2S)$ (right) in the water column of Lake Idro. Each data point is the average of
854	four samplings (January, May, August and November 2011); error bars are standard deviations
855	
856	Figure 3 Rates of total denitrification $(D_T)$ , and denitrification coupled to nitrification $(D_N)$ , nitrate
857	+ nitrite (NO <sub>x</sub> ) and ammonium (NH <sub>4</sub> <sup>+</sup> ) measured in the lake sediment at the four different depths
858	and in the different seasons. Error bars represent standard deviation (n=4).
859	
860	Figure 4 Rates of denitrification measured as a function of $NO_x$ concentration at 120 and 20 m
861	depth (A) and at 3 and 6 m depth (B) in May 2011. The equations of the regression lines are
862	$D_T = 0.39 \pm 0.01 * [NO_x] - 35.4 \pm 31.2$ , $R^2 = 0.99$ , p < 0.001 at 3 m.
863	$D_T = 0.58 \pm 0.16 * [NO_x] - 343 \pm 306$ , $R^2 = 0.87$ ; $p = 0.05$ at 6 m
864	$D_T = 0.013 \pm 0.002 * [NO_x] - 2.3 \pm 4.4, R^2 = 0.93, p < 0.05 at 20 m$
865	$D_T = 0.023 \pm 0.001^* [NO_x] + 1.5 \pm 10.8$ , $R^2 = 0.86$ , $p = 0.07$ at 120 m
866	Note the difference in scale of the y axes.
867	
868	Figure 5 Net Anthropogenic Nitrogen Inputs - NANI (t N y <sup>-1</sup> ) and its components in the lake Idro
869	watershed
870	
871	Figure 6 Daily loads of total nitrogen measured at lake inlet (watershed outlet) and lake outlet from
872	June 2010 to May 2012
873	
874	Figure 7 Summary of nitrogen budgets for the entire watershed, the terrestrial part and the lake.
875	Data are reported as total (t N $y^{-1}$ ) and areal fluxes (kg km <sup>-2</sup> $y^{-1}$ ). Denitrification is treated as a

876 component of the total lake Nr retention.

- **Figure 8** Relationship between denitrification and net NH<sub>4</sub><sup>+</sup> fluxes across the sediment water
- 879 interface in the three lake areas
- **Figure 9** Proportion (%) of the annual Nr load retained by Lake Idro compared to the predicted Nr
- retention of three general models. Equations for these relationships are: %N = 23.4 x T<sup>0.2</sup>
- 883 (Seitzinger et al., 2016), %N = 22 x log<sub>10</sub>T + 57 (Finlay et al., 2013), %N = 5.6 x ln T + 48.3
- 884 (Rissanen et al., 2013), with T=Retention Time

891			
892	Average watershed elevation (m)	1600	
893	Average air temperature (°C)	7	
894	Average rainfall (mm y <sup>-1</sup> )	1500	
905	Lake area (km <sup>2</sup> )	11.03	
895	Z max (m)	124	
896	Lake volume (km <sup>3</sup> )	0.85	
897	Monimolimnion volume (%)	47	
898	Mean TP ( $\mu g L^{-1}$ )	110	
899	Mean TN (µg L <sup>-1</sup> )	960	
900	Watershed area (km <sup>2</sup> )	609	
901	Agricultural land (km <sup>2</sup> )	51	
002	Pastures and meadows (km <sup>2</sup> )	106	
902	Inhabitants	18140	
903	Livestock units	3630	
904	Aquaculture (Mg y <sup>-1</sup> )	1700	

**Table 1.** General characteristics of Lake Idro and its watershed.

**Table 2.** Average density and organic matter (OM) content measured in the 0-5 cm sediment 906 horizon and water temperature (T), oxygen ( $O_2$ ),  $NH_4^+$  and  $NO_x$  concentrations measured in the 907 water above the sediment at the 4 sampling depths.

Sampling depth	Density	ОМ	Т	$O_2$	$\mathbf{NH}_{4^+}$	NO <sub>x</sub>
m	g ml <sup>-1</sup>	%	°C	mg L <sup>-1</sup>	$\mu g L^{-1}$	$\mu g L^{-1}$
3	1.3 - 1.5	3.3 - 4.7	5 - 23	8.6 - 12.1	0 - 42	336 - 868
6	1.1 - 1.2	6.3 - 7.3	5 - 23	8.6 - 14.1	0 - 14	378 - 840
20	1.0 - 1.1	9.1-10.2	5 - 8	4.0 - 11.5	0 - 56	714 - 826
120	0.9 - 1.0	6.3 - 8.5	7 - 7	0 - 0	672 - 1064	<50 - 100

910	Table 3 Distribution of total denitrification $(D_T)$ and denitrification efficiency in the three	

911 bathymetric layers in which the lake was partitioned. Values in parenthesis are standard deviations.

Bathymetric layer	Sediment s	urface	Denitrification		Denitrification Efficiency	Mass transfer coefficient	
	km <sup>2</sup>	%	t N y <sup>-1</sup>	%	%	m y <sup>-1</sup>	
0-10	1.2	10	42 (10)	51	127 (50)	49 (12)	
10-50	2.2	20	30 (3)	36	102 (41)	17 (1)	
50-120	8.1	70	11 (2)	13	4 (3)	6 (1)	
Whole lake	11.5	100	83 (10)	100	21 (15)	16 (3)	

- 915 **Table 4**
- 916
- 917 Average daily values of denitrification measured in sediments at different depths in freshwater
- 918 lakes. Values are expressed as mg N m<sup>-2</sup> d<sup>-1</sup>. Denitrification rates are measured using IPT during
- 919 laboratory core incubations (Nizzoli et al. 2010, 2014; Rissanen et al. 2013; Wenk et al. 2014), net
- 920 N<sub>2</sub> fluxes (Saunders and Kalff 2001; Small et al. 2014) or net N<sub>2</sub> fluxes corrected for N fixation
- 921 (McCarty et al. 2016). PM = polymictic, MO = monomictic, DM = dimictic; ME = meromictic; OX
- 922 = oxic; S-AN = seasonally anoxic; P-AN = permanently anoxic, n.a. = not known.
- 923

Environment	Mix	Ox	Dept h	DT	Source
Lake Champlain - Missisquoi bay	PM	OX	1	4.2 - 238	McCarty et al. 2016
Lake Stanga	MO	OX	2	17 - 134	Nizzoli et al. 2014
Lake Memphremagog	DM	n.a.	2	2.8 - 113	Saunders and Kalff 2001
Lake Lehee	PM	OX	3	14 - 17	Rissanen 2013
Lake Idro	ME	OX	4	16 - 211	This study
Missisquoi bay - Lake Champlain	PM	S-AN	4.5	0 - 41	McCarty et al. 2016
Lake Ormajarvi	DM	OX	5	60 - 182	Rissanen 2013
Lake Suolijarvi	DM	OX	10	67	Rissanen 2013
Lake Paajarvi	DM	OX	12	85	Rissanen 2013
Lake Stanga	MO	S-AN	20	9.8 - 57	Nizzoli et al. 2010
Lake Memphremagog	DM	n.a.	20	9.8	Saunders and Kalff 2001
Lake Erie	DM	OX	30	17.2	Small 2014
Lake Superior	DM	OX	32	7.8	Small 2014
Lake Huron	DM	OX	85	6.7	Small 2014
Lake Lugano	MO	S-AN	95	1.4 - 20	Wenk et al. 2014
Lake Idro	ME	P-AN	120	0 - 7.0	This study
Lake Superior	DM	OX	180	6.2	Small 2014

925

















Total Nitrogen (kg d<sup>-1</sup>)











(beol negorin leunne to %) noitregen load)

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