

# Experimental evidence of the role of nitrogen for eutrophication in shallow lakes: A long-term climate effect mesocosm study

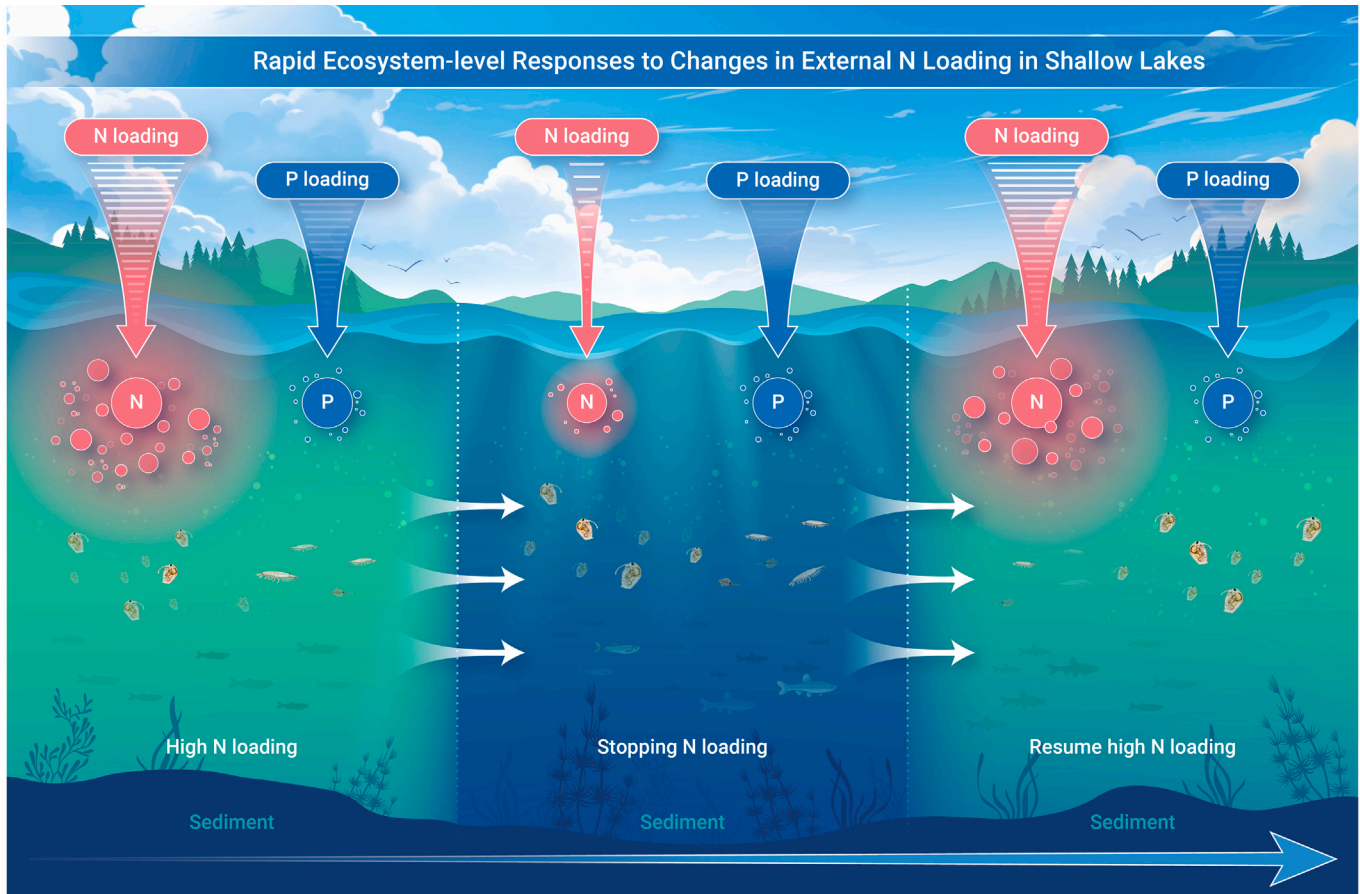
Erik Jeppesen,<sup>1,2,3,4,5,8,\*</sup> Hu He,<sup>6,8,\*</sup> Martin Søndergaard,<sup>1,2</sup> Torben L. Lauridsen,<sup>1,2</sup> Thomas A. Davidson,<sup>1</sup> Eti E. Levi,<sup>1</sup> and Mark J. McCarthy<sup>7</sup>

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## GRAPHICAL ABSTRACT



## PUBLIC SUMMARY

- A 5-year mesocosm study was conducted to examine the effects of external nitrogen (N) loading on shallow lakes.
- Water N concentrations and N-to-phosphorus (P) ratios responded rapidly to changes in external N loading.
- Changes in N loading had major effects on algal biomass and ecosystem metabolism at all temperature scenarios.

# Experimental evidence of the role of nitrogen for eutrophication in shallow lakes: A long-term climate effect mesocosm study

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The effectiveness of controlling nitrogen (N) loading (in addition to phosphorus [P]) to manage the eutrophication of aquatic ecosystems has been debated despite the role of N in producing algal biomass and toxins. Long-term, controlled tests of the efficacy of N loading reductions are largely missing from the scientific record, perhaps due to the historical focus on P control. To address this knowledge gap, we examined the results from a unique, long-term study conducted in 24 flow-through (2.5-month retention time) lake ecosystem-scale mesocosms in Denmark, operating since 2003 at two contrasting nutrient loading levels crossed with three temperature scenarios (ambient, IPCC (Intergovernmental Panel on Climate Change) A2 scenario, and A2+50%). For 1 year, the N loading, apart from groundwater inputs, was stopped in high nutrient loading mesocosms, while P loading was maintained. We followed the changes in key environmental variables and system metabolism for 5 years, including the 2 years prior to N loading reduction and 2 years after N loading resumption. The low nutrient loading treatments, which only received N and P from groundwater, were used as a reference. We found a strong effect of N loading on total N (TN), N oxides (NO<sub>2</sub> + NO<sub>3</sub>), and N:P ratios. After reducing the excess external N loading, which had lasted for 15 years, TN and N oxides declined to similar levels as those in the low nutrient treatments at all temperature scenarios and increased quickly when N loading was resumed. Algal biomass (as chlorophyll *a*) and ecosystem production and respiration were also affected. The results showed (1) a rapid response of water N concentrations to external N loading, (2) major ecosystem effects, including reduced algal biomass and system metabolism, and (3) overall low sensitivity in response to the IPCC temperature scenarios. This study was conducted under semi-natural conditions, providing strong experimental support for the key role of N at the ecosystem level in shallow lakes. Our results have profound implications for lake management and suggest that external N loading reductions may strengthen the recovery of shallow lakes from eutrophication.

## INTRODUCTION

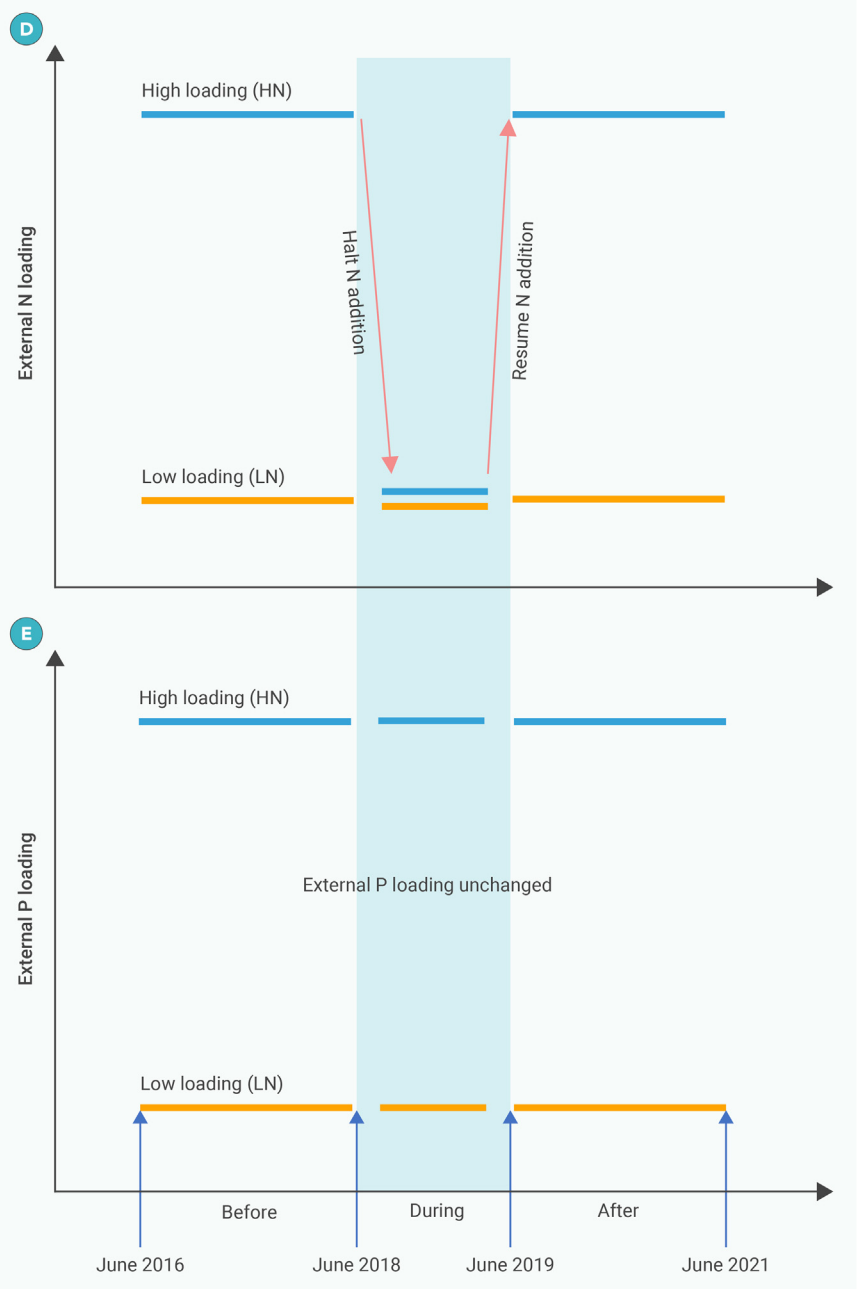
Nitrogen (N) and phosphorus (P) are important nutrients for primary producers in terrestrial and aquatic ecosystems.<sup>1</sup> While N is often considered a key limiting factor for production in terrestrial and marine systems, its role in lakes has been debated. Some have argued that P is the key limiting nutrient in lakes because N limitation will be fully compensated by atmospheric N<sub>2</sub> fixation,<sup>1</sup> while others argue for a dual effect of N and P.<sup>2,3</sup> Several experimental and field studies show that N<sub>2</sub> fixation by cyanobacteria cannot compensate for N deficiency,<sup>4–7</sup> and N losses via denitrification usually exceed N<sub>2</sub> fixation rates.<sup>6</sup> Compositional changes in algae and cyanobacteria in response to reduced N loading do not necessarily lead to higher representations of N<sub>2</sub>-fixing groups since non-N<sub>2</sub>-fixing groups can persist and outcompete N<sub>2</sub> fixers due to other adaptive characteristics, such as a higher capacity for cellular N storage in large-sized groups and competitiveness for regenerated N.<sup>8</sup> Lack of compensation for reduced N inputs by N<sub>2</sub> fixation was also demonstrated in long-term field studies, e.g., studies conducted in 35 lakes from around the globe showed rapid responses of N concentrations (in contrast to P) to external N loading re-

ductions,<sup>9</sup> and similar results come from the well-studied Lake Müggelsee (Germany) and Danish lakes.<sup>10,11</sup>

The role of N is expected to be particularly high in shallow lakes<sup>12,13</sup> due to high denitrification rates and sequestration of nutrients in biomass.<sup>14–16</sup> Growth of submerged macrophytes, abundant in low-nutrient shallow lakes, may also contribute to N loss in the summer via denitrification in attached biofilms, as well as biomass production.<sup>15</sup> However, inorganic P may be higher in the summer in eutrophic lakes due to P release from sediment mediated by high temperatures<sup>17</sup> and stoichiometric imbalances with depleted bioavailable N.<sup>18</sup> Decomposition of newly settled phytoplankton contributes to low oxygen concentrations in sediment, which can enhance iron-bound P release.<sup>17</sup> Organic matter decomposition in lake sediments<sup>16,19</sup> and N regeneration processes in the water column<sup>20</sup> are also consistent sources of bioavailable N. These internal N loads may exceed external N loads, suggesting that N legacies accumulated in sediments and biomass can help support phytoplankton growth and cyanobacterial toxin production during periods of low external loading in the summer.<sup>8,16,19</sup>

Global warming is predicted to have major implications for biogeochemical cycling in shallow lakes, potentially contributing to higher internal nutrient (N and P) loading and loss of N by denitrification, thus altering the N:P balance.<sup>20</sup> Many studies have focused on relationships between N concentrations and phytoplankton biomass,<sup>10,13,21</sup> but interactions between warming and N concentrations are not well understood. In a regression analysis of monitoring data from 504 Danish lakes, the total N (TN) concentration and water temperature were most strongly associated with phytoplankton and potentially toxic cyanobacteria biomasses in the summer and indicated the exacerbating effects of N at higher temperatures.<sup>22</sup> Increasing temperatures may also increase metabolism and lead to lower net system production due to the disproportionately stronger effects of temperature on respiration,<sup>23,24</sup> but how variations in N loading affect the metabolism variables in lakes is unknown.

Most studies of the role of N are based on surveys,<sup>10,25,26</sup> while there are few well-replicated, full-scale, or short-term experiments in mesocosms.<sup>21,27</sup> Longer-term N experiments under controlled nutrient loading conditions at contrasting temperatures are lacking. Here, we used a unique, long-term mesocosm study in Denmark, which has been operating since 2003 at two nutrient loading levels (HN [high nutrient] and LN [low nutrient], representing the algal-dominated turbid state and macrophyte-dominated clear water state, respectively; see [Figure 1](#) and detailed loading data in the [materials and methods](#)) crossed with three temperature scenarios (AMB [ambient temperature scenario], W [warming scenario (A2 scenario from the Intergovernmental Panel on Climate Change<sup>28</sup>)] and EW [extreme warming scenario (A2+50%)]; see [Figure S1](#)). The facility consists of 24 outdoor, freshwater, cylindrical stainless-steel tanks (1.9 m diameter and 1.5 m deep) equipped with paddles for water mixing. They have sediment, macrophytes, plankton, and fish, thus mimicking shallow lake ecosystems. The tanks continuously receive groundwater, resulting in a water retention time of approximately 2.5 months.<sup>29</sup> For further details, see the [materials and methods](#) and Liboriussen et al.<sup>29</sup> External N loading, except for groundwater inputs, was stopped for 1 year in HN mesocosms,



**Figure 1. Experimental facility and setup** (A) the mesocosm experimental facility and the close-up view of (B) high nutrient loading and (C) low nutrient loading tanks, as well as a schematic diagram showing how external (D) N and (E) P loadings changed between three periods in the two nutrient loading scenarios. Photographs were provided by Lone Li-borussen.

while P loading was maintained. Changes in key environmental variables, including phytoplankton chlorophyll *a* (Chl-*a*), gross primary production (GPP), ecosystem respiration (ER), and net ecosystem production (NEP), were observed for 5 years: 2 years before N loading reduction (the “before” period), 2 years after resumption of previous N loading levels (the “after” period), and the year N loading was stopped (the “during” period) (Figure 1). We hypothesized that lower N loading would lead to rapid decreases in N concentrations, phytoplankton biomass (as Chl-*a*), and system productivity and respiration in all temperature scenarios. We also expected that these effects would increase synergistically when combining warming and N loading reductions. This study fills important knowledge gaps on the effects of N loading reductions on shallow aquatic systems and may help guide nutrient management in agricultural and urban watersheds. Filling these knowledge gaps is needed, as achieving global food security through agriculture is more difficult with an increasing global population, climate change, and the recognition that over-application of agricultural fertilizers causes eutrophication in surface waters.

## RESULTS

Time-series plots for key variables are given in Figures S2–S5. Here, we focus on integrated data from the three experimental periods (Figure 1). We used generalized linear mixed models (GLMMs) to explore differences in daily averages for each variable among the three periods by conducting a before-after-control-impact (BACI) analysis.<sup>33</sup> Results showed that the 3-factor interaction term (nutrient  $\times$  temperature  $\times$  periods) was not significant for most chemical variables (e.g., TN, NO<sub>x</sub>, total phosphorus [TP], PO<sub>4</sub>, and Chl-*a*) or the three metabolism metrics (GPP, EP, and NEP; Table S1), nor for the plant volume inhabited as a percentage (PVI %) of submerged macrophytes (macPVI). However, for TN:TP, NO<sub>x</sub>:PO<sub>4</sub>, and PVI of filamentous algae (filaPVI), we found significant 3-factor interactions for EW (Table S1), i.e., for TN:TP, the changes from both before-to-during and during-to-after periods were larger in EW than in AMB mesocosms and for NO<sub>x</sub>:PO<sub>4</sub> from during to after periods. For filaPVI, both W and EW temperature treatments responded more strongly from during-to-after periods than AMB mesocosms.



There were no significant 3-way interactive effects for most variables (Table S1), so GLMMs were used to separately test the interactive effects of nutrient and period for each of the three temperature scenarios.

### Nutrients and phytoplankton Chl-a

The termination of N enrichment (apart from groundwater input) had large effects on measured N concentrations. For both TN and nitrates ( $\text{NO}_x$ ), significant interaction terms (nutrient  $\times$  period1 [from before to during] and nutrient  $\times$  period2 [from during to after]) for all three temperature scenarios indicated that TN and  $\text{NO}_x$  concentrations declined following termination of N enrichment and increased after resumption of N enrichment (Figure 2; Table S3). Notably, in all temperature scenarios, mean  $\text{NO}_x$  concentrations in HN mesocosms did not differ from those in LN mesocosms in the during period (Figure 2; Table S3), and TN in HN mesocosms did not differ from that in LN mesocosms in the EW treatment (Figure 2).

For TP, the interaction terms indicated that termination or resumption of N enrichment did not affect TP concentrations in the AMB treatment (Figure 2; Table S3). In the EW scenario, the interaction between nutrient and period2 (from during to after) indicated that resumption of N enrichment increased TP concentrations in the during-to-after period, while it was marginally significant for the W treatments. Orthophosphate ( $\text{o-PO}_4^{3-}$ ) increased steadily from before to during and during to after in all three temperature scenarios in HN mesocosms (Figure 2; Table S3), suggesting that both termination and resumption of N enrichment elevated  $\text{o-PO}_4^{3-}$  concentrations. In the during period, both TP and  $\text{o-PO}_4^{3-}$  remained higher in the HN compared to LN mesocosms (Figure 2; Table S3).

In the EW scenarios, the interaction for phytoplankton Chl-a (hereafter Chl-a) for the before-to-during period indicated that the termination of N enrichment reduced Chl-a concentrations, while the resumption of N enrichment had no effect on Chl-a (Figure 2). In the AMB and W scenarios, neither termination nor re-establishment of N enrichment impacted Chl-a concentrations, which were the lowest during the low N loading period in HN mesocosms (and also low after resuming N loading in EW) but did not reach the levels observed in LN mesocosms (Figure 2; Table S3).

For TN:TP and  $\text{NO}_x:\text{PO}_4$  interaction terms (nutrient  $\times$  period1 and nutrient  $\times$  period2) in all three temperature scenarios suggested that termination and re-establishment of N enrichment negatively and positively affected these ratios, respectively (Figure 3; Table S3). Mean TN:TP ratios were 6.2 by weight in the during period in HN mesocosms in all temperature scenarios and 4.6 during the summer (Figure S3).  $\text{NO}_x:\text{PO}_4$  approached zero (mean: 0.14) in the summer (Figure S3).

### Metabolism rates

GPP and ER were both lower in the during period compared with before and after, while net ecosystem production (NEP) results were less clear (Figure 4). The interactions (nutrient  $\times$  period1 and nutrient  $\times$  period2) on GPP as well as ER showed varied responses to termination and re-establishment of N enrichment in the three temperature scenarios, but no effect was found for NEP (Figure 4; Table S4). In the during period, GPP and ER did not differ between the HN and LN mesocosms but were substantially higher in HN than LN mesocosms in the before and after periods for all temperature scenarios (Figure 4; Table S4).

### Macrophytes and filamentous algae

Resumption of N enrichment coincided with a decrease in the PVI of filamentous algae in AMB temperature mesocosms (Figure 5; Table S5), as well as an increase in macrophyte PVI in the EW scenarios. No other differences were observed for macrophytes or filamentous algae during the 3-year observation period (Figure 5; Table S5).

## DISCUSSION

We found that a 1-year N loading reduction in HN mesocosms led to a substantial decrease in N concentrations in all temperature scenarios. The results also revealed effects on phytoplankton biomass (Chl-a) and, to various degrees, on system GPP and ER but not on NEP. The 3-factor analyses, however, revealed only marginal effects of temperature on TN:TP,  $\text{NO}_x:\text{PO}_4$ , and filamentous PVI between AMB and EW mesocosms, suggesting that the range of IPCC predictions was less important than the N loading reductions. A recent synthesis of

experimental and field studies from eutrophic lakes also indicated a much stronger effect of nutrient loading than temperature.<sup>35</sup>

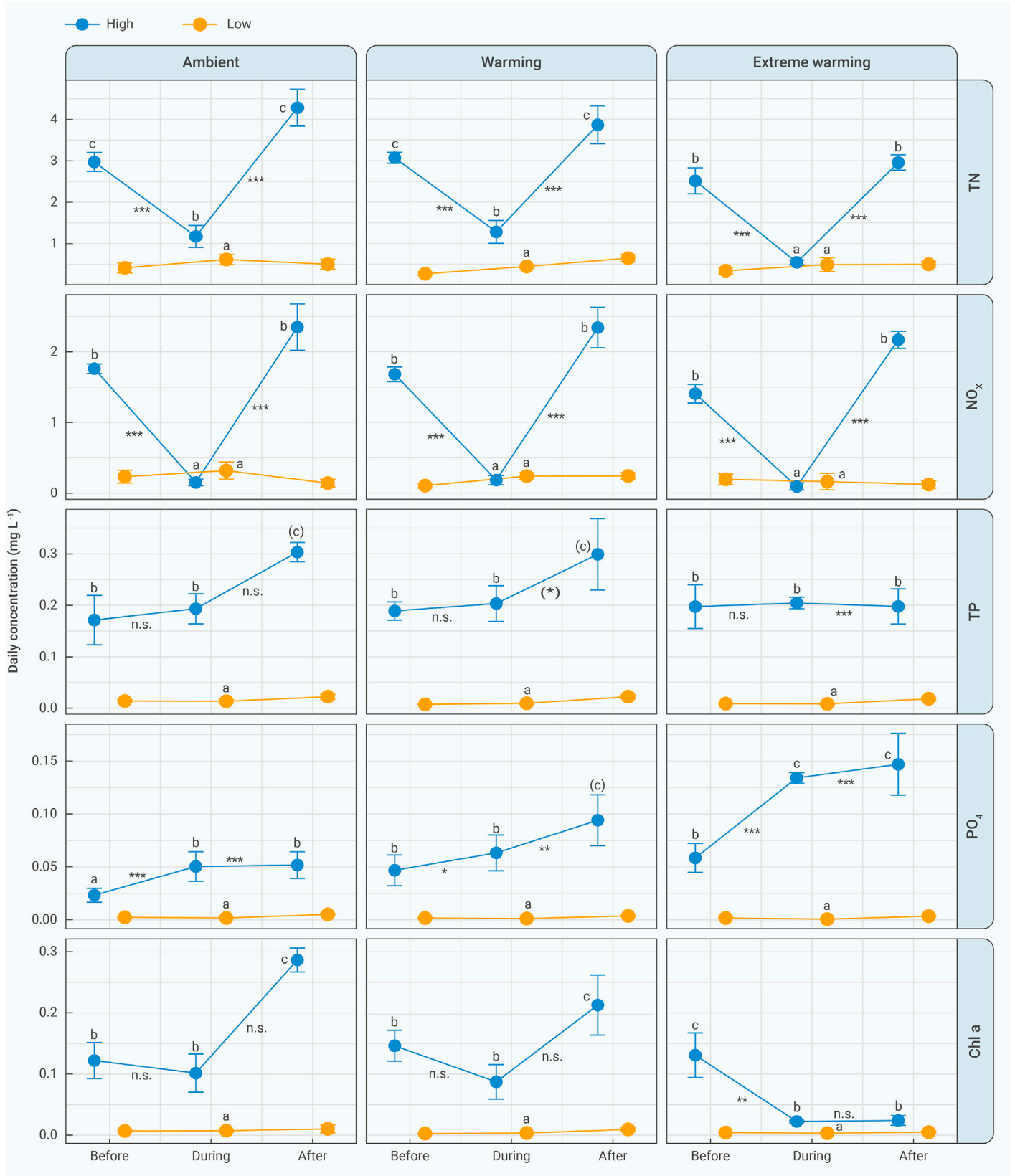
During the N loading reduction period,  $\text{NO}_x$  concentrations in HN mesocosms reached levels measured in LN mesocosms at all temperatures, while TN did not fully reach the levels observed in LN mesocosms, likely because Chl-a (and sequestration of N in phytoplankton biomass) remained higher in HN than LN mesocosms. The decrease in N, in part, reflects dilution. However, with an average retention time of 80 days, it would take 240 days (3 times the retention time) for a conservative element to reach 96% of the equilibrium level for the lower loading. Simple dilution cannot, therefore, explain the rapid TN and  $\text{NO}_x$  decreases observed in annual averages (Figure 2) and, even more clearly, at higher temporal resolution (Figure S2). The rapid response to TN loading reductions is consistent with a 30-year nutrient mass balance study of 10 Danish lakes, showing that N retention (i.e., loss by denitrification and storage), as a percentage of TN loading, was fast and not sensitive to major reductions in external TN loading.<sup>11</sup> A long-term field investigation conducted in shallow Lake Müggelsee (Germany) provides similar evidence after N loading reduction.<sup>10</sup>

Changes in N loading had modest effects on TP concentrations, but TP tended to increase at AMB and W temperatures after N loading resumed. Compared with LN mesocosms,  $\text{o-PO}_4^{3-}$  concentrations in HN mesocosms increased during and after the low N loading period. During the low N loading period, a higher  $\text{o-PO}_4^{3-}$  concentration may reflect reduced Chl-a (and thus algal uptake), as well as in the after period in EW mesocosms, where Chl-a remained low. However, this explanation does not apply for higher  $\text{o-PO}_4^{3-}$  concentrations at AMB and W temperatures after N loading resumed because Chl-a increased again in these mesocosms. Low nitrate concentrations during the low N loading period are also expected to lead to increased  $\text{o-PO}_4^{3-}$ , as seen in AMB and W mesocosms, reflecting reduced redox conditions in the sediment.<sup>17</sup> Potentially, high nitrate (and ammonium) concentrations can, however, also enhance  $\text{o-PO}_4^{3-}$  (and phytoplankton biomass) when P is deficient by stimulating alkaline phosphatase,<sup>27</sup> but  $\text{o-PO}_4^{3-}$  was high in HN mesocosms during all three periods, so this mechanism can be excluded.

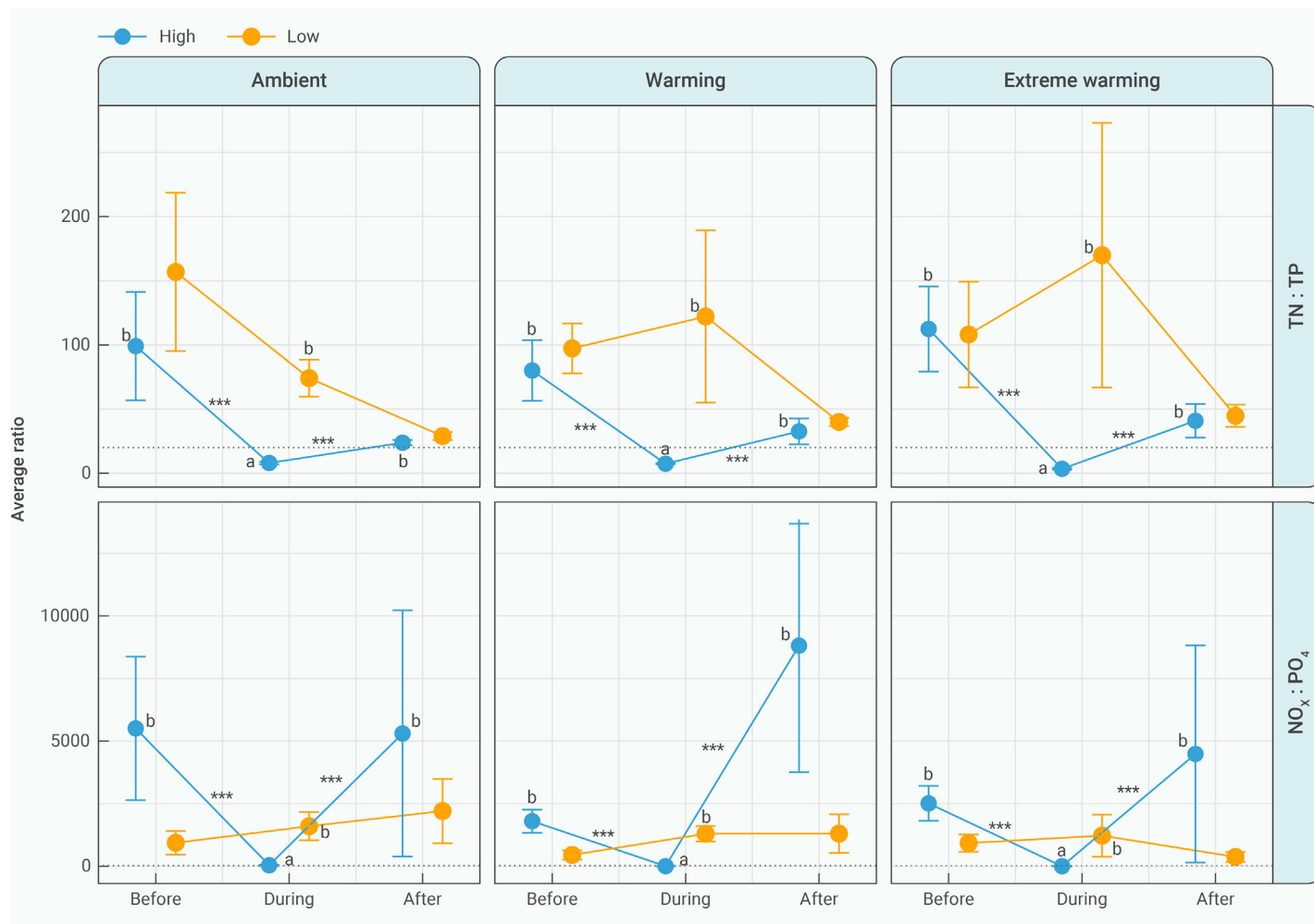
Concentrations and sediment release rates of chemically reduced N forms (ammonium and urea), which are energetically more bioavailable than  $\text{NO}_x$ , were not measured in this study. However, other studies on shallow, eutrophic lakes show that sediment release of these highly bioavailable N forms vastly exceeds sediment P release in most cases, even during low bottom-water oxygen events.<sup>19</sup> We lack the data to speculate on the magnitude of sediment N release in the mesocosms, but it is reasonable to assume, based on evidence from several studies,<sup>16,19</sup> that mesocosm sediments are sources of bioavailable N as ammonium and urea, in addition to P. In support of this assumption, the mixing paddles were shut off to allow the water columns to stratify in a subsequent experiment in these mesocosms in 2021. Both ammonium and urea accumulated in the "hypolimnia" of most mesocosms, but  $\text{o-PO}_4^{3-}$  only accumulated in the bottom waters of a few of the mesocosms (data not shown).

Due to the modest effect of N loading reductions on P, TN:TP ratios decreased in HN mesocosms during the loading reduction period, reaching an average of 6.2 by weight.  $\text{NO}_x:\text{PO}_4$  also decreased, reaching an average of 13.4 in HN mesocosms in all temperature scenarios and a mean value of 0.14 in the summer (Figure S3), indicating that the lack of N may have inhibited phytoplankton growth. Both ratios were lower in HN than LN mesocosms, where TN:TP and  $\text{NO}_x:\text{PO}_4$  ratios were generally high, likely reflecting the high N:P ratio of groundwater inputs (39.6; see materials and methods), which was the only external source of nutrients in LN mesocosms apart from rainwater. The effects of N loading reduction may have been stronger in HN mesocosms if the N:P ratio in groundwater had been lower. Most variables did not respond to N loading reduction in the three temperature scenarios, but TN:TP and  $\text{NO}_x:\text{PO}_4$  did respond in EW compared to AMB mesocosms, which potentially might be due to higher denitrification rates (lower N) and P release from sediment (higher P) at higher temperatures, making these ratios more sensitive to temperature than P and N.

Phytoplankton growth during the low N loading period was not compensated by any  $\text{N}_2$  fixation that might have occurred in the mesocosms, as would be predicted by some authors.<sup>1</sup> Cyanobacteria and chlorophytes dominated the phytoplankton biomass in HN mesocosms during and after the external N loading reduction.<sup>36</sup> Cyanobacteria biomass mainly comprised filamentous and potential  $\text{N}_2$ -fixing *Cuspidothrix* spp. and *Pseudanabaena* spp. and non- $\text{N}_2$ -fixing, colony-forming *Microcystis* spp. The proportion of chlorophytes, including *Micractinium* spp., increased concomitant with a decreased proportion of



**Figure 2. Mean daily average concentrations of nutrients (TN, NO<sub>x</sub>, TP, PO<sub>4</sub>) and Chl a in three periods for two nutrient and three temperature scenarios** We used before-after-control-impact (BACI) analysis to explore changes between three periods (see materials and methods). In each image, the symbols around the line show the significance level (\*\*\*)  $p < 0.001$ , \*\*  $p < 0.01$ , \*  $p < 0.05$ , (\*)  $p < 0.10$ , and n.s.  $p > 0.10$  of the interaction between periods (e.g., period1: during/before or period2: during/after) and nutrients (e.g., high/low nutrient loading), indicating whether the intervention (e.g., termination or recovery of N addition) affected the variable from the former to the latter period. Data are represented as mean  $\pm$  SEM. The letters above the points in HN denote the significance of the differences between the three periods in HN. Different letters in the during period show significant differences between HN and LN.



**Figure 3. Mean daily average TN:TP and  $\text{NO}_x:\text{PO}_4$  ratios in the three periods for two nutrient and three temperature scenarios** The dashed horizontal lines indicate a ratio of 20, below which N may be deficient relative to P. Symbols and letters are the same as in Figure 2.

cyanobacteria at AMB and W temperatures after N loading was resumed.<sup>36</sup> Thus,  $\text{N}_2$  fixation may have increased during the low N loading period in HN, but any effect was apparently weak, as Chl-*a* decreased during the low N loading period and increased after N loading was resumed.

In a parallel study, periphyton biomass development on natural plants (*Potamogeton crispus* and *Elodea canadensis*) and artificial imitations of these plants added to the mesocosms were compared for the reduced N loading period versus before N loading reductions.<sup>37,38</sup> Reduced N loading and concentrations led to increased periphyton biomass and lower genus richness,<sup>37</sup> but the biomass increase was not associated with  $\text{N}_2$ -fixing cyanobacteria. Instead, it comprised large filaments and colonies, particularly green algae, which are favored by decreased phytoplankton biomass and improved light conditions. Thus, there were no indications of important increases in  $\text{N}_2$ -fixing phytoplankton or algae in the biofilm of the plants during the low N loading period in HN mesocosms. Others have also questioned the idea of full compensation of cyanobacteria growth by atmospheric  $\text{N}_2$  fixation.<sup>6</sup>  $\text{N}_2$  fixation is metabolically costly, as it requires 16 ATP molecules to reduce one triple-bonded di-nitrogen molecule into two molecules of ammonium, which are then used by the algae.<sup>39</sup> In addition to P and several metals,  $\text{N}_2$  fixation also depends on high light intensity<sup>40</sup> and, for most freshwater cyanobacteria, the differentiation of specialized cells (heterocysts) to protect the nitrogenase enzyme from oxygen.

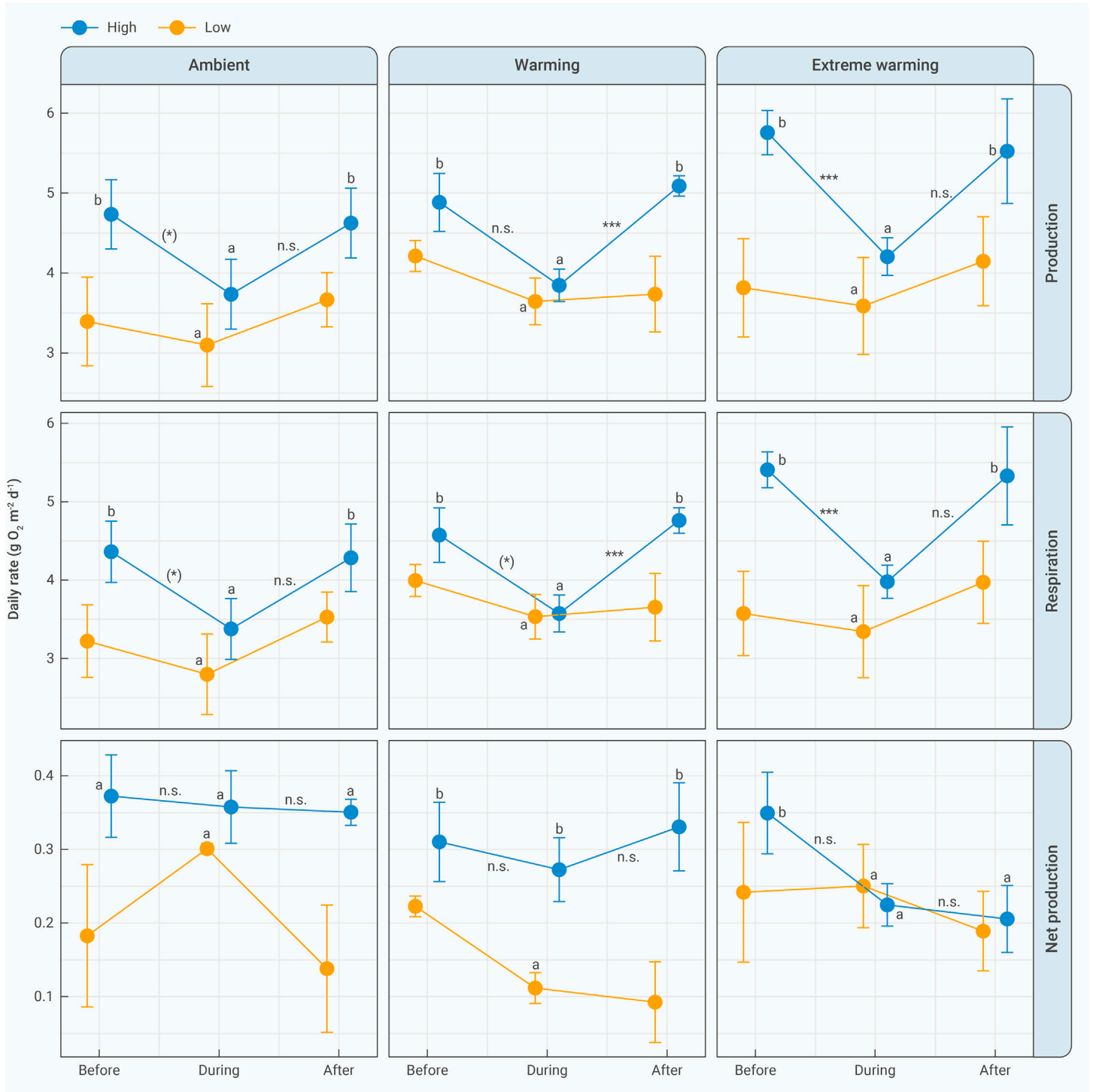
In accordance with earlier studies in these mesocosms,<sup>31,32</sup> we found that GPP and ER were higher in HN than LN mesocosms. Importantly, however, GPP and ER decreased during the low N loading period in HN mesocosms, particularly at W and EW temperatures, despite continued high TP loading. GPP and ER in HN mesocosms decreased to similar levels as those in LN, suggesting a fast and strong effect of changes in N loading for ecosystem function, indicating that ecosystem processes respond rapidly

to changes in loading and are, therefore, sensitive indicators. Interestingly, the response of GPP and ER to the N loading reduction did not differ among the temperature scenarios. Moreover, NEP was not affected by the N loading reduction at any of the temperature scenarios. Our results therefore show a strong response on GPP and ER but not on NEP to N loading reduction, independent of the temperature scenarios.

Macrophytes and filamentous algae did not show a clear response to N loading reduction or resumption. The PVI remained low in HN and high in LN mesocosms during all years. Only the PVI of filamentous algae was higher during low N loading at AMB temperatures in HN, perhaps reflecting improved light conditions due to decreased phytoplankton biomass (Chl-*a*). The 1-year period with low N loading might have been too short for macrophytes to take advantage of the improved light conditions in HN mesocosms. The lack of response from macrophytes and filamentous algae may also help explain why phytoplankton biomass in HN mesocosms during reduced N loading did not reach the levels in LN mesocosms (as otherwise observed for TN and  $\text{NO}_x$ ), as macrophytes have strong direct and indirect effects on phytoplankton in lakes.<sup>26</sup>

## CONCLUSION

We found that water column N concentrations responded quickly to external N loading reduction, with ecosystem effects manifested as reduced phytoplankton biomass (Chl-*a*), GPP, and ER but not NEP. We only found marginally different responses at the three temperature scenarios. Our results have profound implications for lake management and suggest that external N loading reductions may strengthen and quicken the recovery of shallow lakes from eutrophication, both now and equally well in a warmer future. This comprehensive study was conducted under semi-natural conditions, providing strong experimental support for the key role of N at the ecosystem level in shallow lakes.



**Figure 4.** Mean daily average metabolism rates (GPP, ER, and NEP) in three periods for two nutrients and three temperature scenarios. Symbols and letters are the same as in Figure 2.

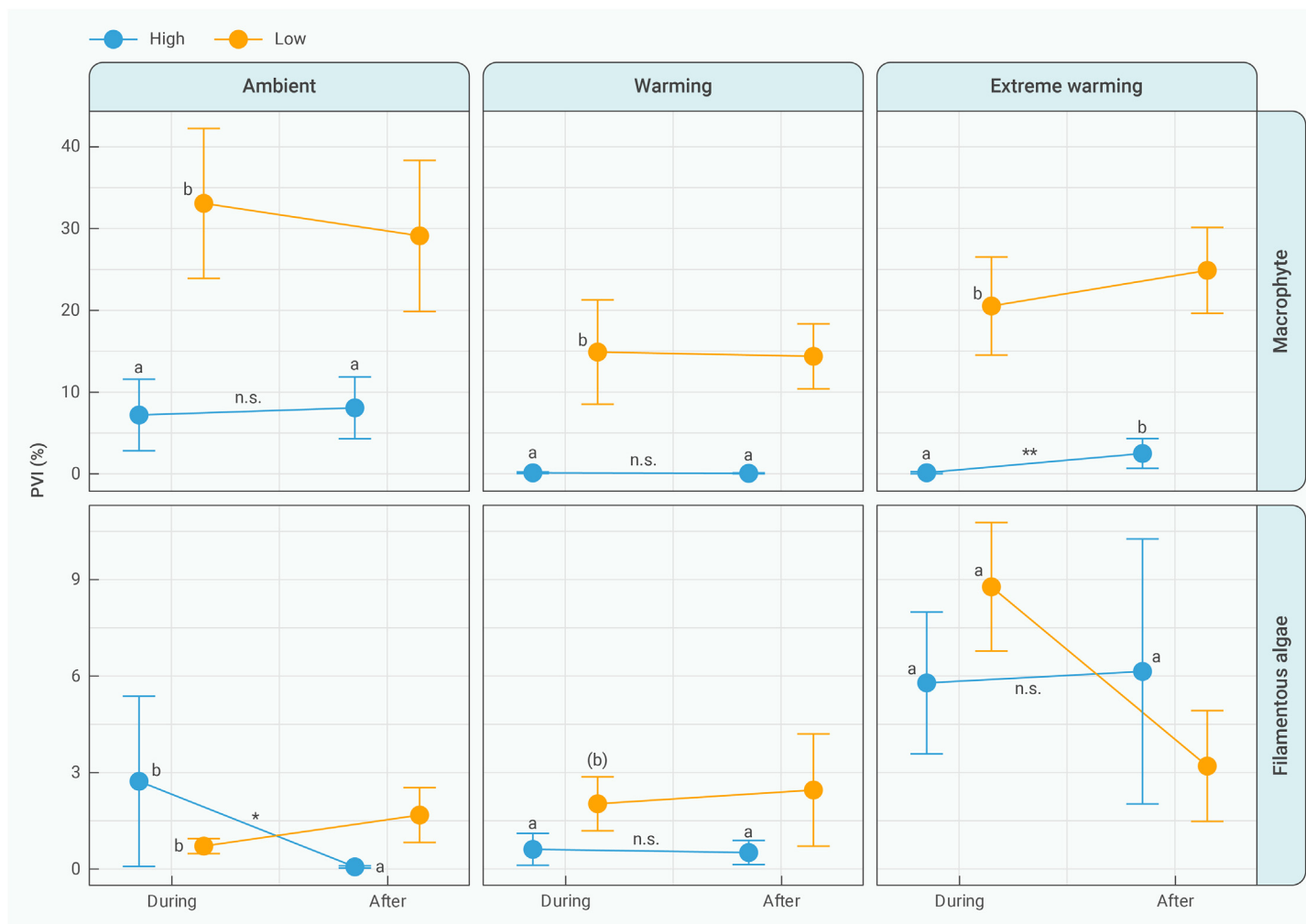
## MATERIALS AND METHODS

### Experimental design

The study was conducted as part of the Lake Mesocosm Warming Experiment (LMWE) at the Aarhus University mesocosm facility located in Lemming, Central Jutland, Denmark (56°140N, 9°310E). The experiment has operated continuously since 2003 and is the longest-running freshwater mesocosm experiment in the world. The facility includes 24 outdoor, freshwater, cylindrical stainless-steel tanks (1.9 m in diameter and 1.5 m deep) equipped with paddles for water mixing. The tanks continuously receive groundwater, resulting in a water retention time of approximately 2.5 months.<sup>29</sup> At the beginning of the experiment (2003), 0.2 m of sediment was placed into each mesocosm, with the sediment consisting of 0.1 m rinsed sand and 0.1 m nutrient-rich lake sediments. Most of the lake sediments orig-

inated from a nearby freshwater pond, while smaller portions came from a plankton-dominated, nutrient-rich lake and a macrophyte-dominated lake with lower trophic status. Male three-spined sticklebacks (*Gasterosteus aculeatus*), captured from local populations, were stocked in each mesocosm in near-natural densities.

The LMWE includes two nutrient treatments (HN and LN) and three temperature regimes in a factorial design, with four replicates for each treatment combination. HN tanks are supplied with 19 mg P and 759 mg N per m<sup>2</sup> each week in the form of Na<sub>2</sub>HPO<sub>4</sub> and Ca (NO<sub>3</sub>)<sub>2</sub> solutions, respectively. All tanks received groundwater with an average concentration of 2.26 (±0.63 SD) mg N l<sup>-1</sup> and 0.057 (±0.10) mg P l<sup>-1</sup> (N:P ratio averaged 39.6 by weight) amounting, on average, to 5 mg P and 207 mg N per m<sup>2</sup> per week. The temperature regimes included ambient water temperature



**Figure 5. Average PVI of submerged macrophytes and filamentous algae in three periods for two nutrient and three temperature scenarios** Symbols and letters are the same as in Figure 2.

(AMB) and two elevated temperature settings according to scenarios A2 (W) and A2+50% (EW) from the Intergovernmental Panel on Climate Change.<sup>28</sup> A more detailed description of the experimental setup is available.<sup>29</sup>

The data used in this study covered a period of 5 years, from June 13, 2016, to June 14, 2021. In the HN treatment (12 tanks across three temperatures), the additional N supply (apart from input with groundwater) was terminated on June 13, 2018, and resumed on June 14, 2019, while the additional P supply was maintained (see Figure 1). No changes were made to the LN treatment (12 tanks across three temperatures), which received groundwater only (Figure 1). The temporal dynamics of water temperatures for the three temperature scenarios (AMB, W, EW) are shown in Figure S1.

### Sample collection and measurement of water parameters

Water for nutrient and Chl-*a* analyses were collected monthly. Sampling was conducted with a 1-m tube sampler (covering the whole water column) at six randomly selected locations within each mesocosm and pooled. A 100- to 1,000-mL subsample was filtered using 1.2- $\mu\text{m}$  GFC filters, and Chl-*a* concentrations were determined on a spectrophotometer after extraction with ethanol. Another 100- to 200-mL integrated subsample was used for determination of nutrient concentrations. TN and TP were measured on unfiltered water, and nitrites and nitrates ( $\text{NO}_x$ ) and orthophosphates ( $\text{o-PO}_4^{3-}$ ) were measured on water filtered to 1.2  $\mu\text{m}$  (GFC filters) using standard methods.<sup>29</sup>

GPP and ER were estimated using the 30-min interval records of dissolved oxygen (DO) concentration, light intensity, and water temperature modified from Odum,<sup>30</sup> as described in Jeppesen et al.<sup>31</sup>:

$$\left(\frac{dDO}{dt}\right) = (K_2 \cdot 1.0241^{(T_w - 20)} \cdot (DO_{sat} - DO_t)) - (\text{Resp}20 \cdot 1.07^{(T_w - 20)}) + \text{Prod},$$

(Equation 1)

where  $T_w$  is the water temperature ( $^{\circ}\text{C}$ );  $DO_{sat}$  is the DO saturation concentration ( $\text{mg L}^{-1}$ ) at  $T_w$ ;  $DO_t$  is the DO concentration ( $\text{mg L}^{-1}$ );  $K_2$  is the gas transfer (piston) velocity for  $\text{O}_2$ , measured in three mesocosms in December 2009 based on the resumption of artificial reduction in  $\text{O}_2$ <sup>32</sup>;  $\text{Resp}20$  is the system respiration at  $20^{\circ}\text{C}$ ; and  $\text{Prod}$  is system productivity. We found little variation in  $K_2$  estimates (0.0080, 0.0086, and  $0.0100 \text{ m h}^{-1}$  at  $20^{\circ}\text{C}$ ) and assumed similar piston velocities in all mesocosms because water column mixing is performed mechanically using the same setup (paddles). We thus used the average of these three estimates for all mesocosms ( $K_2 = 0.0088 \text{ m h}^{-1}$  at  $20^{\circ}\text{C}$ ). Wind speed alters piston velocity; however, at low wind speeds ( $<3 \text{ m s}^{-1}$ ), the effect is negligible, and the mesocosms are well sheltered from wind.  $\text{Resp}20$  was estimated using the secant non-linear method PROC NLIN in SAS 9.3. NEP was estimated as  $\text{NEP} = \text{GPP} - \text{ER}$ .

Macrophytes and filamentous algae were evaluated monthly from June 2018 to June 2021. Macrophyte and filamentous algae abundances were quantified as the percentage of volume of the water column inhabited by plants (PVI %). The percentage of cover (visual inspection) and the height of the submerged plants and filamentous algae were assessed using a ruler, allowing estimation of the proportion of the water column occupied by submerged plants.

### Data analysis

We divided the time-series data into three periods: before (both N and P enriched, June 13, 2016, to June 13, 2018), during (only P enriched, June 14, 2018, to June 13, 2019), and after (both N and P enriched, June 14, 2019, to June 14, 2021). The full time series for each variable is shown in the supplemental information (Figures S2–S5). To reduce the influence of short-term variation, we calculated a daily average for each period of study for each tank as follows. First, we calculated the area under the curve (AUC) in each period using the AUC function in the DescTools R package, applying the “spline” method, yielding the area under the natural cubic spline interpolation. AUC values represent the total accumulation of a



variable (e.g., TN) over a certain time period (e.g., before). Second, we divided AUC by the number of days (e.g., 730 days in the before and after periods and 365 days in the during period) to calculate the average concentrations in a time period.

We used GLMMs to explore differences in daily averages for each variable among the three periods by conducting a BACI analysis.<sup>33</sup> In the BACI analysis, we explored whether there were significant, 3-way interactive effects on each variable via interactive terms of nutrients (HN-LN), temperature (AMB-W-EW), and periods (before-during-after) as fixed factors. Mesocosm identity was included as a random intercept, as the replicated tanks had developed differently over the 15 years prior to our experiment.

As we only found few 3-way interactive effects, we mainly focused on the 2-way interactions (nutrient  $\times$  periods) for each of the three temperature scenarios. Each model was run three times for each variable, one for each of the three temperature scenarios. To ensure that the before-during and during-after comparisons were both implemented, we used P1, P0, and P2 to represent the before, during, and after periods, respectively, so that the during period was set as a reference. The interactions, e.g., nutrient  $\times$  period1 and nutrient  $\times$  period2, describe how the intervention affected changes from the before-during and the during-after periods, respectively. For instance, a positive coefficient of nutrient  $\times$  period2 for the TN concentration indicates that the average difference in TN concentration from the during-after period was more positive in HN than LN treatments. In other words, resumption of N enrichment ("after") increased the TN concentrations. We assumed gamma or Gaussian error distributions for different variables (Table S2). For each model, we implemented residual diagnostics via the DHARMA package<sup>34</sup> and confirmed that assumptions of distribution, homogeneity of variance, and temporal autocorrelation were not violated (Table S2).

## DATA AND CODE AVAILABILITY

Data are available from the corresponding author upon reasonable request.

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#### AUTHOR CONTRIBUTIONS

Conceptualization, E.J., M.S., T.L.L., and T.A.D.; methodology, H.H. and E.J.; investigation, E.E.L., H.H., and E.J.; writing – original draft, E.J. and H.H.; writing – review & editing, M.S., T.L.L., T.A.D., E.E.L., and M.J.M. All authors contributed to and approved the manuscript.

#### DECLARATION OF INTERESTS

E.J. is a steering committee member of *The Innovation* and was blinded from reviewing or making final decisions about the manuscript. Peer review was handled independently of this member and their research group.

#### SUPPLEMENTAL INFORMATION

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