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Réponses des grands lacs périalpins aux pressions anthropiques et climatiques récentes

– Annexes –

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1. Enumération des communautés de cyanobactéries et de *Planktothrix* à partir de l'ADN fossile des sédiments.

❖ *Article : A Quantitative PCR Enumeration of Total/Toxic Planktothrix rubescens and Total Cyanobacteria in Preserved DNA Isolated from Lake Sediments*

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Quantitative PCR Enumeration of Total/Toxic *Planktothrix rubescens* and Total Cyanobacteria in Preserved DNA Isolated from Lake Sediments▽†

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The variability of spatial distribution and the determinism of cyanobacterial blooms, as well as their impact at the lake scale, are still not understood, partly due to the lack of long-term climatic and environmental monitoring data. The paucity of these data can be alleviated by the use of proxy data from high-resolution sampling of sediments. Coupling paleolimnological and molecular tools and using biomarkers such as preserved DNA are promising approaches, although they have not been performed often enough so far. In our study, a quantitative PCR (qPCR) technique was applied to enumerate total cyanobacterial and total and toxic *Planktothrix* communities in preserved DNA derived from sediments of three lakes located in the French Alps (Lake Geneva, Lake Bourget, and Lake Annecy), containing a wide range of cyanobacterial species. Preserved DNA from lake sediments was analyzed to assess its quality, quantity, and integrity, with further application for qPCR. We applied the qPCR assay to enumerate the total cyanobacterial community, and multiplex qPCR assays were applied to quantify total and microcystin-producing *Planktothrix* populations in a single reaction tube. These methods were optimized, calibrated, and applied to sediment samples, and the specificity and reproducibility of qPCR enumeration were tested. Accurate estimation of potential inhibition within sediment samples was performed to assess the sensitivity of such enumeration by qPCR. Some precautions needed for interpreting qPCR results in the context of paleolimnological approaches are discussed. We concluded that the qPCR assay can be used successfully for the analysis of lake sediments when DNA is well preserved in order to assess the presence and dominance of cyanobacterial and *Planktothrix* communities.

Eutrophication of freshwater ecosystems is accompanied by changes in diversity and abundance of the phytoplankton, leading to a decrease in planktonic diversity and, usually, a dominance of cyanobacteria (14, 15). Proliferation of toxic cyanobacteria in aquatic ecosystems has been associated with major ecological disturbances, such as a decrease in aquatic biodiversity, with potential consequences for the entire food web. The toxic potential of several genera of planktonic freshwater cyanobacteria producing microcystins (MCs), the most prevalent cyanobacterial hepatotoxins in freshwaters, may also represent a considerable health risk for both wild and domestic animals as well as for human beings (7). Toxic cyanobacteria can even occur in less eutrophic systems, such as deep mesotrophic lakes in the Alps (7, 29). In the latter case, *Planktothrix rubescens* is currently the dominant species reported for the following European subalpine lakes: Zurich (Switzerland) (55, 56), Garda (Italy) (48), Mondsee (Austria) (27), Nantua (France) (17), and Bourget (France) (23, 24). *Planktothrix* is one of the most important MC-producing genera in temperate lakes (27). Various chemical, physical, and biological parameters are involved in the development of cyanobacteria (12), and the variability of the spatial distribution (biogeography), the determinism of cyanobacterial blooms, and their impact at the lake scale are not understood. Several studies have highlighted the importance of long-term data sets in attempting to elucidate these ecological questions (2, 3, 20, 24, 57). However, existing long-term biomonitoring programs are usually incomplete and started, at best, in the middle of the 1950s, when the majority of the lakes (in temperate regions) were already impacted by cyanobacterial blooms. The paucity of long-term climatic and environmental monitoring data on the water body can be compensated by the use of proxy data from high-resolution sedimentary records (22). Fossilized organic components provide an archive of ancient aquatic microbial communities and hence can be used to reconstruct variations in climate and their impact on biodiversity (9). Preserved DNA can provide information at the species level by phylogenetic comparison, as well as data on quantitative proportions of species, and DNA has been shown to be preserved in the sediments of stratified lakes with anoxic and cold bottom waters (5, 9, 11). A strong DNA

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adsorption to mineral and organic matrices in sediments is also thought to be responsible for enhanced preservation due to protection against degradation by nucleases (4, 10, 44, 47, 49). Consequently, the cells of different microorganisms may often be preserved in deep lake sediments with sufficient fidelity to allow taxonomic identification or ecological inference, thus producing an archive of limnological history.

To date, most preserved DNA analyses have been performed on DNA from permafrost zones, halite crystals, amber, and marine sediments (4, 9, 10, 13, 41). Sediment records have been applied to a range of global environmental issues, including lake acidification, eutrophication, climate change (22), and the effect of human activity on the environment (34). In this sense, molecular biology-based techniques, such as PCR and quantitative PCR (qPCR), represent powerful tools for providing valuable information about *in situ* microbial dominance and diversity. Recently, *Taq* nuclease assays (TNAs) were designed and applied for quantification of total and toxic *Planktothrix* populations in water samples (6, 40, 50). However, to our knowledge, no comparable approach has been reported from the paleolimnological point of view, particularly regarding the quantification of *Planktothrix* and cyanobacteria in the preserved DNA derived from lake sediments. However, the comparability of such studies between environments, or even between different depths in the same sediment core, depends on various factors, e.g., sedimentary processes (ultimately explaining the efficiency of the preservation of organic matter) or methodological issues such as nucleic acid yield and amplification efficiency for each particular sediment type (33). Additionally, studies on preserved DNA are complicated by the extreme sensitivity of analytical techniques to DNA contamination and degradation, requiring adequate test procedures for both experimental and authentication methodology. The sensitivity of qPCR to even a small amount of coextracted inhibitors, such as organic matter in humus-rich sediments, might cause erroneously low estimates of the target. Therefore, these facts necessitate proper validation of the method before applying it to real environmental sediment samples.

The primary goal of this study was to demonstrate that qPCR can be applied successfully to enumerate total cyanobacterial and total/toxic *Planktothrix rubescens* and *Planktothrix agardhii* populations in preserved DNA derived from the sediments of three lakes located in the French Alps. This methodological validation represents an essential step for developing the analytical approach for further application to large-scale analyses of whole deep-sediment cores in order to understand the historical presence of cyanobacteria and their impact on lake ecology.

MATERIALS AND METHODS

Culture sample collection. A total of 21 *P. rubescens*, 17 *P. agardhii*, and 26 other cyanobacterial and algal clonal but nonaxenic strains were used for the testing of primers and probes (see Table S1 in the supplemental material). Strains were either obtained from the Thonon Culture Collection (TCC) (INRA, France) or supplied by international culture collections. All strains were grown under sterile conditions in BG11 medium (46) under continuous light (20 μmol m^{-2} s^{-1}) at 20 ± 2°C. Cells were harvested during the early stationary phase and subjected to counting by microscopy or DNA extraction as detailed below.

Lake sediment samples. Our approach was based on analyses of sediment cores from three lakes located in the French Alps (Lake Geneva [Leman], Lake Bourget, and Lake Annecy) (see Table S2 in the supplemental material). Lake Bourget (45°44′N, 5°52′E; 18 km long by 2.8 km wide; maximum depth, 145 m; mesotrophic) is a hard-water lake at the northwestern edge of the French Alps (19). Five sediment cores were obtained from both the southern basin and the northern basin of Lake Bourget (cores B1 to B5) (see details in Table S2). One sediment core from Lake Annecy (A1) was retrieved at the central point, at a 65-m depth, in 2010. Lake Annecy (45°51′N, 6°10′E; 14.6 km long by 3.2 km wide; maximum depth, 82 m; oligotrophic) is known as "Europe's cleanest lake." Sediment core L1 from Lake Geneva (46°26′N, 6°33′E; 73 km long by 14 km wide; maximum depth, 310 m; mesotrophic), the largest lake on the border between France and Switzerland, at the northern end of the French Alps, was taken at the deepest point of the lake. All cores were retrieved by means of a UWITEC gravity corer (Mondsee, Austria) and dated by radionuclides (^{210}P, ^{137}Cs, and ^{241}Am) and lamination counting as previously described (19, 39).

Four different slices were subsampled along the deep cores taken from Lake Bourget (B5-1, B5-4, B5-10, and B5-24), Lake Annecy (A1-1, A1-4, A1-10, and A1-24), and Lake Geneva (L1-1, L1-4, L1-10, and L1-24); these slices (1-cm height) were taken at 1-, 4-, 10-, and 24-cm core depths, respectively, to compare distinct regions of the cores which may correspond to the different trophic periods in the lakes (oligotrophic to eutrophic) (19, 39). The dating chronology of the sediment is presented in Table S2 in the supplemental material. For the sediment cores B1 to B4 from Lake Bourget, samples were taken only at a 4-cm depth in each core (samples B1-4, B2-4, B3-4, and B4-4) in order to obtain repeated measures, at the same depth, from different cores taken in the same lake. For each sediment sample, an aliquot of approximately 10 g of homogenized wet sediment was dried at 50°C and weighed. The rest of the sample was subjected to molecular analysis.

Due to the risk of contamination of the sediment samples by modern DNA, special laboratory precautions against contamination were applied as described previously (separated stations for sampling, DNA extractions, and PCR, sterile disposable labware, and negative controls for PCR) (9, 34).

Total cell counts from culture samples by microscopic analysis. In order to test the accuracy of qPCR, *P. rubescens* strain TCC 29 cell numbers determined by microscopy were related to those estimated by qPCR with DNA extracts. *P. rubescens* TCC 29 cell densities were determined from freshly grown cultures preserved with Lugol's solution by use of an optical microscope (Olympus BH-2) equipped with a hemocytometer (Malassez cells). Samples were diluted up to 100-fold (depending on the population density), *P. rubescens* TCC 29 filament lengths were measured at a magnification of ×20, and the results were averaged for at least duplicate whole-chamber counting. The number of TCC 29 cells making up the filament was estimated by measuring the total filament length and mean cell length (5 μm as measured at a magnification of ×400). All image combining, processing, and analysis were performed with the standard software package provided by Olympus (BH-2).

DNA extraction from culture and sediment samples. Genomic DNAs (gDNAs) from a wide range of cyanobacterial and other algal cultures were extracted by the DNA extraction protocol with GenElute LPA (linear polyacrylamide) modified by Jardillier et al. (26). Modification included the addition of 1 μl of GenElute LPA (Sigma-Aldrich, Inc.) to aid in DNA recovery.

Genomic DNAs preserved in sediment samples were extracted from 2 or 3 subsamples of 1 g of wet sediment each to avoid any heterogeneity, and resulting DNA extracts were pooled together. DNA was extracted using an UltraClean soil DNA isolation kit (Mobio, Carlsbad, CA) according to the manufacturer's instructions. The DNA extraction was evaluated on the basis of recovery efficiency, reproducibility, resulting DNA integrity, and performance in qPCR analyses. The total DNA concentration was measured using a Quant-iT PicoGreen kit (Invitrogen) on a Rotor Gene 3000 qPCR cycler with software version 6.0 (Corbett Research, Mortlake, Australia). Quantification with lambda DNA standards (Invitrogen) was performed in triplicate. The DNA was stored at −20°C until further analysis.

Preserved DNA quality check by amplification of the 16S rRNA-ITS region. Each preserved DNA extract was analyzed for the presence of the cyanobacterial 16S rRNA-internal transcribed spacer (ITS) gene region by PCR (25) to determine the quality and fragment integrity of the DNA throughout the core and to exclude the possibility of PCR inhibition causing negative qPCR results. Thus, the presence of 16S rRNA-ITS gene amplification products (around 1,500 bp) was used as a reference to standardize the PCR. PCR amplification was performed in a volume of 50 μl containing up to 50 ng of a template DNA, 5 μl of 10× PCR buffer (Bioline), 500 nM (each) forward (CYA371F [5′-CCTACGG GAGGCAGCAGTGGGGAATTTTCC-3′]) and reverse (373R [5′-CTAACCA CCTGAGCTAAT-3′]) primers (Biomers), 1.2 mM $MgCl_2$ (Bioline), 1 U of *Taq* DNA polymerase (Bioline), deoxynucleoside triphosphates (a 200 μM concentration of each; Bioline), and 1 mg ml^{-1} bovine serum albumin (BSA; Sigma). All PCRs were performed using a TProfessional Basic gradient thermocycler

(Biometra) and included an initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, an annealing temperature of 60°C for 1 min, and elongation at 72°C for 1 min. The amplification products of the 16S rRNA-ITS gene region from preserved DNA were sequenced (Beckman Coulter Genomics) as described previously (31). Obtained sequences were checked for chimera formation and compared with similar sequences available in GenBank by a BLAST search (1).

qPCR protocols. Two types of qPCR chemistries were applied in our study: a nonselective fluorescent dye, SYBR green (SG), was explored to quantify total cyanobacteria, while TNAs were applied to enumerate (i) the total population of *Planktothrix* by amplification of two alternatives (the intergenic spacer region of the phycocyanin operon [PC-IGS] region [two alternative primer-probe sets] and the 16S rRNA gene [TNA-16S rRNA]) and (ii) the population of MC-producing *Planktothrix* species via amplification of the *mcyA* region, which is indicative of microcystin biosynthesis (see Table S3 in the supplemental material) (21). Multiplex qPCR assays were then applied to quantify total and toxic *Planktothrix* spp. in a single reaction tube for all sediment samples.

All amplifications were performed in triplicate, using a Rotor Gene 3000 instrument with software version 6.0 (Corbett Research, Mortlake, Australia). Each multiplex reaction mix (25 μl) contained 12.5 μl of 2× Rotor Gene multiplex PCR master mix (Qiagen), a 300 nM concentration of each primer, a 200 nM concentration of each TaqMan probe, and 2 μl of template DNA. No-template controls and respective positive controls were included in all runs. The TaqMan probes had a fluorescent reporter dye (6-carboxyfluorescein [FAM] or 6-carboxyl-X-rhodamine [ROX]) attached covalently to the 5′ end and black hole quencher 1 (BHQ-1) or black hole quencher 2 (BHQ-2) dye (see Table S3 in the supplemental material). The following three qPCR master mixes were evaluated based on their performance during amplification: QuantiTect Probe PCR master mix, with and without ROX passive reference dye (Qiagen), and Rotor Gene multiplex PCR master mix (Qiagen). The singleplex TNA reaction mix (25 μl) consisted of 12.5 μl of one of the 2× PCR master mixes mentioned above, 300 nM (each) forward and reverse primers (Biomers), 200 nM TaqMan probe (Biomers), and 1 to 5 μl of template DNA. TNA reactions were initiated by either a 5-min (for Rotor Gene multiplex PCR master mix) or 15-min (for QuantiTect Probe PCR master mixes) hold at 95°C to activate the hot start polymerase, followed by 40 cycles of a two-step PCR consisting of a denaturation step at 95°C for 15 s and subsequent annealing and elongation steps at 60°C for either 15 s (for Rotor Gene multiplex PCR master mix) or 60 s (for QuantiTect Probe PCR master mixes).

For the SYBR green assay, two alternative primer sets were checked to enumerate total cyanobacteria (25): (i) CYA371F-CYA783R, targeting the 16S rRNA gene, and (ii) CSIF-373R, targeting the 16S rRNA-ITS gene region. qPCRs were performed in a 25-μl volume containing 12.5 μl of 2× QuantiTect SYBR green PCR master mix (Qiagen), 500 nM (each) forward and reverse primers (Biomers), and 2 μl of template DNA. The qPCR program consisted of an initial polymerase activation step (95°C for 15 min) followed by 40 cycles of denaturation at 94°C for 60 s, annealing at 60°C for 60 s, and extension at 72°C for 60 s. The qPCR program was followed by a melting curve determination step (from 70°C to 95°C at a transition rate of 1°C every 5 s) to determine the specificity of the amplification. As an additional post-PCR analysis (51), all qPCR products from the SYBR green-rRNA-ITS assays were analyzed for unspecific PCR amplification by running in a 2% agarose gel for 99 min at 50 V.

Assessment of specificity of qPCR assays. The specificities of primers and TaqMan probes as well as the optimal annealing temperatures for all qPCR assays were evaluated both *in silico* by Primer-BLAST (1) and experimentally by amplification tests with a wide range of target and nontarget algal species (see Table S1 in the supplemental material). The specificity and robustness of each TNA were tested by adding different amounts of DNA originating from other organisms as a background (40). DNAs extracted from *Microcystis* strain HUB 524, *Lyngbya* strain TCC 3a, *Synechococcus* strain TCC 32, and *Anabaena* strain TCC 79 were added to the DNA of *P. rubescens* strain TCC 29 (at 1.25 ng of DNA per template) and mixed at ratios of 1:100 and 1:1, and the resulting mixtures were analyzed by TNAs amplifying the PC-IGS region and the 16S rRNA gene. The TNA targeting the *mcyA* region was considered highly specific (6), and only DNA from *Synechococcus* strain TCC 32 was added to the DNA of *P. rubescens* strain TCC 29 (at 0.5, 5, and 50 ng of DNA per template) and mixed at ratios of 1:100, 1:10, and 1:1. In order to verify the specificity of the qPCR assays performed with sediment samples, the resulting qPCR amplification products (for enumeration of total cyanobacteria and toxic *Planktothrix* spp.) from preserved DNA were sequenced as described above.

Standardization and amplification efficiency. In our study, the minimum information for publication of quantitative PCR experiments (MIQE) guidelines (8) were followed in order to standardize the experimental practice and terminology and to promote consistency between different results (the quantification cycle [C_q] was used instead of the threshold cycle [C_T]).

Tenfold dilution standards were prepared from gDNA for each qPCR assay, ranging from 2.5 to 2.5×10^{-5} ng gDNA μl^{-1}. For each standard, the concentration (number of copies) of diluted DNA was plotted against the mean C_Q value (determined in triplicate), and the slopes of the standard curves were calculated by performing a linear regression analysis with Rotor Gene software (version 6.0; Corbett Research, Mortlake, Australia). The *mcyA*, 16S rRNA, and PC-IGS gene copy numbers of the DNAs of the standard strains of *Planktothrix* were calculated using the equation indicated below, assuming that the *Planktothrix agardhii* strain CYA126/8 genome has only one PC-IGS and one *mcyA* gene and four copies of the 16S rRNA gene (R. Kurmayer, unpublished data). Cell numbers for total cyanobacterial counts were estimated using an average 16S rRNA gene copy number of 3 (18, 41). The approximate genome size of *Planktothrix* spp. was determined to be 5.0 Mb, and the average size of cyanobacterial genomes (4.2 Mb) was used to estimate their total counts (54; http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi?view=1). It was calculated that 25 ng of each gDNA standard corresponded to averages of 4.63×10^6 and 5.48×10^6 cells for *Planktothrix* spp. and total cyanobacteria, respectively, according to the following equation: number of copies per microliter = DNA concentration ($\mu g/\mu l$) $\times$ 10^6 ($pg/\mu g$) $\times$ (1 pmol/660 pg $\times$ genome size [bp]) $\times$ 6.022×10^{23} (copies/mol) $\times$ 10^{-12} (mol/pmol).

C_q values of the sediment samples were determined for each TNA and SYBR green assay by importing the respective standard curve from each qPCR assay and adjusting it to the C_q value of the 10^{-4} or 10^{-5} gDNA standard, used as a respective positive control in each run. The accuracy of the qPCR assay was confirmed by relating the cell numbers determined by microscopy and estimated by qPCR with those determined by TNA-16S rRNA with DNA extracts, and the regression equation defining this relationship was established by standard Excel software.

Assessment of inhibition in sediment samples. TNA-16S rRNA and TNA-PlPC (50) were performed with a series of increasing amounts of genomic DNA of *P. rubescens* strain TCC 29 (0.125, 1.25, 12.5, and 125 ng per reaction mix) in the presence of increasing amounts of sediment DNAs (extracted from the Lake Bourget core at different depths [1, 10, and 24 cm]), mixed at ratios of 1:1, 1:10, 1:100, and 1:1,000. Efficiencies of qPCR performed with the resulting mixtures and also with DNA extracts of *P. rubescens* strain TCC 29 in the absence of sediment DNAs were compared based on the obtained C_q values. To test the effect of sediment addition (potential inhibition) on the qPCR results, the data were subjected to one-way analysis of variance (ANOVA) (the equal variance of groups and the normal distribution of residuals were tested). XLstats software was used to perform this statistical analysis.

Nucleotide sequence accession numbers. The sequences of the amplification products of the 16S rRNA-ITS gene region from preserved DNAs were deposited in the GenBank database under accession numbers JN098337 to JN098376. The sequences of qPCR amplification products from preserved DNAs were deposited in the GenBank database under accession numbers JN703798 to JN703887.

RESULTS AND DISCUSSION

Specificity of qPCR assays. For all TNAs, the assessment of primer and probe specificities was performed previously with respective controls (6, 40, 50); however, in order to confirm their specificities with other isolates, additional *in silico* and experimental amplification tests were performed in our study. The results revealed that *Planktothrix*-specific primers and TaqMan probes were specific for *P. rubescens* and *P. agardhii* but not for other species of cyanobacteria. The PC-IGS and 16S rRNA gene fragments were amplified from all *P. agardhii* and *P. rubescens* strains tested, whereas the *mcyA* gene fragment was amplified only from MC-producing *Planktothrix* strains (see Table S1 in the supplemental material). All nontarget DNAs could be amplified by targeting the cyanobacterium-specific 16S rRNA-ITS gene region but gave no products when those DNAs were amplified with *Planktothrix*-specific primer-probe sets. The presence of as much as 0.0125 to 1.25 ng of *Microcystis*, *Lyngbya*, *Synechococcus*, and *Anabaena*

TABLE 1. qPCR assay specificity check for *Planktothrix rubescens* based on C_q values obtained for mixtures of target and nontarget species DNAs

Organism(s)[a]	Strain(s)	Ratio of mixture	C_q value (mean ± SD)				
			TNA-PlPC	TNA-16S rRNA	TNA-MAP with dilution factor of:		
					1	0.1	0.01
P. rubescens	TCC 29		21.52 ± 0.04	19.57 ± 0.05	16.82 ± 0.07	20.58 ± 0.11	24.83 ± 0.08
P. rubescens and *Microcystis*	TCC 29/HUB 524	1:1	21.74 ± 0.07	19.61 ± 0.03			
	TCC 29/HUB 524	1:100	21.94 ± 0.03	19.89 ± 0.19			
Microcystis	HUB 524		33.11 ± 0.04	33.82 ± 0.07			
P. rubescens and *Lyngbya*	TCC 29/TCC 3a	1:1	21.95 ± 0.13	20.15 ± 0.04			
	TCC 29/TCC 3a	1:100	21.81 ± 0.03	19.98 ± 0.08			
Lyngbya	TCC 3a		34.12 ± 0.07	33.91 ± 0.03			
P. rubescens and *Synechococcus*	TCC 29/TCC 32	1:1	21.78 ± 0.02	19.58 ± 0.08	16.68 ± 0.06	20.12 ± 0.04	24.89 ± 0.02
	TCC 29/TCC 32	1:10			16.97 ± 0.05	20.59 ± 0.08	24.53 ± 0.23
	TCC 29/TCC 32	1:100	22.03 ± 0.02	19.95 ± 0.09	16.74 ± 0.05	20.22 ± 0.05	24.78 ± 0.03
Synechococcus	TCC 32		32.17 ± 0.15	32.27 ± 0.07	31.72 ± 0.22	—[b]	—[b]
P. rubescens and *Anabaena*	TCC 29/TCC 79	1:1	22.32 ± 0.12	20.14 ± 0.09			
	TCC 29/TCC 79	1:100	21.84 ± 0.06	19.96 ± 0.12			
Anabaena	TCC 79		32.59 ± 0.04	32.56 ± 0.05			

[a] Mixes of *P. rubescens* strain TCC 29 and background DNAs originating from other organisms (*Microcystis* strain HUB 524, *Lyngbya* strain TCC 3a, *Synechococcus* strain TCC 32, and *Anabaena* strain TCC 79).
[b] —, below the detection limit.

DNAs in the qPCR mixtures did not show a significant effect on C_q values of the samples which had only *P. rubescens* DNA (Table 1). For *mcyA*, the majority of nonspecific templates did not show any amplification, but in some cases, late amplification signals ($C_q > 31$) were observed for nontarget species, which could be abundant in the lake sediments (e.g., *Synechococcus*, *Microcystis*, and *Anabaena*). Nevertheless, the sequencing results for the *mcyA* amplicons from the qPCR runs with sediment samples from 1-, 4-, and 24-cm core depths (16 sequences for each depth) confirmed the specificity of TNA-MAP (6), demonstrating the specific amplification of the target *mcyA* gene of *P. rubescens* and *P. agardhii* only from sediment samples.

The application of SYBR green with optimal primer sets can be as sensitive as qPCR using TaqMan probes (35), but it is much cheaper to operate. In our study, SYBR green assay with the CYA371F-CYA783R primer set did not show sufficient specificity over the wide range of annealing temperatures examined (52 to 60°C). The alternative primer set, CSIF-373R, appeared to be highly specific for cyanobacteria, and a single amplification product with a characteristic melting temperature was observed when both genomic DNAs from a variety of algal cultures and preserved DNAs from sediment samples were analyzed. Importantly, the characteristic melting temperatures of different cyanobacterial standard strains, including *P. rubescens* strains TCC 29 and TCC 38 (86 ± 0.1°C), *P. agardhii* strains SAG 5.81 and CCAP 1459/15 (86.1 ± 0.1°C), *Anabaena* strain TCC 79 (85.8 ± 0.2°C), *Microcystis* strains HUB 5.3 and HUB 524 (85.9 ± 0.1°C), and *Synechococcus* strains TCC 32 (86.2 ± 0.3°C) and TCC 173 and 175 (87.3 ± 0.6°C), corresponded to those for DNAs amplified from sediment samples (86.1 ± 0.2°C). This result confirms that specific amplification occurred with preserved DNAs from sediment samples. The sizes of the PCR products varied among the different cyanobacterial genera (275 bp to 350 bp) (25), which resulted in small differences (<1.5°C) in the characteristic melting temperatures. When a DNA mix containing known amounts of specific and nonspecific templates was analyzed by qPCR, only specific amplification was observed, based on the presence of a single melting peak. Post-PCR analyses by inspection of an agarose gel containing each sediment sample showed no unspecific products, which confirmed the results of melting curve analyses. Primer dimers were not formed during qPCR experiments, as shown by the negative-control samples and postamplification melting curve analyses for every run. Moreover, since the application of SYBR green with relatively large (~300-bp) qPCR products might be questionable for environmental samples, the resulting rRNA-ITS gene amplification products of qPCR runs were sequenced to obtain the ultimate proof of the specificity of this assay for sediment samples. The sequencing results (44 sequences) obtained for depths of 1, 4, 10, and 24 cm revealed a wide range of cyanobacterial diversity, and no unspecific sequences were observed. The majority of clones were affiliated with *Synechococcus* spp., *Planktothrix*, *Anabaena*, and *Microcystis* spp. Thus, qPCR with SYBR green chemistry was found to be specific even with sediment samples, was reliable and robust enough to prevent false-positive results, and therefore could be applied successfully to enumerate total cyanobacteria in sediment samples.

Sensitivity and amplification efficiency of qPCR assays performed with culture and preserved DNA samples. Standard curves based on serial dilutions of genomic DNA were con-

structed for all four qPCR assays, and they were not extrapolated beyond the ranges of dilution, which were defined as 23.2×10^5 to 23 and 27.4×10^5 to 27 copies per template for each of the target regions detected by TNAs and SYBR green reactions, respectively. The amplification efficiencies (E) for all qPCR assays were in the range of 92 to 103% (Table 2). Since quantification errors typically occur toward both ends of a calibration curve, cell quantification for sediment samples was achieved by direction toward the central region of the standard curves, which is found to be the most resistant against background effects (28). A significant relationship between the *Planktothrix* cell numbers estimated by microscopic counting and those estimated by TNA-16S rRNA was found (Fig. 1C). The cell number per milliliter estimated by qPCR with DNA extracts was $1.11 \times 10^7 \pm 9.81 \times 10^5$, compared to $2.08 \times 10^7 \pm 0.56 \times 10^6$ for enumeration by direct microscopic counting. The regression equation was as follows: $y = 4.38x - 1{,}184$ ($R^2 = 0.97$; $P < 10^{-4}$; $n = 18$), where y and x are the log cell numbers determined by microscopic and TNA-16S rRNA methods, respectively.

Controls for evaluating the performances of the multiple assays, amplifying each target individually and comparing the results with those for the multiplex assays, were performed for both cultures and sediment samples. Resulting C_q values for all assays were highly comparable, independent of the sample type and target gene (TCC 29 had C_q values of 16.66 ± 0.45 [singleplex] and 16.39 ± 0.18 [multiplex], and B5-1 had values of 27.14 ± 0.40 [singleplex] and 26.52 ± 0.09 [multiplex], as determined by TNA-PlPC). The different qPCR mixes tested did not reveal significant differences in performance, demonstrating similar C_q values for both culture and sediment samples as determined by either TNA-PlPC or TNA-16S rRNA (data not shown). However, Rotor Gene multiplex PCR master mix, optimized specifically for Rotor Gene cyclers, had a shorter analysis time and was therefore used for all further qPCRs.

When two alternative TNAs for enumeration of total *Planktothrix* populations via the PC-IGS region of *Planktothrix*, i.e., TNA-PC and TNA-PIPC, were compared, the latter demonstrated a higher sensitivity of detection, showing lower C_q values (12.80 ± 0.35 for TCC 29 and 10.86 ± 0.24 for TCC 83-1 with TNA-PlPC versus 15.38 ± 0.37 for TCC 29 and 13.52 ± 0.07 for TCC 83-1 with TNA-PC). Thus, all qPCR assays with sediment samples were performed by TNA-PlPC. Total *Planktothrix* enumeration by either 16S rRNA or PC-IGS gene-targeting primer-probe sets revealed comparable results for both sediment and culture samples, showing the high reproducibility and reliability of these genetic markers. Generally, C_Q values obtained by TNA-16S rRNA were the lowest among the other TNAs for all preserved DNA samples (Table 2). This was likely due to the multiple copy number of the 16S rRNA gene (four copies of the 16S rRNA gene are found in *P. agardhii* CYA126/8). It was feasible to detect small numbers of *Planktothrix*, down to 3 copies per template, by TNA-16S rRNA assay, while such detection was not reliable by TNA-PlPC. Thus, qPCR targeting the 16S rRNA gene of *Planktothrix* spp. has certain advantages over the application of PC-IGS-based qPCR, since it has increased sensitivity and enables the detection of rare organisms in a sample (34, 35, 50).

TABLE 2. Parameters for standard curves of qPCR amplifications of *Planktothrix rubescens* obtained from culture (TCC 29 and TCC 83-1) and sediment (B5-1 and B5-24) samples

Parameter[a]	Value														
	TNA-16S rRNA				TNA-PIPC				TNA-MAP				SG-rRNA ITS			
	TCC 29	TCC 83-1	B5-1	B5-24	TCC 29	TCC 83-1	B5-1	B5-24	TCC 29	TCC 83-1	B5-1	B5-24	TCC 29	TCC 83-1	B5-1	B5-24
Slope	−3.37	−3.34	−3.27	−3.29	−3.35	−3.31	−3.2	−3.36	−3.25	−3.15	−3.39	−3.36	−3.51	−3.40	−3.52	−3.35
R^2	0.998	0.998	0.998	0.998	0.998	0.999	0.996	1	0.997	0.996	0.994	1	0.998	0.998	0.998	0.999
E (%)	98.6	99.3	102.1	101.0	98.6	99.9	102.3	98.0	103.2	101	97.0	98.0	93.6	96.8	92.0	99.0
y intercept	35.67	36.61	32.89	36.26	38.14	36.26	34.57	36.65	38.75	38.32	39.39	39.57	36.02	32.54	36.73	36.48
Dilution factor	$1–10^{-5}$	$1–10^{-5}$	$1–10^{-2}$	1, 0.1	$1–10^{-4}$	$1–10^{-4}$	$1–10^{-2}$	1, 0.1	$1–10^{-4}$	$1–10^{-4}$	$1–10^{-2}$	1, 0.1	$1–10^{-5}$	$1–10^{-5}$	$1–10^{-2}$	1, 0.1

[a] R^2, correlation coefficient; E, amplification efficiency.

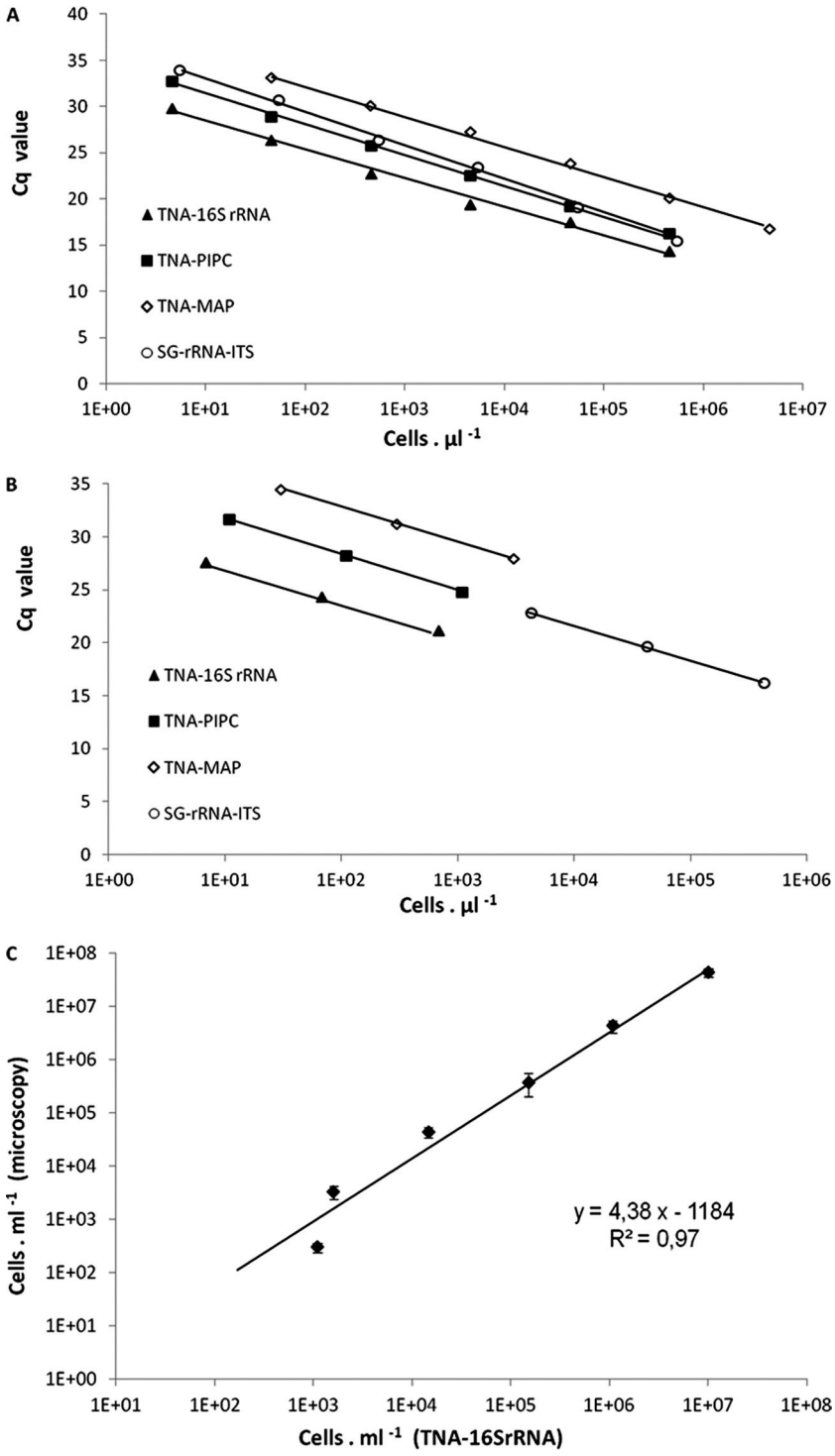

FIG. 1. Examples of qPCR standard curves obtained for *Taq* nuclease assays (TNA-16S rRNA, TNA-PlPC, and TNA-MAP) and the SYBR green assay (SG-rRNA-ITS), generated after amplification of predetermined concentrations (log scale) of MC-producing *P. rubescens* strain TCC 29 (A) and Lake Bourget sediment sample B5-1 (B). For the description of all curves, see Table 2. (C) Correlation between *P. rubescens* strain TCC 29 cell densities estimated by microscopic counting and those estimated by TNA-16S rRNA. Error bars, which are hidden by the symbols in almost all cases, give the standard deviations for three independent amplifications.

Assessment of inhibition due to coextracted materials from sediment samples. Due to the potentially high humic acid content in sediment samples, an absence of inhibition should be demonstrated when performing qPCR with such samples. The approach applied in this case was based on the assumption that inhibitors are diluted out when a log-linear relationship is achieved between C_q and the dilution factor (33). No inhibition effect was noticed when preserved DNAs from sediment sam-

ples from different depths along the sediment core were mixed with *P. rubescens* strain TCC 29 at any ratio, as assessed by either TNA-16S rRNA or TNA-PlPC. Further ANOVA showed no significant effect of sediment DNA addition on C_q values at any dilution rate ($P = 0.935$ and $P = 0.584$ for TNA-16S rRNA and TNA-PlPC, respectively). C_q values of all mixes were 15.62 ± 0.14 and 11.06 ± 0.33 by TNA-PlPC and TNA-16S rRNA, respectively, while C_q values of strain TCC 29 in the absence of sediment samples were 15.46 ± 0.27 and 10.86 ± 0.51, respectively. When both culture and preserved DNAs were diluted 10-, 100-, and 1,000-fold and mixed at a ratio of 1:1, C_q values of those mixes were not changed compared with those of TCC 29 alone at the respective dilution rate. Additionally, a C_Q value of 11.19 ± 0.73 was achieved for all nondiluted culture and sediment samples mixed at a ratio of 1:1, as determined by TNA-16S rRNA, which was close to the C_Q value for TCC 29 alone (10.86 ± 0.51). Importantly, the amplification efficiencies of all qPCR assays performed with preserved DNAs from sediment samples (0.87 to 1.16) (Table 2; Fig. 1B) were as high as those with the respective standards (0.91 to 1.05) (Table 2; Fig. 1A). Similar qPCR amplification efficiencies ensured that no PCR-inhibiting contaminants were present in the sediment samples. Based on the obtained results, we concluded that qPCRs were not inhibited, which is a prerequisite for reliable enumeration of both toxic and total *Planktothrix* spp. and total cyanobacteria in preserved DNA.

Quantification of total/toxic *Planktothrix* spp. and total cyanobacteria in preserved DNAs from lake sediments. Sediment sampling sites were selected based on the fact that Lake Geneva, Lake Annecy, and Lake Bourget are deep large lakes (with anoxic bottom waters over at least the last few decades) from the same region but show different histories in terms of their trophic status and, consequently, different diversity and quantities of cyanobacteria (16, 24). Thus, if DNA is preserved in the laminated sediments of these lakes, DNA-based analyses of sediment samples should reveal these differences regarding planktonic communities, and more particularly, qPCR analyses are likely to reflect the abundance and dominance of a wide range of cyanobacterial species in water columns.

Lake sediments contained 0.24 (L1) to 5.95 (B5-10) μg DNA per gram of dry sediment. Lake Annecy sediments had smaller amounts of genomic DNA extracted than those of Lake Bourget and Lake Geneva, probably due to low productivity. The DNAs from lake sediments resulted in amplification of 1,500-bp PCR products from the 16S rRNA-ITS gene region; thus, as reported before by Coolen et al. (9) for other ecosystems, fragmentation of this region of DNA during burial in the sediments did not occur, or at least was rare enough to favor the preservation of a sufficient quantity and quality of DNAs from several cyanobacterial groups. Moreover, the amplification products of the 16S rRNA-ITS gene region were sequenced, and around 100 sequences per core depth were obtained (O. Savichtcheva et al., unpublished data). To date, sequencing analyses with preserved DNAs from sediments have not been performed often enough, indicating the necessity and novelty of our approach. In this study, we focused mainly on demonstration of the quality and integrity of a preserved DNA fragment, rather than discussing the diversity of various cyanobacterial groups, by sequencing the 16S rRNA-ITS gene region. The sequencing results obtained for depths of 1, 4, 10, and 24 cm revealed a wide range of cyanobacterial diversity, and no chimeric sequences were observed. The majority of clones were affiliated with *Synechococcus* spp. but also included *Cyanobium*, *Anabaena*, *Nostoc*, *Microcystis*, and *Planktothrix* spp. Thus, these results demonstrate sufficient quality and fragment integrity of the preserved DNA.

We determined the amounts of DNA derived from *Planktothrix* and cyanobacteria in sediment cores by qPCR. Overall, qPCR assays performed with preserved DNAs from different lake sediments revealed clear differences in the quantitative compositions of total and toxic *Planktothrix* and total cyanobacteria (Fig. 2). Thus, qPCR enumeration showed high cyanobacterial counts in Lake Bourget sediments and very low ones in Lake Annecy sediments, which is in good correspondence with available data on lake water monitoring (16, 24, 43). Lake Bourget and Lake Geneva were indeed affected by eutrophication in the second half of the 20th century, and these systems are now recovering toward an oligotrophic status, while Lake Annecy has not been impacted by such strong eutrophication, and its oligotrophy has been preserved over the past decade (42). Lake Geneva underwent strong eutrophication in the early 1960s, but following the implementation of phosphorus reduction measures in the 1970s, the phosphorus concentration decreased from ~90 μg liter^{-1} in the mid-1970s to ~30 μg liter^{-1} in 2005 in Lake Geneva (53) and from 120 to 26 μg liter^{-1} between 1980 and 2001 in Lake Bourget (24).

Among all the lakes, Lake Bourget had the highest counts for total (as determined by both 16S rRNA and PC-IGS gene-based qPCR) and toxic (sample B5-1) *Planktothrix* populations as well as for total cyanobacteria (sample B5-1). Total *Planktothrix* quantifications based on the 16S rRNA and PC-IGS genes showed comparable results and demonstrated similar trends for all sediment cores (Fig. 2A). Generally, *mcyA*-based counts exceeded those of 16S rRNA or PC-IGS gene-based assays (Fig. 2A). The fact that sequencing results for the *mcyA* amplicons confirmed the amplification of only *Planktothrix* spp. rejects the idea that the primers targeting this conserved condensation domain (21) might be able to coamplify other MC-producing taxa. At present, the highest *mcyA* proportions (>100%) in sediment samples cannot be explained. From these preliminary results, it was concluded that the proportion of the *mcyA* gene has remained high in Lake Bourget since the 1950s and in Lake Geneva since the 1980s. In the future, alternative qPCR assays to enumerate toxic *Planktothrix* spp. should be applied to validate those results.

For Lake Bourget, the proliferation of MC-producing *P. rubescens* has been reported previously (7, 24); the dominance of this taxon in Lake Bourget has been known at least since 1996 and is associated mainly with the process of restoring this ecosystem. In Lake Annecy sediments, no *Planktothrix* spp. were detected by qPCR, and *Planktothrix* has never been reported for planktonic assemblages in this lake. The presence of *P. rubescens* has been detected in the pelagic compartment of Lake Geneva, but generally in small amounts (compared to those in Lake Bourget). *Planktothrix* spp. have been detected in Lake Geneva, especially during the years 1963 to 1985 (16). Importantly, we observed the same trend by performing qPCR assays with lake sediments, which confirmed the lower *Planktothrix* counts in Lake Geneva than in Lake Bourget.

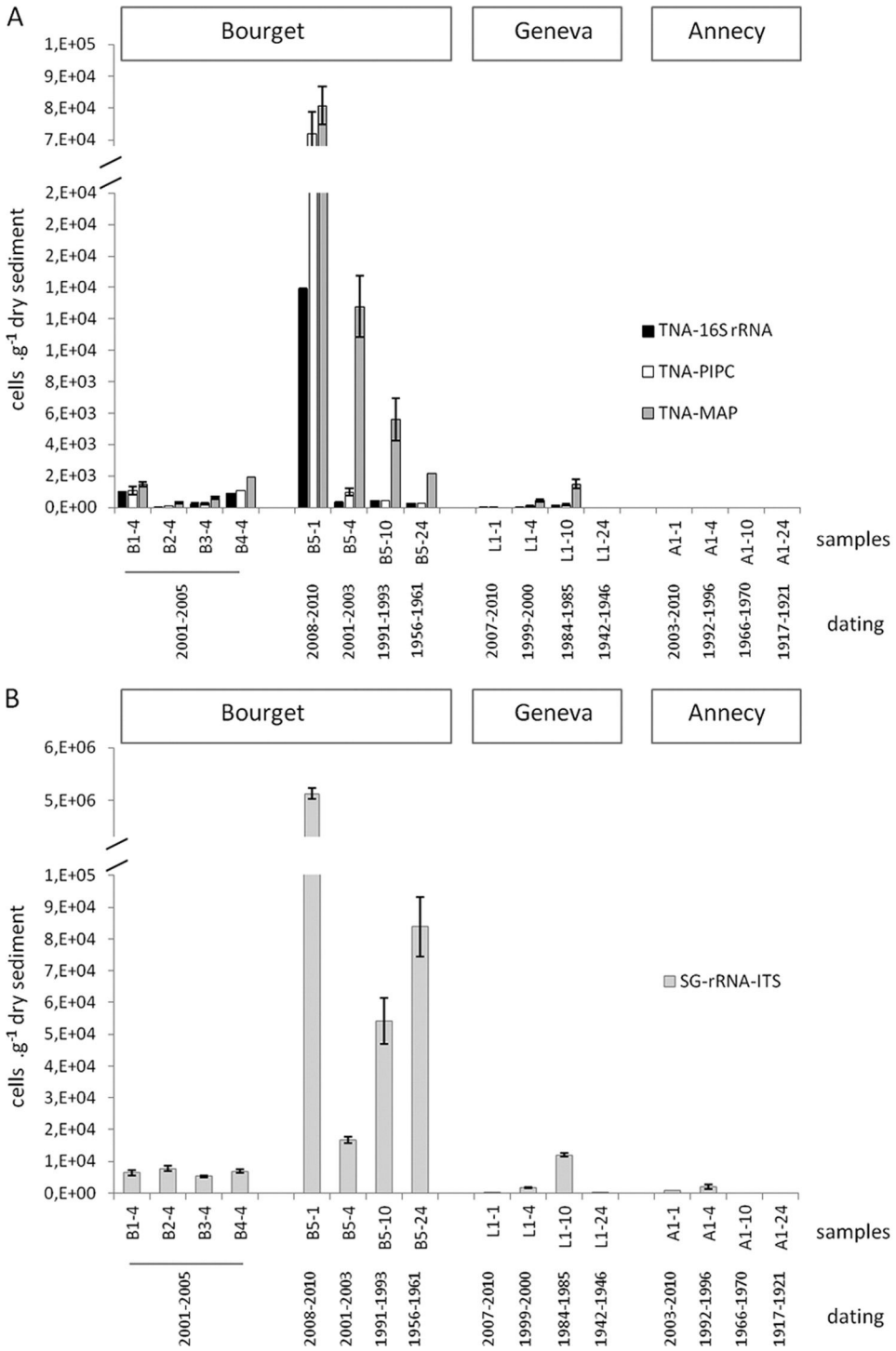

FIG. 2. Abundances of total (TNA-16S rRNA and TNA-PlPC) and MC-producing (TNA-MAP) *Planktothrix* (A) and total cyanobacteria (SG-rRNA-ITS) (B), estimated by qPCR, in the sediment samples from three lakes located in the French Alps (Lake Geneva, Lake Bourget, and Lake Annecy). Error bars, which are hidden by the symbols in some cases, give the standard deviations for three independent amplifications.

Within the Lake Geneva sediment core, total and toxic *Planktothrix* spp. were detected at 1-, 4-, and 10-cm depths (for dating, see Table S2 in the supplemental material) but not at the 24-cm core depth, while other cyanobacteria were detected down to the 24-cm layer. Interestingly, total cyanobacterial qPCR counts increased gradually from 1 to 10 cm, consistent with the period of strong eutrophication observed in the late 1980s (53), and decreased again at the 24-cm core depth (consistent with a period of pre-eutrophication according to data from the long-term monitoring of deep perialpine lakes).

Within the Lake Bourget sediment core, particularly high *Planktothrix* and cyanobacterial counts were observed at a 1-cm core depth (Fig. 2), and they decreased with the depth but remained at high levels at 4-, 10-, and 24-cm core depths. This suggests that the presence of *P. rubescens* is ancient in this lake (the 24-cm depth in sediment corresponds to the period from 1956 to 1961 [see Table S2 in the supplemental material]). Some older data on planktonic counts (performed occasionally) confirm the presence of this taxon in Lake Bourget in 1913 (32).

In the Lake Annecy sediment core, cyanobacteria were detected by qPCR at 1- and 4-cm depths but not at 10- and 24-cm core depths (Fig. 2). The values were generally low, with the highest quantification recorded for the 4-cm layer (corresponding to the period from 1992 to 1996).

The data obtained in our study are consistent with the past dynamics of cyanobacteria and likely reflect the temporal variations in *Planktothrix* and cyanobacterial species in these deep lakes. Thus, combining paleolimnology and molecular tools, the amounts of cyanobacterial and *Planktothrix* compositional turnover in sediments from different lakes could be analyzed, and distinct regions of the sediment core, corresponding to different trophic periods in the lake (oligotrophic to eutrophic), could be identified, inferring the recent history of the lake ecosystems.

Limitations of the use of qPCR for paleolimnology, with future perspectives. One of the major limitations in the use of the qPCR approach for paleolimnology is the efficiency of DNA preservation, which could vary from 1 year to another, especially depending on the length or stability of anoxia. Even though it is difficult to prove that no shifts in degradation processes occurred from 1 year to the other during the past century in the three studied lakes, it could be expected that rather good preservation conditions have occurred during the last century in these three cold, anoxic aquatic sediments (19). Paleolimnological studies performed recently on Lake Bourget and Lake Annecy (19, 39, 42) provided converging information about the efficient storage of organic material in these lakes during the last century. Additionally, we showed that high-quality DNA could be extracted from these sediments, and since a short DNA region is targeted in performing qPCR, the qPCR method should be considered even with partial degradation of preserved DNA. Consequently, we assume that full analyses of these sediment cores by using the qPCR approach and sequencing in order to target cyanobacteria (and potentially other phytoplanktonic taxa, such as diatoms) are of special interest to reconstruct the comparative history of these lakes (19). Moreover, to avoid the obstacles related to the different DNA preservation levels within sediment layers, it would be useful to apply the relative counts (e.g., the ratios of *Planktothrix* counts to total cyanobacterial counts) instead of their absolute numbers.

On the other hand, not all cellular material is transported equally well to sediments, and there are species- or cell-specific variations in the level of (post)depositional degradation of intracellular DNA (10). We assume that obtained DNAs represent preserved DNAs from *Planktothrix* and other cyanobacteria residing in the water column rather than DNAs derived from physiologically active microorganisms residing in the sediments. *P. rubescens* is characterized by its location in the metalimnic layer of the water column, and internal waves appear to have a major impact on its spatial distribution and proliferation, influencing the growth of this species by a direct impact on light availability (12). This species is particularly well adapted for growing at low light intensities and also at low temperatures (56). In contrast, other cyanobacterial colonies (especially *Microcystis* spp.) have a benthic life cycle, where they form blooms during the summer, sink and reach sediments in autumn, remain in the sediments as vegetative cells during winter, and provide an inoculum for the water column in spring (30). For *Planktothrix*, such a benthic life cycle has not been reported. All of these factors could introduce a certain level of discrepancy and error to the qPCR-based approach performed with preserved DNA, especially where comparisons between different phytoplanktonic groups (e.g., diatoms, cyanobacteria, and chlorophyceae) are concerned. We should thus be cautious in using DNA as a quantitative indicator of past phytoplankton community structure. The combined application of DNA and traditional biomarkers, such as lipids or pigments, would also provide converging information about the importance of phytoplankton groups stored in sediments.

Further studies on the application of qPCR to sediment samples, as well as development of the data set by sequencing of the 16S rRNA-ITS gene region, should be conducted. Only a few characteristics about cell viability of cyanobacteria in sediments have been studied yet; thus, the identification of the active microbial population within sediment samples can be addressed by targeting rRNA content directly rather than targeting DNA. Moreover, the preservation (during sedimentation processes and after deposition on sediments), origin, and size of DNA in sediments should not be neglected. Even though it is expected that similar preservation conditions might occur in various anoxic aquatic sediments, further work examining whether the DNAs from different species are equally well preserved should be performed.

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2. Détermination des espèces de phosphore (organiques et minérales) à partir de la spectrométrie XANES appliquée aux archives sédimentaires : une reconstitution de la pédogenèse

❖ *Article : Phosphorus speciation by XANES spectrometry as a tool to reconstruct soil genesis from lake sediments*

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Phosphorus speciation by XANES spectrometry as a tool to reconstruct soil genesis from lake sediments

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Abstract

Sediment records of Lake Anterne (2063 m asl, North French Alps) covering the last ~10 000 years have proved, in a previous study, their capacity to record soil cover changes. In this study we are using P species recorded in lake sediments as a tracer of soil development through times within the corresponding catchment. To tackle this question, five lake sediment samples, corresponding to different steps of soil development, were chosen for P speciation analyses. Furthermore a soil sequence from the catchment was analyzed to better constrain our interpretations of the lacustrine archive. Synchrotron techniques (P K-edge XANES and XRF mapping) were applied on lake sediments, soils and references (mineral and organic) to identify the main P species. Results show that soil development raises the P species diversity. At the onset of the Holocene, when bedrocks and leptosols dominated the catchment cover, P was under form of apatite. Then, the soil genesis processes triggered the dissolution of apatite and the formation of a large amount of compounds containing phosphorus both in organic and mineral forms. The phosphorus geochemistry related to the main step of soil genesis (early leptosols with predominance of apatite, low

weathered cambisols with P mainly adsorbed on iron oxides, highly weathered podzols with large amounts of P on Al/Fe organic complexes) is clearly recorded in the lake sediments. P K-Edge XANES spectrometry is particularly relevant as a qualitative method to study phosphorus speciation in soils and lake sediments with high precision.

Keywords

Phosphorus speciation, lake sediment archive, soil genesis, XANES

Introduction

In terrestrial ecosystems without external inputs of P by fertilization (pre-human), the speciation of P depends on glaciers activity and state of soil evolution (Walker and Syers, 1979; Filipelli and Souch, 1999, Slaymaker et al., 2003; Filippelli et al., 2006). On a newly-exposed lithic surface, nearly all the P is present in primary phosphate minerals (apatite, monazite, lazulite…). Dissolved phosphorus released from the weathering of these minerals is then i) fixed (adsorbed, chimisorbed or occluded) onto particles, specially on secondary minerals generated by pedogenic processes (e.g. iron, manganese and aluminium oxyhydroxides, edge of clay mineral, CaCO3 particles or Al- and Fe-organic complexes), and co-precipitated mainly with Ca, Al, or Fe, all these compounds constituting the complex fraction called “non-apatite inorganic P” (NAIP) and ii) partially used by the biota leading to the formation of organic P (OP) (Filipelli and Souch, 1999; Filippelli, 2008; Filippelli et al. 2009). P is associated with primary minerals both in the fine and coarse fractions of the sediment but Stone and English (1993) noted that NAIP concentrations increased with

51 decreasing grain size, whereas apatite inorganic P (AIP) contents decreased with decreasing
52 grain size. The reducible oxyhydroxides have large binding capacities for phosphate, due to
53 their immense surface area and numerous delocalized positively charged sites (Froelich,
54 1988; Turner et al., 2007). Under anaerobic conditions, reductive dissolution of these
55 compounds releases phosphate (hydromorphic soil wetlands, or sediments in lake bottom…).
56 Organic P can be partially released as phosphate to soil solution after oxidation of organic
57 matter by bacterial and fungal activities (Bucher, 2007; Chen et al., 2008).
58
59 Therefore, with time and soil development, P is increasingly released from primary
60 phosphate minerals (mainly apatite) and incorporated in the others forms, i.e. the non-apatite
61 inorganic phosphorus and the organic phosphorus. Over time, the total amount of P available
62 in the soil profile decreases, as soil P is lost both in soluble and particulate forms through
63 surface and subsurface runoff (Walker and Syers, 1979; Filipelli and Souch, 1999). In rare
64 case, a steady state is reached with equilibrium between loss of P and new P weathered from
65 apatites at the base of the soil column. Recently several studies have observed
66 transformations of P fractions under ecosystem change (from forest to grassland or the
67 reverse) (Turner et al., 2007; Grossmann and Mladenoff, 2008; Chen et al., 2008; Fillipeli et
68 al., 2009) which shows that terrestrial P cycle is controlled by complex interactions between
69 climate, soil development and vegetation type. These changes have also implications on
70 lacustrine ecosystems. Indeed, the potential impact of particulate-P inputs on trophic level of
71 receiving surface waters depends on the composition and speciation of these particles
72 (Anderson et al., 2008; Norton et al., 2011).
73
74 As the soil is a non-stratified natural object and has geochemical composition that
75 integrates all the conditions acting since the soil development, it is a poor recorder of its own

evolution. Therefore, the model of P fraction evolution in relation with soil and ecosystem development was in fact widely established and tested using downstream lake sediments (Slaymaker et al., 2003; Filippelli, 2006; Filippelli, 2008; Filipelli et al. 2009; Norton et al. 2011). This approach is based on the following assumptions (Filippelli et al., 2009). First, lake sediment archives allow examining an integrated record of catchment-scale processes associated with P cycling on the landscape. Secondly, it allows discrete temporal resolution at a given site, providing an actual record of local processes including landscape stability, soil and ecosystem development. Thirdly, in case of oligotrophic headwater lakes dominated by allochthonous sediment production, lake sediments are usually largely unaffected by diagenetic processes related to sedimentary decomposition of organic matter, and hence have a relatively stable P geochemistry after deposition.

Using the same approach, we propose to study qualitatively the evolution of P species diversity trapped in Lake Anterne sediments, in relation with the soil development. This high altitude site presents the interest that soil development history was already reconstructed since the early Holocene, using mineral and organic geochemical analyses on lake sediments and sources (Giguet-Covex et al., 2011). This previous work constitutes a framework for the interpretation of phosphorus species variability found in lake sediments. Furthermore, to complete and compare with the data obtained on lake sediment archives, analyses on rock and different soil horizons, representing a pedogenesis sequence, are also used. Together, these results will allow better understanding P transformation linked to soil development and evaluating changes linked to biological and/or chemical processes during the sediment transfer or inside the lake.

Phosphorus speciation: a brief overview

Phosphorus speciation is usually carried out by chemical sequential extractions (Dorioz et al., 1998; Kaiserli et al., 2002; Perrone et al., 2008). Such fractionation analyses, based on the different reactivities of solid substrates, have different extractants. The different techniques of extractions require delicate and labored work and some results still raise questions: (1) the specificity of a given extracting agent with respect to a chemical form is always relative, (2) some authors (Barbanti et al., 1994) highlighted redistributions and re-adsorption processes during the extractions, and (3) the relationships between these operationally defined fractions and key properties, such as bioavailability, are poorly understood and subject to debate (Jarvie et al., 2002). Furthermore, these techniques cannot determine mineral species in association with P. Another approach using TEM-EELS has been developed (Poulenard et al., 2008) to improve our knowledge in inorganic P fractionation, but not provide information about organic species. On the contrary, ^{31}P-NMR (Nuclear Magnetic Resonance) techniques can only identified specific organic P compounds (Carman et al., 2000; Turner et al., 2007; Brandes et al., 2007). In this context, we propose to use the P K-edge XANES method to distinguish both the organic and inorganic chemical forms of P. This approach is particularly innovating to study qualitatively P speciation at high resolution (µm) in lake sediments. To our knowledge, only some studies using x-ray at P K-edge have been done on marine sediments (Brandes et al., 2007), peat (Kruse et al., 2008) and soils (Hesterberg et al., 1999; Beauchemin et al., 2003). This method was also applied to characterize P species of different filter materials used for wastewater treatment (Eveborn et al. 2009). All these studies show the promising potential of XANES spectroscopy to characterize chemical species of phosphorus in complex environmental samples. Furthermore, this method is non-destructive, needs very few material quantities and is very sensitive. The high resolution in energy allows also detecting very low phosphorus concentration. Finally, the phosphorus dispersion in samples and the shapes of particles can

be investigated as we can realize high resolution map of elemental distribution. This spatial distribution of P was few exploited in the literature (Brandes et al. 2007; Monnier et al. 2011). The analytical approach applied here consists in the comparison between K-XANES spectra acquired on P particles and on different appropriate organic and mineral references. Furthermore, spectra suggesting blending of several compounds are fitted using linear combination to determine the mixing.

Site location and characteristics

Lake Anterne is a high altitude lake (2063 m asl) located in north French Alps. Its small catchment (2.55 km^2) presents very steep slopes, which are made of easily erodible rocks: mainly calcshales (Bajocian) and black shales (Bathonian-Oxfordian) (Fig.1A). Today, three main types of soils were identified in the catchment: leptosols (~50% of the catchment area), cambisols (~25%) and acid soils (pozosols/stagnosols, ~15%) (Giguet-Covex et al., 2011).

Being done the presence of easily erodible material on steep slopes, lake sedimentation is dominated by erosion processes. The lake is frozen during 6 to 7 months of the year (from November/December to June). Detrital inputs thus occur in summer/autumn. Diatoms were found in sediment traps, which attest also the existence of a lacustrine productivity. However, silica frustules are not preserved in sediments. These characteristics suppose that lake sediments can record phosphorus species coming from soils through catchment erosion processes but also produced by biological activity inside the lake.

Materials and Methods

Reference compounds

Mineral and organic phosphate references were analyzed for the interpretation of XANES data obtained on natural samples. The table 1 gathers all the phosphorus compounds used as reference, including formula, and origin.

Phosphorus minerals. The following inorganic compounds were selected for the study: hydroxyapatite, chloroapatite, monazite, variscite, lazulite, vivianite. These minerals were obtained from the National Museum of Natural History (France), except the monazite reference available on the ID21 beamline (ESRF, non-radioactive mineral).

Synthetic compounds. Phosphated iron oxide samples were prepared following the procedure described by (Huang, 2004). Phosphate adsorption was done both on hematite and goethite (natural and not pure samples) according to the required experimental conditions. 500 mg of iron oxide powder were immersed in 50 mL of phosphate solution (NaH_2PO_4 (0,03 mmol/L), NaCl (0,1 mol/L), sodium acetate (0,06 mol/L)) during 10 days. The pH were measured and adjusted at 6.6 considered as optimized pH by Huang (2004). Then, samples were filtered, rinsed with ultrapure water and dried at 105°C during 24H. Phosphated calcium carbonate and aluminium oxide were also prepared from 500 mg of crushed mineral, immersed in 50 mL of phosphate solution (Na_2HPO_4 (0,01 mol/L)). Humic-Al-phosphate complexes were synthesized from 25 mL of humic acid solution (100 mg/L), 25 mL of phosphate solution (Na_2HPO_4, 2.10^{-2} mol/L) and aluminium oxide (20 g/L) added until precipitation. Phosphate linked to clay minerals (montmorillonite) by organic (humic)

coating was prepared after to have left aside, during one night, 500 mg of montmorillonite in 25 ml of a calcium chloride solution ($CaCl_2$, 2.10^{-2} mol/L). The next day, 25 mL of humic acids and 25 mL of phosphate solution (NaH_2PO_4, 2.10^{-2} mol/L) were added. These four preparations were filtered 4 days later, rinsed with ultrapure water and dried at 60°C during 24H. Any measurement was realized to get the amount of phosphate adsorbed.

Commercial organic references of phosphorus. As organic compounds, molecules of ATP, organic acid (Phenylphosphonic acid), phospholipids (3-*sn*-Phosphatidylethanolamine) and phosphoamine (*O*-Phosphorylethanolamine) were chosen (Sigma-Aldrich). The ATP represents the most abundant molecule with P in the life world (Filippelli, 2008).

Natural standards. In order to have also more representative signatures of organic phosphorus in living organisms susceptible to be found in our system, samples of diatoms (*Cyclotella Meneghiniana*) and cyanobacteria (*Planktotrix Rubescens*) were also analyzed. These natural standards come from culture realized at INRA-CARRTEL laboratory (France). The fingerprint of phosphorus in the top layer of soils, i.e. litters coming from meadow, mixed forest and conifer forest were also analyzed.

Soil samples

The soil samples, collected for this study, represent a pedogenetic sequence, from the rock (parent material, calcshales (S3)) to the developed soil characterized by a cambisol (S1) and going through a leptosol (S2). Two samples were taken from the cambisol profile: the surface horizon (S1-hor A) and the horizon just below (S1-hor B) (Fig. 1). Today, the Anterne catchment is made of alpine grassland, but pollen and macrofossil data indicate the existence of conifers during the first half of the Holocene (David, 2010; Giguet-Covex et al., 2011). That is why we analyzed different litters.

200 ***Lake sediment samples***

201

202 Five lake sediment samples were selected taking into account the environmental
203 evolution of the catchment (Giguet-Covex et al., 2011). The sample ANT#1, dated at 10000
204 cal. BP, corresponds to a phase where rocks and leptosol constituted the main material
205 eroded due to the quasi-absence of vegetation and thus of developed soils. The sample
206 ANT#2 is dated at 8450 cal. BP. It is in a period of vegetation and soil development, which
207 reduce the catchment erosion. In 5550 cal. BP (ANT#3), the catchment has probably reached
208 its maximum of vegetation cover. These environmental conditions with low erosion and
209 mainly of soil surface horizons favour the appearance of anoxic conditions in the lake
210 bottom. The two other samples (ANT#4 and ANT#5) correspond to periods of high erosion
211 rates in 4910 and 2125 cal. BP, respectively, linked to wetter climate and human impact on
212 soil stability. The climatic change is characterized by erosion of both developed soil surface
213 horizons and leptosols while during the "anthropogenic" period developed soils are mainly
214 eroded.

215

216 ***Sample preparation for XANES and XRF analyses***

217 Soil and lake sediment samples were oven-dried at 60°C and powdered. Samples of
218 rock, leptosol and of standards were also powdered. These powders of standard and natural
219 samples were prepared as pellet of 3 mm of diameter protected by film of ultralene®. The
220 diatoms and cyanobacteria liquid samples were filtered and then rinsed several times with
221 distilled water in order to eliminate the phosphorus contained in the breeding ground. Then,
222 filters were oven-dried at 60°C. Analyses at P K-edge XANES were realized directly on
223 small pieces of filters protected by ultralene® film.

224

225 ***XRF analyses***

226 The phosphorus concentration (expressed as oxide weight, P_2O_5) of each sample was
227 determined by x-ray fluorescence (XRF) on fused beads. Analyses were performed with an
228 XRF spectrometer at the University Claude Bernard, Lyon (FRANCE).

229

230 ***µ-XRF/XANES***

231 X-Ray fluorescence based on synchrotron radiation (SR-XRF) was realized at the
232 ID21 beamline at the ESRF (www.esrf.fr/UsersAndScience/Experiments/Imaging/ID21) at
233 2.2 keV (P K-edge) to detect the repartition of P and lower elements and to combine
234 µXANES measurements (Susini et al., 2002).

235 The setup of the ID21 beamline consists of a fixed exit double crystal monochromator
236 equipped with Si(111) to tune the beam energy. Sample was tilted by an angle of 30 ° with
237 respect to the incident beam. Chemical maps were obtained by means of a microbeam
238 focused thanks to a Fresnel zone plate to 0.2 x 0.8 μm^2, in area of 100 x 100 μm^2. The micro-
239 fluorescence signal was collected in the horizontal plane perpendicular to the incident beam
240 direction by using a SDD (silicon drift detector). A videomicroscope, placed perpendicularly
241 to the sample, enables the observation of the sample under visible light, and the selection of
242 region of interest.

243 P K-edge XANES spectra were collected under high-resolution conditions in energy
244 (0.2 eV) between 2.13 and 2.2 keV. The XANES spectra were acquired in fluorescence mode
245 (SDD). Unfocalized beam (200 µm) was used for the acquisition of reference compounds,
246 whereas spatially resolved XANES (or µXANES) spectra were acquired thanks to the
247 microbeam (0.2 x 0.8 μm^2).

248

249 ***Data treatment***

The P K-edge XANES spectra were normalized using conventional methods and then compared to the P-based minerals database containing all the phosphorus reference compounds detailed previously (Fig. 2). Assumption was checked by fitting some of P K-edge XANES spectra from various points in natural samples (soils and sediments) using linear combination of reference spectra (DeAndrade et al., 2011). This technique estimates the contribution of each reference (mineral or organic phosphorus) to the fitted spectrum of an unknown phase (Fig. 3). The use of this technique was validated for mixtures of phosphorus associated with calcium, aluminium and iron (Ajiboye et al., 2007).

The treatment of the 2D XRF map consists both in the classical fitting for the fluorescence spectra and then reconstruction of chemical map (PyMCA software, ESRF) (Sole et al., 2007).

Results

Phosphorus standards

The positions of white line peaks for mineral references (minerals containing phosphorus and phosphorus adsorbed onto mineral surface) vary between 2.1517 and 2.1534 keV (Fig. 2). Spectra of all mineral references present specific pre- and/or post-edge features which guaranty the possibility to differentiate one mineral from another. In particular, monazite is characterized by a broad white line, which in our case is subdivided in two peaks and by four unique post-edge resonances between 2.1591 and 2.1797 keV. PO_4 adsorbed on goethite presents a pre-edge feature, between 2.1477 and 2.1506 keV. This feature, already underlined by other studies (Hesterberg et al., 1999; Beauchemin et al., 2003; Eveborn et al., 2009), is weaker in the K-edge XANES spectrum for PO_4 adsorbed on hematite. It is also noticeably on the vivianite K-edge XANES spectrum (Brandes et al., 2007). It is linked to the oxidation state of iron associated with phosphate (Ingall et al. 2011). PO_4 adsorbed on

goethite presents unique post-edge features with absorbance higher than all the other references. Samples of apatite-group (chloroapatite, hydroxyapatite) and of PO_4 adsorbed on calcium carbonate exhibited a post-edge shoulder between 2.1548 and 2.1593 keV. (Hesterberg et al., 1999; Beauchemin et al., 2003; Kruse et al., 2008; Brandes et al., 2007). A similar post-edge shoulder is also observed for PO_4 adsorbed on hematite. However, it is less pronounced than for apatite minerals. The two following resonances between 2.1613 and 2.1822 are located at the same positions for apatite minerals and PO_4 adsorbed on hematite. P K-edge XANES spectra of lazulite and variscite have similar trends with, in particular, a broad peak between 2.1655 and 2.1755 keV. P K-edge XANES spectra of PO_4 on Al oxide and PO_4 adsorbed or co-precipitated with Al-humus complexes present similar XANES spectra as variscite and lazulite, with the same broad peak (Fig. 2; Beauchemin et al., 2003; Eveborn et al, 2009). Their white lines have just higher intensities. PO_4 associated with humic-montmorillonite complexes is characterized by a P K-edge XANES spectrum with a thin white line at 2.1533 keV and an asymmetric post-edge resonance at 2.1679 keV.

P K-edge XANES spectra of organic references present clear differences with the mineral ones. They had principal K-edge peak energies (white line) between 2.1524 and 2.1528 keV. All spectra present very broad post-edge resonance. However, differences appear in the energy position of these resonances, which would have to provide a mean to distinguish those (Brandes et al., 2007). As suggested by Brandes et al. (2007), the shifts in these energy positions may be attributed to the size of phosphate chains. Indeed, polyphosphates have peak higher in energy than monophosphates. The phosphonic acid is the only spectrum, of our organic standards, exhibiting a pre-edge characteristic.

As predicted, the P K-edge XANES features of the litter samples could be attributed to organic compounds. Linear decompositions of XANES spectra suggest mixing between cyanobacteria (~60 %) and phospholipids (~40 %) for the meadow and mixed forest and

between cyanobacteria (~78 %) and algae (22 %) for the conifer forest. These results could signify that the P K-edge XANES fingerprint of cyanobacteria more largely reflects microbial communities. Consequently, high proportion of phosphorus in litters appears to be linked to microbial activity. Microbial intracellular P is usually mainly made of nucleic acids (60 %), acid soluble P-ester (20 %) and phospholipids (5 %) (Stewart and Tiessen, 1987). However, the proportions determined through linear combinations have to be taken with caution because they depend on organic phosphorus standards used in the study (Beauchemin et al., 2003), which does not reflect the complete pool. In particular, inositol phosphorus (storage of organic phosphorus in plants), representing an important contribution to soil organic P (one third, Stewart and Tiessen, 1987), is not taken into account in our study.

Soil samples

The noise of most soil sample K-edge XANES spectra is relatively high and sometimes there are diffraction peaks at high energies (> 2.17 keV). However, being done the specific characteristics of each species below this energy, it is possible to recognize the phosphorus forms.

Calcshale (S3) presents low phosphorus content (0.07% of P_2O_5). Phosphorus particles measure between 5 and 10 µm as shown by x-ray fluorescence imaging (Fig. 4) and correspond to apatite according to the shape of XANES spectra (Fig. 4, shoulder in the post-edge region). In leptosol (i.e. the first stage of soil weathering S2), phosphorus content increases (0.19% of P_2O_5). XANES spectra show the presence of apatite as in calcshale (S3) (particles ***a*** and ***b***, Fig. 4). We observe also a new phosphorus form which is less concentrated in phosphorus (particle ***g,*** Fig. 4) and whom XANES spectrum differs from all

the references analyzed. Furthermore, the poor quality of the linear fit confirms the presence of an additional compound relative to references taken into account in our study.

The deep cambisol horizon (S1-hor B) is made of various phosphate species (Fig. 5). According to the shape of P K-edge XANES spectra obtained, the different particles are mainly made of apatite (particle ***i***, which is the most concentrated in P), phosphate adsorbed on hematite with a possible contribution of organic phosphorus (meadow litter) (particle ***h***) and phosphate adsorbed on aluminium oxide or humic-Al-phosphate complex (particles ***j*** and ***k***, Fig. 5 and 3A). There is also the unknown phosphorus form (particle ***l***) already observed in leptosol. This soil sample is more concentrated in phosphorus (0.23% of P_2O_5) than the leptosol. The cambisol surface horizon (S1-hor A) contains apatite (particles ***m*** and ***n***, Fig. 5 and 3B), phosphate adsorbed on hematite (particles ***o*** and ***p***, Fig. 5 and 3C) and a large proportion of the unknown phosphorus particles (***q***, ***r***, ***s*** and ***t***, Fig. 5). Although the results from linear decomposition have to be taken with caution, they suggest the presence of organic phosphorus (30 to 60 % of P from litter) associated with particles of phosphate adsorbed on iron oxides (hematite, Fig. 3C).

Well-defined grains characterize the undetermined phosphorus particles. This feature and the shape of XANES spectra show that organic phosphorus specie is unlikely. This form is thus inorganic phosphorus (P adsorbed or co-precipitated or associated with a mineral), different from humic-clay-phosphate complexes, humic-Al-phosphate complexes, and phosphate adsorbed or co-precipitated with iron or aluminium oxides and calcite.

Lake sediment samples

347 The oldest sample (ANT#1, 10 000 cal. BP) has the lowest phosphorus concentration
348 of the lake sediment samples (0.15% of P_2O_5). The punctual μ-XANES analyses show that
349 this phosphorus is only under form of apatite (particles ***a***, ***b***, ***c***, Fig. 6).
350 The sample corresponding to 8450 cal. BP (ANT#2) presents 0.16% of P_2O_5. The
351 main phosphorus particles (***d***, ***e*** and ***f***) are also apatite (Fig. 6). Another group of particles (***g***
352 and ***h***) presents pre-edge and post-edge characteristics similar to the ones of phosphorus
353 adsorbed on iron oxides (mainly goethite) (Fig. 3D and 6). The linear deconvolution of
354 spectra suggests also a contribution of organic phosphorus (around 20 % of diatom-type, Fig.
355 3D).
356 In sample ANT#3, containing 0.19% of P_2O_5 and dated at 5550 cal. BP, phosphates
357 are mainly adsorbed on goethite (particles ***l*** and ***m***; Fig. 6) and on hematite (particle ***n***; Fig.
358 6). A contribution of organic phosphorus (30-50 %) on particles of phosphate adsorbed on
359 iron oxides is suggested by the linear deconvolution. We observe also humic-Al-phosphate
360 complexes (particles ***j*** and ***k***). In this sample ANT#3, there is also organic phosphorus (48 %
361 of diatom and 30 % of conifer litter; particle ***i***, Fig. 6).
362 At 4910 cal. BP, the sample ANT#4 (0.17% of P_2O_5) contains monazite (particle ***p***)
363 and humic-Al-phosphate complexes (particles ***q*** and ***r*** Fig. 7). The XANES spectrum of
364 particle ***s*** is different from our references. However, the shape of the spectrum suggests a
365 primary phosphate mineral. The sample ANT#5, corresponding to 2125 cal. BP, is made of
366 apatite (***u*** and ***v***, Fig. 7), phosphate adsorbed on hematite (particle ***t***) and humic-Al-phosphate
367 complexes (particle ***w***). Its phosphorus concentration is 0.18 % (P_2O_5).
368
369
370 **Discussion**
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372

373 ***Soil sequence and phosphorus species***

374

375 On the contrary to the classical model of P geochemistry evolution in soils proposed
376 by Walker and Syers (1976), the phosphorus concentration increases across de pedogenetic
377 sequence, i.e. in developed soils relative to rock and leptosol. This is explained by the
378 pedogenetic process of carbonate dissolution, which concentrates the phosphorus (Giguet-
379 Covex et al., 2011). Across the sequence, we observe an increase in phosphorus form
380 diversity (Fig. 8), as predicted by the model (Walker and Syers, 1976; Filipelli and Souch,
381 1999). The rock contains only phosphorus under form of apatite. Then, in the first step of
382 pedogenesis, mainly characterized by carbonate dissolution, a new form of phosphorus
383 appears (Fig. 8). XANES spectra of this specie are very different from our references and
384 from spectra measured in other studies (Beauchemin et al., 2003; Brandes et al., 2007;
385 Eveborn et al., 2009; Ingall et al., 2011). This specie presents a high contribution in cambisol
386 surface horizons and is absent in lake sediments (Fig. 8), which suggests an unstable phase,
387 transformed during the transport toward the lake or inside the lake.

388 These observations led us to two different hypotheses about the possible identification
389 of this compound. First, this form could be opal-associated P in phytoliths. Indeed, as
390 diatoms are not preserved in lake sediment, it is reasonable to think that phytoliths formed by
391 plants in soils and coming from erosion, are not preserved anymore in the lake. The presence
392 of opal-associated P in phytoliths is not mentioned in the literature. Nevertheless, it was
393 recently underlined in diatom frustules, which has significant implications for the marine P
394 cycle (Latimer et al., 2006). In other hand, organic carbon is frequently found in relatively
395 large amounts in phytoliths (Parr and Sullivan, 2005). P sequestration in phytoliths seems
396 thus likely. Being done the important production and conservation of phytoliths in topsoils of

herbaceaous sub-alpine environments (Carnelli et al., 2001; 2004), a high accumulation of opal-associated P in topsoils of the cambisol (S1-hor A), seems logical. The presence of this form could have important consequences for the lake productivity and our knowledge of the relationships between the lake and the soil and vegetation development in the catchment. However, if in some cases, XRF maps show very high concentrations of Si for these compounds, there are also cases where no (or very few) silica seems associated with these particles (for example particle ***g*** in Fig. 4), which contradicts our first hypothesis. The second hypothesis is P associated with Mn oxide. Manganese oxides have been frequently described as phosphate scavengers (Yao and Millero, 1996). The anoxic conditions prevailing in the sediment during winter (with ice cap) could lead to the reduction of Mn oxides and then to the lack of such form in lake sediment. Unfortunately, our XRF map does not allow testing this hypothesis. In both case, further works are need to better identify this form, which seems to have a key role in the relationships between soils and lake.

The both cambisol horizons are mainly made of phosphate associated with metals (iron oxides and secondarily humic-Al-phosphate complexes) and of undetermined phase. Some primary phosphate minerals (apatite) are also found. These apatite minerals are relics of the parent material. Linear decompositions of XANES spectra associated with phosphate adsorbed on hematite often suggests also a contribution of organic phosphorus. This could signify that phosphates are, in reality, linked to Fe-organic complexes as this is the case for aluminium. The liberation of iron and aluminium during the pedogenesis process and the integration of organic matter coming from the litter allow the formation of these phosphates. Jörg (2010) suggests that humic-metal-P associations are important in soils, which supports our interpretation. This P specie is a source of plant-available phosphate (Jörg, 2010). Furthermore, other studies have shown complexation of inositol phosphate (phytic acid accounting for up to one third of the soil organic P) with amorphous metal oxides (Fe and Al)

(Stewart and Tiessen, 1987 and references therein; Turner et al., 2007). The formation of such associations is favoured by the development of podzols as the podzolisation processes is largely due to the formation of such Al/Fe humus complexes (Skjemstad et al., 1992; Sauer et al., 2007).

Lake sediment record of P species

Besides the information obtained by soil sequence study, lake sediments bring indication of the timing of processes leading to the phosphorus species transformation in soils. At the onset of the Holocene, phosphorus is only under form of apatite as in rock and leptosol, which is concordant with the absence (or almost) of developed soil and vegetation cover shown by the previous study (Giguet-Covex et al., 2011). During the period of soil development (ANT#2, 8450 cal. BP, Fig. 8), phosphates adsorbed on goethite are recorded in lake sediments. The contribution of organic phosphorus, suggested by the linear decomposition, is interpreted as reflecting the presence of phosphate adsorbed on Fe-organic complexes. At 5550 cal. BP, the soil and vegetation covers are in the period (7850-5550 cal. BP) of their maximal expansion of the whole Holocene. The phosphorus species are more diversified. We can find phosphate adsorbed on Fe (or more probably humic-Fe-phosphate complexes), humic-Al-phosphate complexes and organic phosphorus (Fig. 8). The two first components are present in developed soils (mainly horizon B, Fig. 8). However, a formation of these phosphate-metal-organic complexes inside the lake, from lacustrine organic matter and Al and Fe inputs linked to catchment erosion, cannot be excluded. Secondary Al and Fe-bearing phases are characteristic of acidified soils such as podzol (Kopáček et al., 2009; Mourier et al. 2010; Norton et al., 2011), which suggests that lake sediments record a period

447 of podzol development. The absence of apatite is also consistent with this assumption as the
448 apatite solubility increases under acidic conditions (Lindsay et al. 1979). The origin of the
449 organic phosphorus observed alone (aquatic or terrestrial from litters) cannot be determined
450 with our data. Nevertheless the presence of organic phosphorus coming from algae suggested
451 by several linear deconvolutions of lake sediment samples (in particular for XANES spectra
452 mainly composed of phosphate associated with Fe) and their absence in fitting of soil
453 samples, could underline the preservation of lacustrine organic P in sediments. Consequently,
454 at least a part of the organic phosphorus recorded in lake sediments cannot be only
455 interpreted as reflecting directly P transformations driven by soil development. This result
456 shows the importance of in-lake processes on P species recorded in lake sediments, whereas
457 the system is mainly controlled by catchment erosion.
458 The second half of the Holocene is characterized by important soil erosion processes,
459 triggered first by a climate reversal toward wetter and colder conditions (sample ANT#4,
460 4910 cal. BP) and then by human activities at the end of Iron Age (sample ANT#5, 2125 cal.
461 BP) (Giguet-Covex et al. 2011). Primary phosphate minerals (monazite or apatite) and
462 humic-Al-phosphate complexes are trapped in lake sediments during both periods. However,
463 the period of climatic reversal presents more humic-Al-phosphate complexes. The
464 anthropogenic phase records also PO_4 adsorbed on hematite. Phosphorus species found at
465 4910 cal. BP, suggest erosion of both podzols and low weathered soils, while during the
466 anthropogenic phase, cambisols seem to mainly contribute to erosion. These assumptions are
467 consistent with the story of soil cover in the catchment inferred from greochemcial study of
468 the lake sediment and interpreted as the result of climatic condition changes and human
469 activities, respectively (Giguet-Covex et al., 2011). Furthermore, the erosion of cambisols
470 after a period of intense erosion of soil cover developed formerly in forest conditions

(podzol), underlines the existence of regressive soil genesis step (*sensu* Johnson et al., 1990) during the second half of the Holocene.

Model of phosphorus evolution linked to soil development

The results obtained in this study indicate that phosphorus trapped in lake sediments reflects, at least in part, the soil evolution in the catchment, as the lake sediment record is concordant with the schema drawn by the soil sequence and thus with the model proposed by Walker and Syers (1976) and picked up again by others (Stewart and Tiessen, 1987; Filipelli and Souch, 1999; Slaymaker et al., 2003; Filippelli et al., 2009). Development of soil led to a gradual transformation of phosphorus in primary minerals to i) phosphorus adsorbed on reactives primary constituents (iron oxides) and ii) phosphorus co-precipitated in secondary components. In the case of a subalpine zone, these secondary constituents are essentially amorphous phases or organometallic complexes. Simultaneously, organic phosphorus increases as the result of P incorporation from the vegetation. In our study, except in litters, this organic phosphorus is rarely found alone. It seems to present a high affinity to form complexes with metals. The growing of this vegetation is allowed by the liberation of phosphate during the weathering process. Firstly, the N-fixing vegetation is installed which provides the other important nutrient, the nitrogen (which does not exist in rocks), to the system and favours the arrival of other plants and trees. Humic-clay-phosphate complexes were not observed in soils and lake sediments. This phosphorus fraction, already observed in river sediments (Poulenard et al., 2008), does thus not seem to play an important role in our mountain system. However, the undetermined phosphorus fraction found in high quantity in soils shows that a part of P cycle in soils is not well understood. Further investigations will be

496 necessary to determine the mineral associated with the phosphorus. As this undetermined
497 phase is not found in lake sediments, it is probably a very easily assimilated form which
498 contributes to dissolved P inputs to the lake water and thus to the primary productivity. Since
499 the natural evolution of P in catchment controls, in large part, the lake ecosystem
500 development (Anderson et al., 2008; Heggen et al., 2010; Norton et al., 2011), good
501 assessment of P cycle in mountain environment can help us to improve our knowledge of lake
502 ecosystems functioning and their modifications over time in connection with catchment
503 evolution.
504
505
506 ***XANES for P fractionation in soils and Lake sediment***
507
508 The main interest of P K-edge XANES is the possibility to study, at the same time, the
509 diversity of organic and inorganic P species. The analytical approach of the study, with
510 focused measurements on P particles of different P concentration, is useful to evaluate the
511 diversity of P species. In particular, it allows studying the interactions between organic and
512 inorganic P. However, this approach does not allow accessing to quantitative information
513 about theses species. It would have been necessary to make unfocused analyses on bulk
514 samples. But, results of linear combinations, in this case, have to be taken with cautious due
515 to the possibility to have complex mixing and to the insensitivity to P species of low
516 concentration (Beauchemin et al., 2003). The both approaches thus appear complementary.
517 Linear deconvolution of XANES spectra obtained for each P particles has also to be taken
518 with cautious. Indeed, the goodness of the fitting is inherently restricted by the data quality
519 (signal/noise ratio) and how well the chosen set of standards represents the real species in the
520 sample of unknown composition (Beauchemin et al., 2003). However, the second problem

has a limited effect in our case as the "source to sink approach" allows us knowing, at least in part, the species susceptible to be brought to the lake by erosion processes and coming from the primary production. Finally, synchrotron techniques present the disadvantages that we are limited in the number of samples we can analyze. In particular, for paleolimnological or paleoenvironmental studies, this method is not enough to reconstruct and understand changes linked to climatic, environmental or anthropogenic changes. It has to be coupled with classical sequential extractions of P species.

Conclusion

P K-edge XANES spectroscopy was used successfully to study phosphorus species evolution in relation with soil development. Important information was also provided thanks to the "source to sink approach" applied through analyses of rock, soil and lake sediment samples. The key results of our study are (1) the highlight of P species diversity increase due to pedogenetic processes, (2) the determination of the presence of podzols in lake sediment samples corresponding to 4910 and 5550 cal. BP, and (3) the underlining of an undetermined phase in soils which probably affects the concentration of phosphorus available in lake water and thus the productivity. Future investigations will be necessary to reveal this phosphorus fraction and understand its role in P cycle of mountain catchment-lake systems.

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3. Reconstitution du régime de l'oxygène dans le Lac d'Annecy à partir des assemblages de chironomes de 3 archives sédimentaires spatialisées depuis 150 ans

❖ *Article : Chironomid assemblages in cores from multiple water depths reflect oxygen-driven changes in a deep French lake over the last 150 years*

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Chironomid assemblages in cores from multiple water depths reflect oxygen-driven changes in a deep French lake over the last 150 years

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Abstract

We sampled modern chironomids at multiple water depths in Lake Annecy, France, before reconstructing changes in chironomid assemblages at sub-decadal resolution in sediment cores spanning the last 150 years. The lake is a large, deep (z_{max} = 65 m), subalpine waterbody that has recently returned to an oligotrophic state. Comparison between the water-depth distributions of living chironomid larvae and subfossil head capsules (HC) along three surface-sediment transects indicated spatial differences in the influence of external forcings on HC deposition (e.g. tributary effects). The transect with the lowest littoral influence and the best-preserved, depth-specific chironomid community characteristics was used for paleolimnological reconstructions at various water depths. At the beginning of the 20th century, oxygen-rich conditions prevailed in the lake, as inferred from *M. contracta*-type and *Procladius* sp. at deep-water sites (i.e. cores from 56 and 65 m) and *Paracladius* sp. and *H. grimshawi*-type in the core from 30 m depth. Over time, chironomid assemblages in cores from all three water depths converged toward the dominance of *S. coracina*-type, indicating enhanced hypoxia. The initial change in chironomid assemblages from the deep-water cores, occurred in the 1930s, at the same time that an increase in lake trophic state is inferred from an increase in total organic carbon (TOC) concentration in the sediment. In the 1950s, an assemblage change in the core from 30 m water depth reflects the rapid expansion of the hypoxic layer into the shallower region of the lake. Lake Annecy recovered its oligotrophic state in the 1990s. Chironomid assemblages, however, still indicate hypoxic conditions, suggesting that modern chironomid assemblages in Lake Annecy are decoupled from the lake trophic state. Recent increases in both TOC and the Hydrogen Index (HI) indicate that changes in pelagic functioning have had a strong indirect influence on the composition of the chironomid assemblage. Finally, the dramatic decrease in HC accumulation rate over time suggests that hypoxic conditions are maintained through a feedback loop, wherein the

accumulation of (un-consumed) organic matter and subsequent bacterial respiration prevent chironomid re-colonization. Our results support the idea that study of cores from multiple water depths is relevant in a large, deep lake compared to the sampling of a unique core to assess the extent and the dynamic of ecological changes.

Keywords: Paleoenvironmental reconstructions, Re-oligotrophication, Pelagic-benthic links, Hypoxia

Introduction

Paleolimnological studies provide valuable information on environmental changes in lakes over extended time scales. The increasing influence of anthropogenic forcing on these systems has led to a growing body of studies that focus specifically on ecological changes that lakes have undergone over the last century (Battarbee et al. 2011). Among the numerous sediment variables that can be used as proxies to infer ecological changes in lakes, biological remains, especially chironomid head capsules (hereafter HC), have been used extensively to infer past climate and environmental variables such as temperature (Millet et al. 2012), oxygen concentration (Quinlan and Smol 2002; Brodersen and Quinlan 2006) and lake productivity (Woodward and Shulmeister 2006).

Most of these paleolimnological investigations were carried out in relatively small lakes. For example, Larocque et al. (2001) studied chironomid-temperature relationships in a set of lakes (n=100) with surface areas < 20 ha. Small, shallow lakes were favored in climatic and environmental reconstructions because chironomids respond more directly to environmental variables in these lakes than they do in large and deep lakes (Eggermont and Heiri 2011). In such small systems, a single sediment core from the deepest part of the lake has been hypothesized (Hofmann 1988) or shown (van Hardenbroek et al. 2011) to integrate biological remains from the entire lake basin. A detailed analysis of the spatial variability of chironomid remains in shallow Norwegian lakes (Heiri 2004), however, highlighted the existence of spatial patterns in the distribution of HC, although most taxa were present in sediment from the maximum depth.

Recent studies highlighted important depth-related differences in the composition of biological remains in samples from surficial sediment in shallow (Engels and Cwynar 2011; Cao et al. 2012) and deep lakes (Eggermont et al. 2008; Kurek and Cwynar 2009). In the

large, deep lakes, results indicate the bathymetric structure of the chironomid community is effectively preserved in the sediment. These water-depth-dependent differences in assemblage composition are common in chironomid communities and are related to variability in environmental conditions such as oxygen availability (Quinlan and Smol 2002) or habitat structure, e.g. macrophyte cover (Langdon et al. 2010). Reconstructions using chironomid assemblages from different depths should therefore more accurately describe the chironomid assemblage composition at the whole-lake scale, and enable inference of temporal changes, which are both of primary interest in environmental policy (Water Framework Directive [WFD] 2000). Furthermore, anthropogenic effects on living organisms may differ along the depth gradient. For example, increased nutrient loading often induces collapse of deep-water chironomid assemblages as a consequence of hypolimnetic hypoxia, whereas in the littoral zone, assemblages can be richer and more abundant because of increased macrophyte coverage under moderate eutrophication (Langdon et al. 2010; Millet et al. 2010). These spatially variable responses, observed mainly in chironomid densities, have multiple effects on the lake ecosystem. Changes in chironomid densities influence sediment structure and mineralization processes (Olafsson and Paterson 2004) and also have implications for fish populations (Vander Zanden et al. 2003). Thus, the depth-specific dynamics of chironomid assemblages must be considered to better specify the ecological consequences of anthropogenic forcings on the structure and function of food webs in large, deep lakes (Free et al. 2009).

A prerequisite for multiple-depth assemblage reconstruction is that HC assemblages from cores collected at various depths accurately reflect the chironomids that lived at those depths all around the lake, i.e. there has been limited vertical HC transport. The suitable location for such chironomid reconstructions will differ depending on the lake in question, because of its morphometry, water circulation, tributaries and wind effects (Schmäh 1993;

Brodersen and Lindegaard 1997). Comparison of the depth distributions of larvae and HC along depths transects can be used to test whether HC reflect accurately the bathymetric distribution of the live larvae. If the HC assemblage is similar to that of living larvae at multiple depths, the depth-specific chironomid assemblage reconstructions will be more reliable. Such an approach can substantially improve the robustness of chironomid-based reconstructions, but to our knowledge, this multiple-transect approach has not been used previously, coupled with a HC-living larvae comparison.

In this study, we evaluated chironomid assemblages at three different depths in re-oligotrophied Lake Annecy, a large, deep pre-alpine lake that underwent eutrophication during the second half of the 20th century. Our objectives were:

1) to compare the bathymetric distributions of HC and living larvae along three water-depth transects to assess differences in chironomid assemblage composition and identify a suitable location within the lake to perform chironomid reconstructions.

2) to evaluate the gain in ecological information provided by a multiple-depth coring approach (community composition, depth-specific temporal changes) compared with the ecological information obtained from a single core retrieved from the deepest region of the lake.

3) to assess the extent of ecological changes on a whole-lake scale using depth-specific (i.e. local) chironomid assemblage changes. These changes were interpreted considering the direct influence of changes in the organic matter content of the sediment as well as oxygen constraints.

Study area

Lake Annecy is a large (2,740 ha), deep (65 m) hard-water lake located in the French pre-Alps (45°50' N, 6°40' E, 447 m a.s.l.) (Fig. 1). The lake basin is surrounded by calcareous rocks of Mesozoic age. It comprises two sub-basins, the "Grand Lac" to the north and the "Petit Lac" to the south, which are separated by a submerged bar reshaped by glaciers (Nomade 2005) (Fig. 1). The local climate is characterized by dry, snowy winters and warm summers associated with high rainfall (100-150 cm yr^{-1}). The lake is monomictic and mixing usually occurs from December to February (Balvay 1978). The land cover of the watershed is constrained by the strong altitudinal gradient (alt. max. = 2,351 m). Forests (63% of the total watershed area) are mainly found on steep slopes, whereas pastures and cultivated areas (21%) are restricted to flat lowlands. The urbanized area of the watershed (13%) is mainly concentrated along the lake shore, where tourism has developed since the 1970s. The current total population in the catchment is 186,000.

Lake Annecy is currently oligotrophic and total phosphorus concentrations (TP) in the epilimnion have been about 7-8 µg L^{-1} during winter circulation since the 2000s (INRA/SILA data). Prior to the 1940s, the phytoplankton community of Lake Annecy was dominated by taxa such as *Cyclotella* spp., which are typical oligotrophic diatoms (Leroux 1908). A decrease in the oxygen content of the hypolimnion was first reported in 1937 by Hubault (1943), signaling the earliest signs of eutrophication, followed by algal blooms in the 1940s (Servettaz 1977). Monitoring data (INRA/SILA) indicate that Lake Annecy was in a mesotrophic state in the mid-1970s, with TP levels reaching approximately 15 µg L^{-1} during winter mixing (Perga et al. 2010). A remediation plan, including wastewater collection and treatment, was implemented in 1957 and completed in 1967. Since the 1970s, remediation has reduced TP in the water column, leading to a return to oligotrophy in the 1990s (TP < 10 µg L^{-1}).

The fish community of the lake has been managed since the late 19th century, when Whitefish (*Coregonus* sp.) and Arctic char (*Salvelinus alpinus*) were introduced (Leroux 1908). The coregonid population was supported by sporadic introductions from 1900 until the 1930s and by annual introductions every winter from 1936 to 1997. Currently, the fish resources of the lake are exploited by two professional fishermen and ~2,000 recreational anglers.

Materials and methods

Coring and sampling of surface sediment

Living chironomid larvae were studied in 48 sediment samples retrieved from four water depths (2, 30, 56 and 65 m) along three transects (Fig. 1). The top 5 cm of sediment was sampled using an Ekman grab in spring 2008 and autumn 2009 (two samples/depth/transect/year). This sampling strategy was designed to avoid under-representation of chironomid taxa arising from different species phenologies. Sediment samples were preserved in the field using 60% ethanol.

For the chironomid HC analysis (i.e. assemblage reconstructions), nine sediment cores were retrieved at the points on the transects where larvae samples were obtained, at 30, 56 and 65 m water depth, using a gravity corer (Uwitec, Austria) (Fig. 1). Cores were not retrieved at 2 m depth because not enough sediment had accumulated or sediments were disturbed by resuspension. An additional sediment core was obtained from the deepest area of the lake (65 m) in 2009 and devoted to chronology (Fig. 1).

Chironomid analysis

In the laboratory, surficial sediment obtained using the Ekman grab was sieved through a 250-µm-mesh sieve. Chironomid larvae were hand-sorted from the residue under a stereomicroscope at 40× magnification. Chironomid larvae were treated with cold KOH

solution (10%) overnight and were mounted on slides ventral side up. Identification of larvae was performed using Wiederholm's (1983) guide.

HC were extracted from the top cm of the nine sediment cores and from samples taken at 0.5-cm intervals in cores from the selected transect "Sévrier" (Sev65, Sev56 and Sev30), spanning the last 150 years. The procedure involved treatment with HCl (10%) and KOH (10 %) and sieving of the sediment through 100- and 200-µm-mesh (Walker 2001). Chironomid HC were hand-sorted from the sieved residue under 40-70× magnification and mounted ventral side up on microscope slides using Aquatex® mounting agent. Identification of specimens to the genus or species-group levels followed Wiederholm (1983) and Brooks et al. (2007) and was performed under 100-1000× magnification.

Core chronology

To establish a chronology for the cores, a reference chronology was developed on the LDAref core from the deepest part of the lake. The activities of ^{210}Pb, ^{226}Ra, ^{137}Cs and ^{241}Am were measured in approximately 1 g of dried sediment by gamma spectrometry, using very-low-background, high-efficiency well-type Ge detectors in the Modane Underground Laboratory (Reyss et al. 1995). Six standards were used to calibrate the gamma detectors (Cazala et al. 2003), and a 24–48 h count time was required to achieve a statistical errors <10% for excess ^{210}Pb in the deepest samples and for the 1963 peaks of ^{137}Cs and ^{241}Am. Excess ^{210}Pb activity was calculated as the difference between total ^{210}Pb activity and ^{226}Ra activity (supported ^{210}Pb).

Correlation between the cores from the selected transect ("Sévrier") was achieved following different procedures. Cores Sev65 and Sev56 were correlated to the reference core using visible lithological tie points. The shallow-water core, Sev30, had no visible lithological

markers. It was therefore correlated to the reference core using high-resolution spectrophotometry. The first derivative value at 555 nm was chosen as a proxy for clay mineral composition (Damuth and Balsam 2003). Clay minerals in Lake Annecy are derived from pedogenic processes in the catchment (Manalt 2001), and because they represent the smallest grain-size fraction, they are easily transported far into the lake from the river mouth, via discharge of the main river. Spectrophotometric variations are assumed to be synchronous and were useful for core correlation throughout the lake.

Organic matter analysis

The total organic carbon (TOC) content and the Hydrogen Index (HI) were measured in the cores of the selected transect (Sev30, Sev56 and Sev65) using Rock-Eval pyrolysis (Espitalié et al. 1985a) with a Model 6 device (Vinci Technologies). The sample resolution was set to 0.5-cm intervals for Sev65 (57 samples) and 1-cm intervals for Sev56 and Sev30 (29 and 30 samples, respectively). Analyses were carried out on 50-100 mg of crushed, dried samples. TOC (%) is indicative of the amount of organic matter (OM) in the sediment. Because OM content of the sediment is negatively correlated with oxygen concentrations at the sediment interface, TOC is a relevant proxy for a major environmental constraint on chironomid communities (Sæther 1979; Brodersen and Quinlan 2006) and considered to be a local driver of chironomid assemblages. HI is expressed as the H/C ratio of organic matter and represents the amount of hydrocarbon products released during pyrolysis (in mg HC g^{-1} TOC). HI provides insights into the origin of the OM in the sediment and the degree of OM degradation. High HI values are indicative of fresh, autochthonous OM, whereas low HI values are indicative of strongly degraded autochthonous or allochthonous OM, with high lignin content. HI was therefore used to assess temporal variability in the quality of sediment TOC (Meyers and Lallier-Vergès 1999).

Numerical and statistical analyses

For the living larval community, counts from the three transects were merged by depth and the abundance of each taxon was relative to all counts. Chironomid counts from the three transects were merged to provide a robust assessment of the depth-distribution of larvae. Transect-specific patterns likely reflect, at least in part, a sampling effect of low replication, rather than true differences in environmental conditions.

For the chironomid HC, the bathymetric distributions (from 30 to 65 m) of the subfossil communities were defined for each transect. At each depth, the relative abundance of each subfossil taxon was obtained by dividing the number of HC from each taxon by the overall number of HC found on the transect.

For the chironomid assemblage reconstructions, HC counts were transformed into accumulation rates using the age-depth models. HC accumulation rates (HCAR) are expressed as number of HC 10 cm^{-2} yr^{-1}.

Chironomid assemblage zones (CAZ) were determined for each of the three water depths using the Bray-Curtis dissimilarity index and Coniss, a program for stratigraphically constrained cluster analysis (Grimm 2004). Taxa with <5 occurrences were excluded from the analysis. The number of statistically significant zones was assessed using the broken stick model (Bennett 1996). Only HC originating from deep-living chironomid species, according to Brooks et al. (2007), were utilized to define the chironomid assemblage zones in the deeper core and to assess depth-specific assemblage changes. Variability of HCAR and TOC content between CAZ was assessed in the cores using analysis of variance (ANOVA) followed by Tukey HSD *post hoc* tests.

An analysis of similarity (ANOSIM), using the "Bray-Curtis" dissimilarity index, and a correspondence analysis (CA) were performed to explore the influence of water depth on the live chironomid larvae and HC assemblages and select the most suitable transect for the chironomid assemblage reconstructions. Eighteen taxa were considered, after removal of unidentified specimens and taxa that were absent in either the larvae or HC datasets. This CA comprised 12 samples (3 depths × 3 transects for HC samples + 3 depths for the summed larvae samples). The larval sample from 2 m depth was included in the ordination as a passive sample. This prevents excessive influence of the 2-m larvae sample in the CA because of its high species richness. The littoral influence on the subfossil assemblage compositions at 30, 56 and 65m was therefore discriminated using the 2-m species assemblage composition. A second CA was used to assess HCAR variability over time at the various depths.

All statistical analyses were performed in R (R Development Core Team 2011). The packages ade4 (Dray and Dufour 2007) and FactoClass (Pardo and DelCampo 2007) were used for the CA. Ade4 was also used to perform the inter-class analysis. Rioja (Juggins 2009) and vegan (Oksanen et al. 2011) were employed for CONISS and ANOSIM.

Results

Chronology

The $^{210}Pb_{exc}$ profile for Lake Annecy exhibited regular exponential decay with depth in the sediment core (Fig. 2a). The CFCS model (Constant Flux, Constant Sedimentation, Krishnaswami et al. 1971) was chosen for this study because recent sediments were characterized by constant linear sedimentation, shown by the linear relations between log activity of ^{210}Pb and both cumulative mass ($r^2 = 0.98$) and depth ($r^2 = 0.98$). Regardless of the

model used, ^{210}Pb-based chronologies should be confirmed by independent methods (Smith 2001). Our ^{210}Pb chronology is supported by ^{137}Cs profiles (Robbins and Edgington 1975). The CFCS age model indicated a mean sedimentation rate of 2.04 ± 0.01 mm yr^{-1} for Lake Annecy. The ^{137}Cs activity shows a sharp peak at 4.2 cm without any increase in ^{241}Am activity, thus corresponding to fallout from the 1986 Chernobyl accident. This yields an average sedimentation rate of 1.83 ± 0.01 mm yr^{-1} for Lake Annecy between 1986 and 2009. The well-resolved ^{137}Cs peak at 9.8 cm, coinciding with an ^{241}Am peak, confirms that sediment at this depth was deposited during the period of maximum atmospheric fallout from nuclear weapons testing, in 1963 (Michel et al. 2001). This age-depth relationship yields an average sedimentation rate of 2.13 mm yr^{-1} in Lake Annecy from 1963 to 2009. Good agreement among the mean sedimentation rates derived from ^{210}Pb$_{ex}$, ^{137}Cs and ^{241}Am profiles enabled us to estimate a mean sedimentation rate of 2.00 mm yr^{-1} over the last century (Fig. 1a).

Correlation of the three cores indicated that sedimentation rate in each core was roughly constant. Mean sedimentation rate in two of the cores were similar to the reference core (Sev65, 2.00 mm yr^{-1}; Sev30, 1.98 mm yr^{-1}) (Fig. 2b and d). Sev56, however, showed a lower mean sedimentation rate (1.39 mm yr^{-1}) (Fig. 2c).

Living larvae vs. subfossil remains in surface sediment: transect selection

A total of 1,104 chironomid larvae and 645 HC were extracted from surface sediment samples that integrated *ca.* 5 years according to the age/depth models (Fig. 2). According to the ANOSIM, there were no significant differences with respect to water depth between larvae and HC on any transect ($p > 0.05$). However, there were clear differences among transects

with respect to similarity of samples across depths. The "Sévrier" transect had the highest similarity (p = 0.8) followed by the "Annecy" transect (p = 0.2) and the "Saint Jorioz" transect (p = 0.1). This analysis indicates the "Sévrier" transect is the best for using chironomid assemblages from different depths to do reconstructions. The CA biplot depicts these differences in the larvae/HC distributions. The CA axis 1 (F1) and axis 2 (F2) captured 58.3 % of the variability in chironomid larvae and the subfossil assemblage (Fig. 3). Along CA axis 1 (F1), two chironomid groups were defined. Taxa such as *Cladopelma*, *Dicrotendipes*, *Cladotanytarsus*, *Cricotopus*, *Cryptochironomus*, *Thienemanniella* and *Ablabesmyia* showed high F1 scores. Close association of these taxa with the larval sample obtained from a depth of 2 m indicated that they were major components of the littoral larval community. In contrast, *Micropsectra*, *Chironomus*, *Sergentia*, *Pagastiella* and *Parakiefferiella* were associated by low F1 scores and were related to samples from deeper water (30, 56 and 65 m). CA axis 2 (F2) segregated the specific larval composition of the three studied depths. However, the larvae assemblage at 30 m had an intermediate F2 score compared to the larvae assemblages at 56 and 65 m, suggesting that a depth gradient was not expressed along the F2 axis. However, because the sampling depths effectively differed along this axis, F2 scores nonetheless allowed differentiating sampling depths according to their chironomid assemblages. The larvae assemblage at 56 m was mainly composed of *Chironomus* and *Procladius*, whereas the assemblage at 65 m was characterized by *Micropsectra*, *Sergentia* and *Parakiefferiella* (Fig. 3). The HC sample for the "Saint Jorioz" transect at 56 m was noteworthy in being segregated from other samples, with low F2 scores associated with *Eukiefferiella* and, to some extent, *Polypedilum*.

The F1 and F2 scores of the CA were extracted for both larval and HC samples and considered synthetic (both qualitative and quantitative) descriptors of the chironomid

structure of the samples. At each depth, the difference in the CA scores between larval and HC samples was calculated and averaged for each transect (Table 1). Differences in the F1 scores between larvae and subfossil samples reflected the influence of littoral deposits on the subfossil assemblages at 30, 56 and 65 m (Fig. 3). Differences in F2 scores between larvae and subfossil samples are related to depth of the chironomid sample. The "Sévrier" transect had the lowest mean difference in F1 and F2 scores. Thus, it was the transect least influenced by littoral HC deposition and most suitable for reflecting the depth distribution of larvae. It was therefore selected to reconstruct chironomid assemblage changes over the last 150 years at three water depths: 30 m (Sev30), 56 m (Sev56) and 65 m (Sev65).

Downcore chironomid assemblage reconstructions and OM

Figure 4 illustrates the temporal dynamics of the main chironomid taxa at the three studied depths. For each depth, chironomid assemblage zones (CAZs) obtained from the constrained cluster-analysis (CONISS) highlight assemblage shifts.

Among the 52 taxa found in 67 samples from the core in 65 m of water (Sev65), five were found as living larvae inhabiting the deep zones of the lake, at 56 and/or 65 m (*Chironomus*, *Sergentia*, *Micropsectra*, *Procladius*, *Paratendipes*), and two others (*H. grimshawi*-type and *Paracladopelma* sp.) are associated with the deep zone in lakes (Wiederholm 1983; Brooks et al. 2007). Considering these seven taxa, including the two *Micropsectra* types, three CAZs were identified by cluster analysis (Fig. 4a). CAZ1 spanned from the late 1870s to the early 1930s. The mean HCAR was 7.9 ± 5.9 HC 10 cm^{-2} yr^{-1} and was dominated by *M. contracta*-type and *Procladius* sp. *Paracladopelma* sp. and *H. grimshawi*-type also occurred sporadically during this time period. The onset of CAZ2 in the early 1930s was marked by a decrease of more than 50% in *M. contracta*-type and *Procladius* sp. HCAR (ANOVAs, $p < 0.001$) and exhibited variations such as the increase in *M. contracta-type* between the 1970s

and the 1990s. During CAZ2, *S. coracina*-type HCAR increased significantly (ANOVA, $p < 0.001$). *C. anthracinus*-type appeared at the beginning of CAZ2 and and *Paratendipes* at its end. Throughout CAZ3, from 2000 to 2008, *S. coracina*-type HCAR decreased, whereas HCAR of *M. contracta*-type, *M. radialis*-type and *Procladius* sp. fell to nearly zero. The total HCAR of profundal taxa declined to 0.85 ± 0.38 HC 10 cm^{-2} yr^{-1} during CAZ3. TOC concentrations increased significantly between the CAZs (ANOVA, $p < 0.001$), ranging from 0.65 ± 0.23 % in CAZ1 to 1.61 ± 0.08 % in CAZ3. HI also increased significantly (ANOVA, $p < 0.001$) and ranged from 183.2 ± 58.6 in CAZ1 to 312.7 ± 6.0 in CAZ3.

In the core from 56 m water depth (Sev56), 40 taxa were identified in the 63 samples, and three CAZs were distinguished by cluster analysis (Fig. 4b). The chironomid assemblage of CAZ1, from *ca.* 1850 to 1915, mainly consisted of *M. contracta*-type, *Procladius* sp. and *Paracladopelma* sp. From *ca.* 1915 to 1945, CAZ2 was mainly characterized by an increase in *M. contracta*-type HCAR (ANOVA, $p < 0.001$). After *ca.* 1945, a sharp decrease in *M. contracta*-type HCAR, a disappearance of *Paracladopelma* sp. and an increase in the HCAR of *S. coracina*-type was observed in CAZ3. TOC concentrations differed significantly among the three CAZs (ANOVA, $p < 0.001$). TOC was lower during CAZ2 (0.90 ± 0.23 %) compared to CAZ1 (1.29 ± 0.37 %) and CAZ3 (1.87 ± 0.59 %). In the core from 65 m water depth, HI significantly increased over time, ranging from 88.3 ± 23.1 during CAZ1 to 282.3 ± 57.5 at the end of CAZ3.

In the core from 30 m water depth (Sev30), 79 genera or species-groups were identified within the 48 samples. Cluster analysis indicated 2 CAZ, with a major change in the chironomid assemblage composition *ca.* 1950 (Fig. 4c). The total chironomid HCAR decreased from 123 ± 30 HC 10 cm^{-2} yr^{-1} during the period between 1850 and 1950 (CAZ1) to 40 ± 16 HC 10 cm^{-2} yr^{-1} during the period between 1950 and 2008 (CAZ2). The HCAR of

all the taxa characteristic of CAZ1 (*Paracladius* sp., *H. grimshawi*-type, *M. contracta*-type) dramatically decreased at the CAZ1/CAZ2 transition. During CAZ2, *Procladius* sp., *S. coracina*-type and, to a lesser extent, *M. contracta*-type remained rather stable. TOC concentrations were significantly higher in CAZ2 than in CAZ1 (t test, $p < 0.001$) and ranged from 0.77 ± 0.08 % in CAZ1 to 1.26 ± 0.18 % in CAZ2. HI increased significantly from 283.05 ± 22.80 in CAZ1 to 387.43 ± 29.40 in CAZ2 (t test, $p < 0.001$).

The CAZs defined for each core were used to depict changes in the chironomid assemblage on a whole-lake basis over the last 150 years. The different CAZs and their transition points, which occurred in the 1910s, 1930s, 1950s or 2000s depending on water depth, were used to define five different Whole-Lake Assemblage Types (WLATs). A CA was performed using all the samples from the three cores, using taxa with at least five occurrences (167 samples, 49 taxa) to evaluate and test the influence of (1) sampling depth and (2) the WLATs on whole-lake chironomid HCAR variability. The two first axes of the CA described 41.4 % of the chironomid HCAR variability (Fig. 5). Chironomid HCARs differed significantly both between sampling depths and between WLATs, explaining 19.82 % and 16.80 % of the chironomid HCAR variability, respectively (permutation tests, $p < 0.01$).

During the first three WLATs, from the 1850s to the 1930s, chironomid assemblages were mainly segregated along CA axis 1 (F1) according to depth. Sev30 was characterized by *Paracladius* sp. and *H. grimshawi*-type and differed from Sev56 and Sev65, which were characterized by *M. contracta-type* and *Procladius* sp. During the latter two WLATs, from the 1950s to the 2000s, the F1 scores of the chironomid assemblages from the various depths converged toward similar values (-1 to -0.5), indicating a progressive decrease in the depth-related specificity of chironomid assemblages. The increase in *S. coracina*-type HCAR in the

chironomid assemblages at all depths was especially responsible for this uniformity in chironomid assemblage. During WLAT4 and WLAT5, the chironomid assemblages at all depths were also characterized by several taxa associated with the littoral zone, such as *Dicrotendipes* sp., *Ablabesmyia* sp., and *Zalutchia* sp. This increase in littoral influence during these two WLATs was related to the decrease in HCARs in the initial profundal community that occurred after the 1930s. The CA F1 therefore greatly reflected successive changes in chironomid assemblages, whereas CA F2 highlighted the increasing influence of littoral HC inputs over time resulting from the progressive extirpation of the original chironomid assemblages at the various depths.

Discussion

This study explored ways to improve analysis of changes in chironomid assemblages in large deep lakes using the approach of multiple-depth reconstructions coupled with larvae/HC spatial comparisons. This approach was undertaken to better define the chironomid assemblage composition and its temporal dynamics at the whole-lake level, compared to reconstructions based on a single core from deep water.

Comparison of modern and subfossil chironomid assemblages: site selection

Our results highlighted transect-specific differences in the ability of subfossil samples to accurately represent the water-depth distribution of living larvae. Sampling of living larvae involved two seasons to account for species phenology. Spring sampling was completed before the massive emergences of most chironomid species. Autumn sampling improved the assessment of the living community and may have also improved determinations of species that might have been at early stages and thus unidentifiable during spring sampling. Our study did not aim to make an exhaustive assessment of the whole lake community, which would

have required a larger sampling effort. Instead, our sampling strategy was designed to compare the water-depth distributions of larvae and HC, and we found that the dominant taxa in HC samples were also found as larvae. The "St Jorioz" transect displayed the greatest differences in the bathymetric distribution between HC and living larvae. The local influence of a tributary and the steep slope of the lake bottom at this location likely facilitate re-deposition of HC from the littoral to the profundal zone. This may explain the poor ability of this transect to provide a reliable picture of depth-specific chironomid assemblages or their temporal variability. In contrast, the bathymetric distributions of HC and larvae were more similar in the "Annecy and "Sévrier" transects. Both were less influenced by HC export from the littoral zone and more representative of depth-specific chironomid assemblages. These two transects had lower slopes and no nearby tributary. The "Annecy" transect had a poorer match between HC and larvae, despite a gentler depth gradient than the "Sévrier" transect. The wind effect may explain this result, as the "Annecy" transect is located at the extreme end of the lake and is more subject to wind-induced HC redeposition than the "Sévrier" transect.

The "Sévrier" transect was selected for chironomid paleo-reconstructions at various depths because its HC/larvae bathymetric distributions were most similar although differences were detected. For example, HC of littoral taxa such as *Psectrocladius*, *Dicrotendipes* and *Cladopelma* were found in sediment samples at 30 and 56 m, whereas larvae of these taxa were only found at a depth of 2 m. These findings are in agreement with results reported by Brodersen and Lindegaard (1997), who compared HC species composition from surface sediment samples with samples of both trapped adult midges and living larvae in Lake Stigsholm (Denmark) and found differences in chironomid assemblage composition depending on the sampling method used. These differences can perhaps be attributed to the passive behavior of HC compared to larvae. Living larvae may aggregate in lakes (i.e. patchy distribution, Lobinske et al. 2002), whereas the spatial distribution of HC, while conserving

the composition of chironomid assemblages (Cao et al. 2012), is more likely to be subject to passive deposition, thus evenly distributing initially aggregated abundances of chironomid larvae. Furthermore, sediment samples reflect a time-integrated measure of the chironomid assemblage that, in a sense, average inter-annual variations in chironomid densities. Finally, the temporal integration of sediment samples facilitates collection of rare species that are especially difficult to find as live larvae in the modern community and would require excessive efforts to document. The sampling effort here, which consisted of 48 samples and was undertaken to define the modern community, was reasonable for characterizing the water-depth distribution of this community. Use of relative abundance to compare larval and HC bathymetric distributions seem reasonable, until adequate conversion factors become available to translate HC abundances into living larvae abundances.

Palaeolimnological development of Lake Annecy

Pre-disturbance assemblages (1850-1930)

Based on detection of different CAZs at depths in the core, five WLATs were identified in Lake Annecy for the period of interest (Fig. 6). The first significant change in chironomid assemblages occurred in the 1910s, at 56 m. This change was restricted to a relatively short time interval associated with a decrease in TOC values. However, it was only characterized by an increase in *M. contracta*-type HCAR and if this relationship (*M. contracta-type*/TOC) seems relevant, the reason why this pattern was only found at 56 m remains obscure. Nevertheless, this pattern was far from being the dominant feature during the period studied and considering the CA of the subfossil samples, WLAT1 and WLAT2 remained rather stable from the 1850s to the 1930s. Prior to ~1930, chironomid assemblages at each of the depths examined in this study were typical for a pre-modern perturbation state and can be described as follows:

- *M. contracta*-type and *Procladius* sp. assemblages in the deepest zones with moderate densities, as inferred from the HCARs of the two taxa (2.55 ± 1.51 HC 10 cm^{-2} $year^{-1}$ and 1.08 ± 0.61 HC 10 cm^{-2} $year^{-1}$, respectively)

- *Paracladius* sp., *H. grimshawi*-type and *M. contracta*-type assemblages in the littoral zone, with higher densities (5-10 fold higher for each dominant taxon)

Except for *Procladius* sp., which tolerates high organic matter, the dominant taxa are indicative of oxygen-rich conditions (Brodersen and Quinlan 2006; Brooks et al. 2007) and low TOC content (i.e. <1%) at all the studied depths. These taxa are also considered typical of oligohumic-oligotrophic lakes (Sæther 1979). These results corroborate the early work of Leroux (1908) on phytoplankton, which indicated oligotrophic conditions in Lake Annecy at the beginning of the time period studied. Before the 1930s, the oligotrophic state of Lake Annecy was associated with a well-oxygenated hypolimnion. At that time, Lake Annecy exhibited efficient trophic functioning, with most of the energy (i.e. OM) flowing through the food web, resulting in little organic matter accumulation in the sediment.

Substantial differences in chironomid assemblages at various water depths at the beginning of the time period studied, corroborate work of Engels and Cwynar (2011) and Luoto (2012), which indicated that sediment samples collected from the deepest part of a deep lake display restricted spatial integration of HC. Furthermore, we found that the dominant taxon at 30 m (*Paracladius*) were poorly represented in the sediment from the deepest part of the lake (i.e. Sev65). This indicates that characteristics of shallower zones are not quantitatively reflected in sediment from the deep zone of this lake. Nevertheless, richness was higher at 65 m than at 56 m, suggesting that a larger fraction of the whole-lake chironomid assemblage is found in the deepest part of the lake. The bottom contour at 56 m enables HC to drift downward, partly explaining this result. Depth-specific assemblage

differences likely reflect the large size and great depth of Lake Annecy (2,740 ha, z_{max} = 65 m).

H. grimshawi-type, considered a deep-living taxon (Sæther 1975), was rarely recorded in the deep-water cores (56 and 65 m), but was abundant in the core from 30 m. Furthermore, several taxa (*M. contracta*-type) were represented at all depths, despite the fact that they are commonly encountered in deep water (Brooks et al. 2007). These peculiarities of the chironomid fauna in Lake Annecy likely come from a combination of environmental factors that support the lake's distinctive chironomid assemblage (Brodersen and Quinlan 2006).

1930-1950: Eutrophication-driven changes

The first major change in the chironomid assemblage occurred in the deep zone (i.e. Sev65) around the 1930s. This change was characterized by an increase in the HCAR of hypoxia-tolerant *S. coracina*-type, which coincided with a decrease in the HCAR of the oxyphilous *M. contracta*-type, indicating decrease in oxygen at the lake bottom (Fig. 6). This inference is supported by monitoring data (Hubault 1943) that indicate anoxia at 60 m in mid-summer 1937. This hypoxic zone progressively expanded upward. The number of oxyphilous taxa decreased at 56 m (Sev56) in *ca.* 1945 and at 30 m (Sev30) in the 1950s. Expansion of the hypoxic layer was correlated with an increase in TOC concentrations and HI in sediment cores over time (Fig. 6). The increase in TOC concentrations in the sediment promoted microbial respiration and consequent oxygen depletion (Jones et al. 2008). From the 1930s until the 1960s, the lake received an increase in nutrients from wastewaters (Perga et al. 2010). During this period, chironomid assemblages were affected by a decrease in oxygen driven by increasing trophic state of the lake (i.e. eutrophication).

From nutrient control to "pelagic functioning" control after 1950

Since the 1960s, local authorities have undertaken various measures to control nutrient inputs to Lake Annecy, including the establishment a wastewater treatment plant. Such initiatives reduced nutrient concentrations in the water column (SRP < 0.01 mg L^{-1}), but no recovery is evident in the chironomid assemblages, at any depth. Paradoxically, chironomid assemblages have shifted over time to a more homogenous ("depth-independent") state, dominated by the *S. coracina*-type (i.e. WLAT4, WLAT5); this was illustrated by the convergence of CA F1 scores at the three studied depths (Fig. 6). These recent assemblage shifts, to namely WLAT4 (30 m) and WLAT5 (65 m), provide insights into the spatio-temporal variability of oxygen constraint at the sediment interface, that is, development of hypoxic conditions at 30 m and an increase in oxygen constraint at 65 m.

The increase in oxygen constraint in the deep zone following the return of oligotrophy was unexpected. Measurements of the sediment indicated that neither TOC concentrations nor the HI has decreased since the 1960s; in fact, these variables have increased continuously to the present. Although maturation of OM during early diagenesis can influence temporal patterns of TOC, and especially HI (Espitalié et al. 1985a), the strength of these patterns enhances our confidence in the inferred progressive increase in phytoplankton-derived input to the sediment since the 1960s. Our results are in agreement with those of Meriläinen et al. (2000), who reported a "*decoupling*" between pelagic conditions and benthic conditions after mitigation efforts went into effect. In the 1930s, oligotrophy in Lake Annecy was accompanied by efficient trophic functioning in the pelagic zone. Today, however, the oligotrophic state is characterized by less efficient trophic functioning, leading to organic matter accumulation in sediments and hypoxia. There is evidence that predation by stocked *Coregonus* in Lake Annecy has driven a decrease in the size of *Daphnia* over the last 60 years, with a consequent increase in export of pelagic primary producer biomass to the sediment (Perga et al. 2010). In this context, the structure of the chironomid assemblage, since

the return of oligotrophy, appears to be strongly coupled with pelagic functioning. Our study points to an indirect, top-down cascade effect, from the pelagic to the benthic food web. This pelagic-benthic linkage is seldom reported in field studies (Hargrave 2006) because the effects of fish on chironomid communities are mostly considered direct effects of predation (Mousavi et al. 2002).

The stability of chironomid assemblages over the last two decades suggests that the oxygen constraint initially promoted by "nutrient enrichment" was progressively replaced by changes in "pelagic functioning." This temporal succession of constraints ("constraint substitution") indicates that a consideration of changes in the food web structure might help researchers understand unexpected lake trajectories following the return of oligotrophic conditions (Jeppesen et al. 2005). Beisner et al. (2003) assessed the factors controlling resilience in north-temperate, clear-water lakes and suggest that a non-negligible part of the variation in resilience predictions is likely related to the food-web structure.

Ecological consequences: negative feedback loops

Detritivore diversity and abundance are important functional features of lake ecosystems and changes in the structure of the benthic food web may affect connections with higher-level food webs (Vander Zanden et al. 2005) and have dramatic repercussions on the recycling of OM (Olafsson and Paterson 2004). The dramatic decrease in chironomid abundance over time (~20-fold) suggests important changes in energy flow in this system over the last 150 years. More precisely, the contribution of the benthic food web to the higher trophic levels likely decreased. This pattern is similar to that described by Vander Zanden et al. (2003), who found a reduced contribution from the benthic food web to the higher trophic levels (i.e. fish) during the last century, following introduction of the freshwater shrimp *Mysis relicta* in Lake Tahoe, California. Further, the lower abundance of chironomids in Lake Annecy likely facilitated

maintenance of hypoxic conditions through negative feedback loops, despite re-oligotrophication, thus hampering the ecological recovery of chironomid assemblages. Simply, initial input of OM triggers a decrease in benthic fauna as a consequence of increased bacterial respiration. In this context, "benthic fauna" refers to chironomids and meiofauna involved in OM mineralization (Nascimento et al. 2012), taxa that are negatively impacted by hypoxic conditions (Kansanen 1981). The response is promotion of bacterial respiration of OM that would have been consumed by the vanished fauna. This phenomenon becomes amplified over time. Negative feedback loops that link benthic fauna and anoxic conditions have been reported mainly in marine "dead zones" (Diaz and Rosenberg 2008). However, Goedkoop and Johnson (1996) showed that chironomids and meiofauna in the profundal zone assimilated 2.4-6.0 % and 1.7-7.2 % of the deposited phytodetritus, respectively, from the spring bloom in Lake Erken. The invertebrate contribution to OM assimilation is rather high once the low chironomid densities (*ca.* 300 ind m^{-2}) and restricted period of activity are considered. Our results cannot provide a quantitative estimate of chironomid control over OM accumulation and consequent hypoxic conditions because conversion factors between HC abundances and larvae are not well established the carbon requirements of each chironomid larval stage are lacking for many taxa. The dramatic decrease in chironomid HC abundance, especially at 30 m depth does, however, suggest the possible facilitation of hypoxic conditions by a decline in the invertebrates, perhaps explaining the time lag between responses in the pelagic and benthic communities.

Conclusions

Comparison of water-depth distributions of HC and larvae on transects highlighted differences that are likely a consequence of bottom slope, input streams and wind effects.

Such a comparison should be undertaken to increase the robustness of reconstructions at multiple water depths. Before the 20th, oligotrophic conditions were inferred by two different taxa (*M. contracta*-type at 65 m and *Paracladius* sp. at 30 m). These taxonomic differences highlight the influence of depth on the dominant taxon and support the usefulness of multiple-depth sampling to reach an assessment of the chironomid fauna on a whole-lake scale. Furthermore, the depth-specific temporal changes in chironomid assemblages suggested that the changes in environmental conditions do not constrain the chironomid fauna similarly at all depths. Hence, despite chironomid assemblages provide similar information toward the trophic status of the lake, a multi-depth sampling approach can be recommended to track the dynamic and the extent of environmental changes such as the expansion of hypoxic zones.

In Lake Annecy, chironomid assemblages were highly correlated with sediment TOC concentrations through time. If, however, chironomid assemblage shifts from the 1930s and 1950s were associated with eutrophication, it is possible that the top-down effects of *Coregonus* sp. on pelagic functioning may have cascaded down to the benthic food web from the 1960s until the 2000s. The loss of benthic consumers could have induced sustained hypoxia in the sediment. Therefore, changes in the structure of the food web might explain chironomid assemblage changes in Alpine lakes following re-oligotrophication. Our results highlight the difficulties in defining the ecological state of lakes according to the Water Framework Directive (WFD 2000), when using both pelagic and benthic indicators.

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Tables

Table 1. Differences in the CA F1 scores between sediment samples (living chironomid larvae) and subfossil samples (HC from the first centimeter of each core) for the 3 studied transects at 3 depths.

		Larvae / HC sample difference in CA scores					
		Axis 1			Axis 2		
	Transects	Annecy	Sévrier	St Jorrioz	Annecy	Sévrier	St Jorrioz
Depths	65 m	0.48	0.28	1.21	0.24	0.10	0.08
	56 m	0.58	0.74	1.09	0.97	0.86	1.61
	30 m	0.87	0.55	1.69	0.13	0.08	0.26
	Mean ±SD	0.64 ± 0.20	0.52 ± 0.23	1.33 ± 0.31	0.44 ± 0.46	0.34 ± 0.44	0.65 ± 0.83

Figure captions

Fig. 1 Geographic location of Lake Annecy. The positions of the various sediment sampling sites (living larvae analysis) and cores (HC analysis) are indicated by black circles. An additional core, LDAref, was used to produce the reference chronology and is indicated by a black square

Fig. 2 (a) Age-depth model of LDAref from radionuclides (^{210}Pb, ^{137}Cs and ^{241}Am). Seven litho-stratigraphic markers (N°1 to N°7) were selected on LDAref to allow correlation with the other cores. **(b-c)** Age-depth models of the SEV65 and SEV56 cores were correlated to LDAref according to visible markers. **(d)** Age-depth model of the SEV30 core was correlated to SEV65 using spectrophotometry

Fig. 3 Correspondence analysis (CA) biplot depicting the associations between chironomid genera and the relative compositions of both larvae and HC assemblages at three depths (30, 50 and 65 m) for the three transects studied. The larval sample taken from a depth of 2 m was

included in the ordination as a passive sample. Larvae samples are indicated as "Larvae," followed by the depth at which they were sampled. Subfossil samples are identified by the letter C, followed by the transect abbreviation (sev = "Sévrier", ann = "Annecy", jo = "St Jorioz") and the sampling depth

Fig. 4 Stratigraphy plots reflecting the temporal dynamics (HCAR) of the main chironomid taxa identified at 65 m **(a)**, 56 m **(b)** and 30 m **(c)**. TOC and HI are also presented, providing evidence for the relationship between chironomid assemblage changes and both qualitative and quantitative changes in the OM of the sediment. Low (one HC) and sporadic occurrences are indicated by black circles. Zonation used to identify the different CAZs for each of the studied depths was accomplished using the broken stick model following a CONISS analysis, considering the Bray-Curtis similarity index. At 65 and 56 m, only truly deep taxa, according to Brooks et al. (2007), were reported. At 30 m, the eight most abundant taxa that had clear temporal trends and accounted for 71.3% of the overall HCAR are reported

Fig. 5 Correspondence analysis (CA) depicting the taxonomic changes in chironomid assemblages over time, reconstructed at the three studied depths. **(a)** HC samples grouped according to both sampling depths and WLATs defined by the stratigraphies. For each group, the numbers 30, 56 and 65 refer to the three studied depths, in m. The numbers 1, 2, 3, 4 and 5 refer to the different WLATs. b) Distribution of the 20 taxa with the highest scores on both the CA F1 and CA F2 axes

Fig. 6 Synthesis of the gradual temporal changes in chironomid assemblages for each of the studied depths and the definition of the different WLATs. From left to right, progressive chironomid assemblage shifts (CAZs) for the different depths, definition of the chironomid changes at the whole-lake level (WLATs), CA F1 scores, summarizing the taxonomic changes for each studied depth over time and the assemblage composition convergence in

recent years, gradual changes in TOC concentration, temporal variability in the trophic state of the lake, emphasizing the increase in the trophic state from historical data obtained in the 1940s and the re-oligotrophication achieved since the 1990s

Figure
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Figures

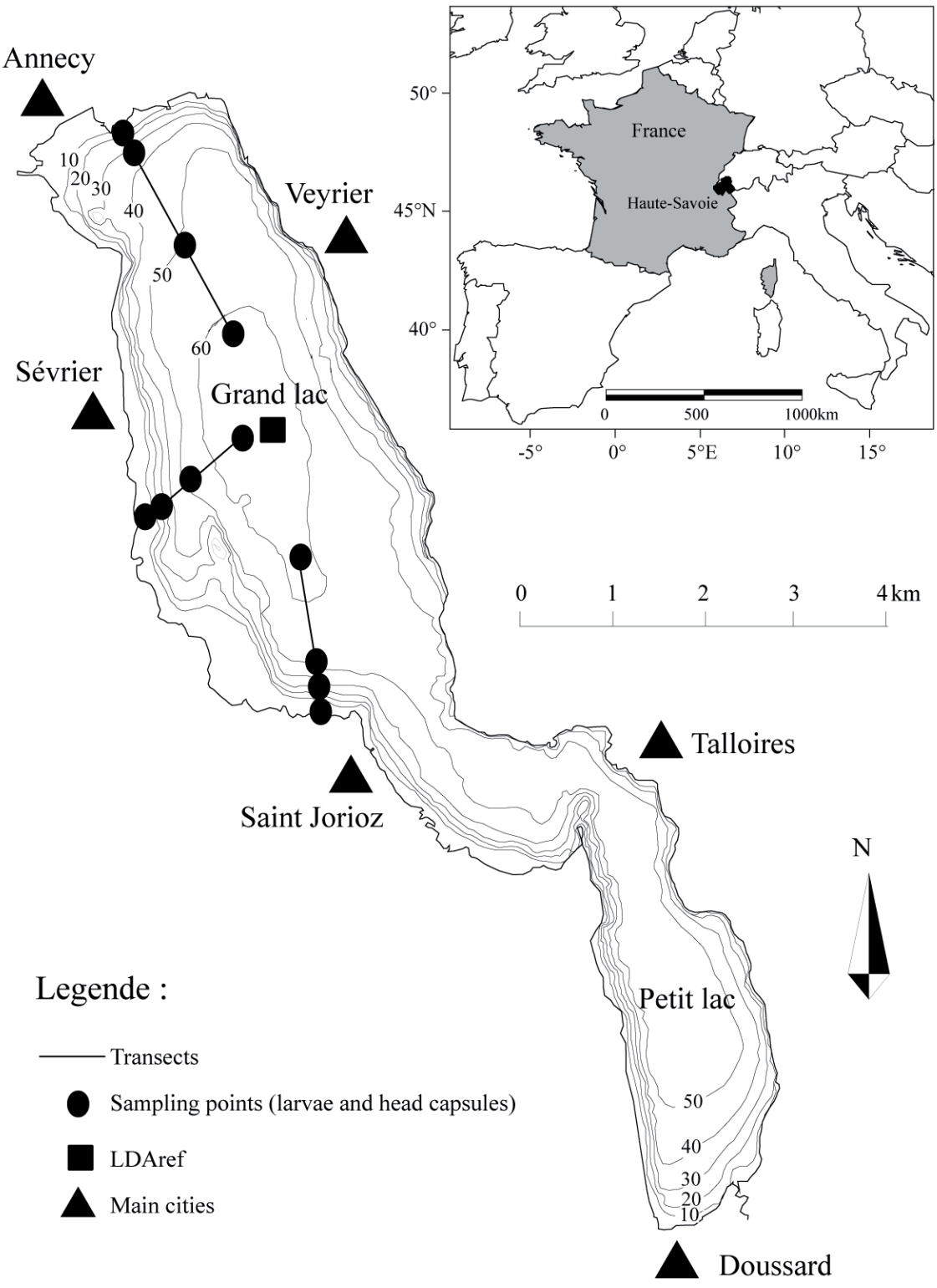

Fig. 1

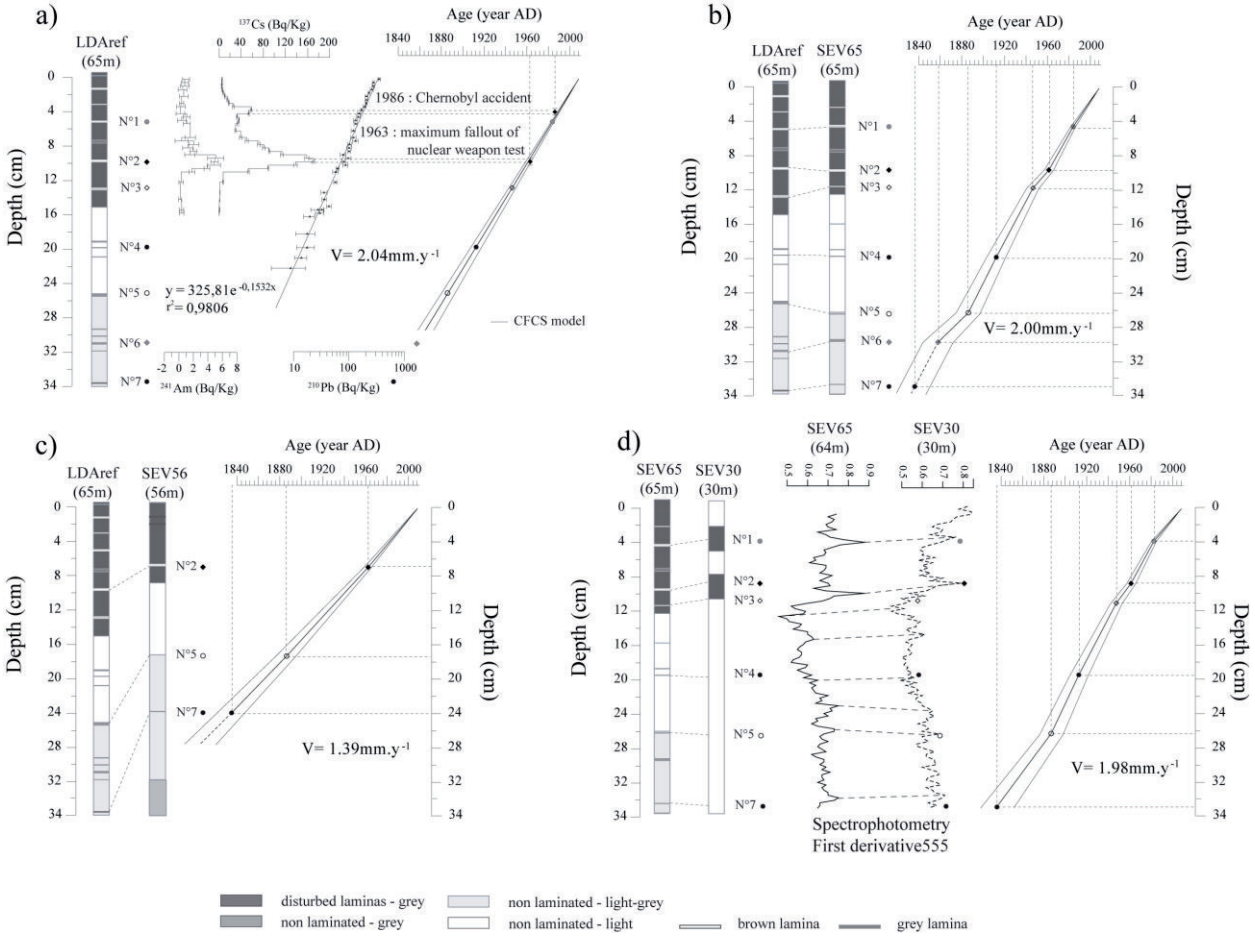
a)
LDAref (65m)
137Cs (Bq/Kg)
Age (year AD)
1986 : Chernobyl accident
1963 : maximum fallout of nuclear weapon test
V= 2.04mm.y-1
y = 325,81e-0,1532x
r2 = 0,9806
CFCS model
241Am (Bq/Kg)
210Pb (Bq/Kg)
Depth (cm)
b)
LDAref (65m)
SEV65 (65m)
V= 2.00mm.y-1
c)
LDAref (65m)
SEV56 (56m)
V= 1.39mm.y-1
d)
SEV65 (65m)
SEV30 (30m)
SEV65 (64m)
SEV30 (30m)
Spectrophotometry First derivative555
V= 1.98mm.y-1
disturbed laminas - grey
non laminated - grey
non laminated - light-grey
non laminated - light
brown lamina
grey lamina

Fig. 2

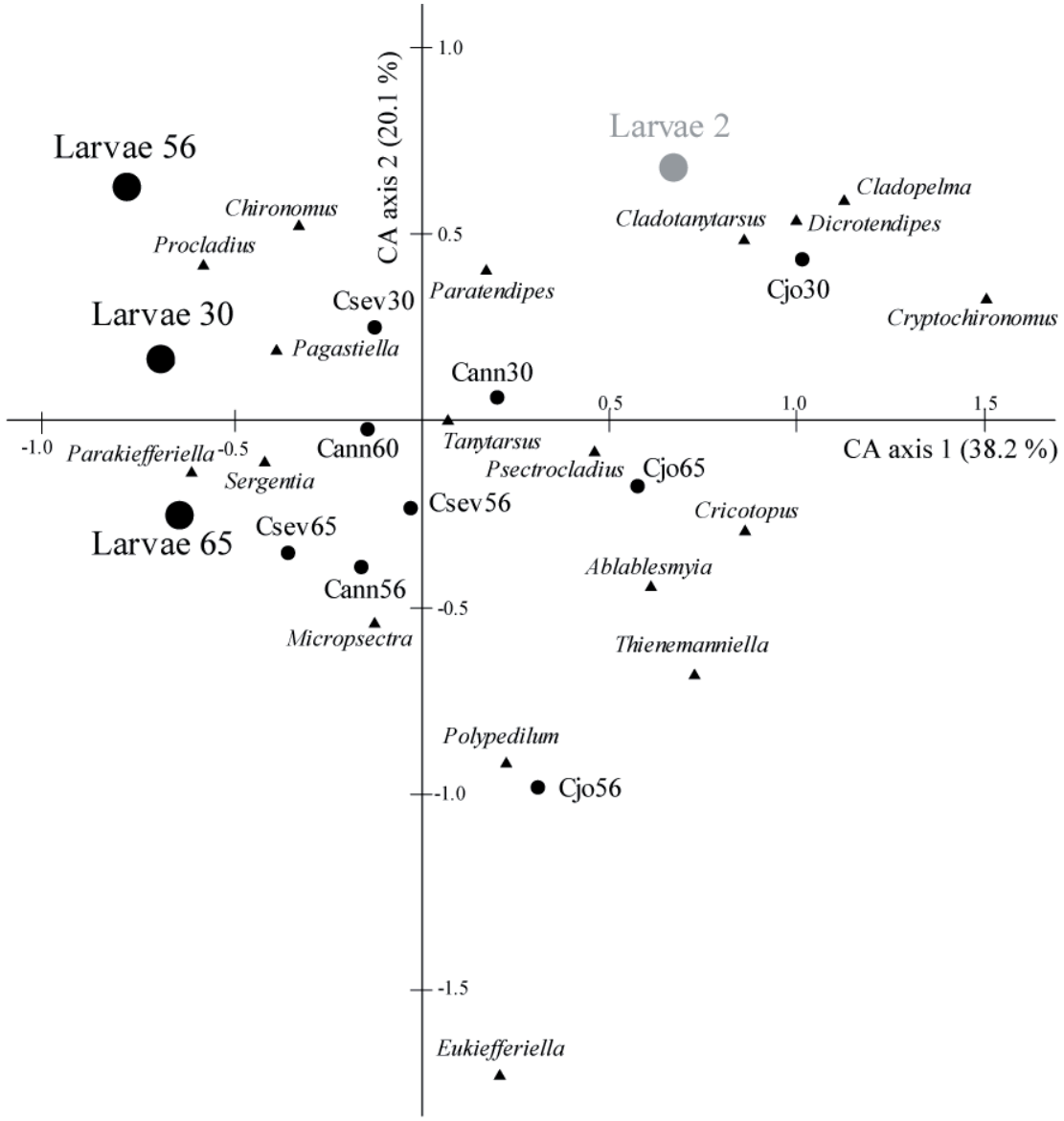
CA axis 2 (20.1 %)
CA axis 1 (38.2 %)
1.0
0.5
-0.5
-1.0
-1.5
0,5
1,0
1,5
-1.0
-0.5
Larvae 56
Larvae 30
Larvae 65
Larvae 2
Chironomus
Procladius
Parakiefferiella
Sergentia
Pagastiella
Csev30
Cann30
Cann60
Csev56
Csev65
Cann56
Micropsectra
Paratendipes
Tanytarsus
Psectrocladius
Cjo65
Cricotopus
Ablablesmyia
Thienemanniella
Polypedilum
Cjo56
Eukiefferiella
Cladotanytarsus
Cladopelma
Dicrotendipes
Cjo30
Cryptochironomus

Fig. 3

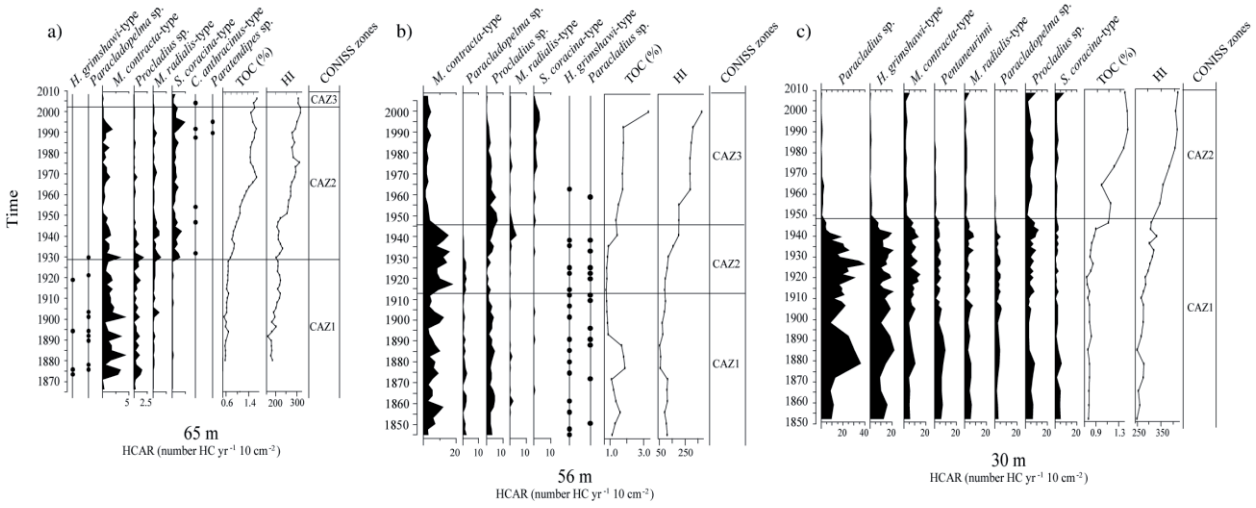
a)
H. grimshawi-type
Paracladopelma sp.
M. contracta-type
Procladius sp.
M. radialis-type
S. coracina-type
C. anthracinus-type
Paratendipes sp.
TOC (%)
HI
CONISS zones
Time
CAZ3
CAZ2
CAZ1
65 m
HCAR (number HC yr⁻¹ 10 cm⁻²)
b)
M. contracta-type
Paracladopelma sp.
Procladius sp.
M. radialis-type
S. coracina-type
H. grimshawi-type
Paracladius sp.
TOC (%)
HI
CONISS zones
CAZ3
CAZ2
CAZ1
56 m
HCAR (number HC yr⁻¹ 10 cm⁻²)
c)
Paracladius sp.
H. grimshawi-type
M. contracta-type
Pentaneurini
M. radialis-type
Paracladopelma sp.
Procladius sp.
S. coracina-type
TOC (%)
HI
CONISS zones
CAZ2
CAZ1
30 m
HCAR (number HC yr⁻¹ 10 cm⁻²)

Fig. 4

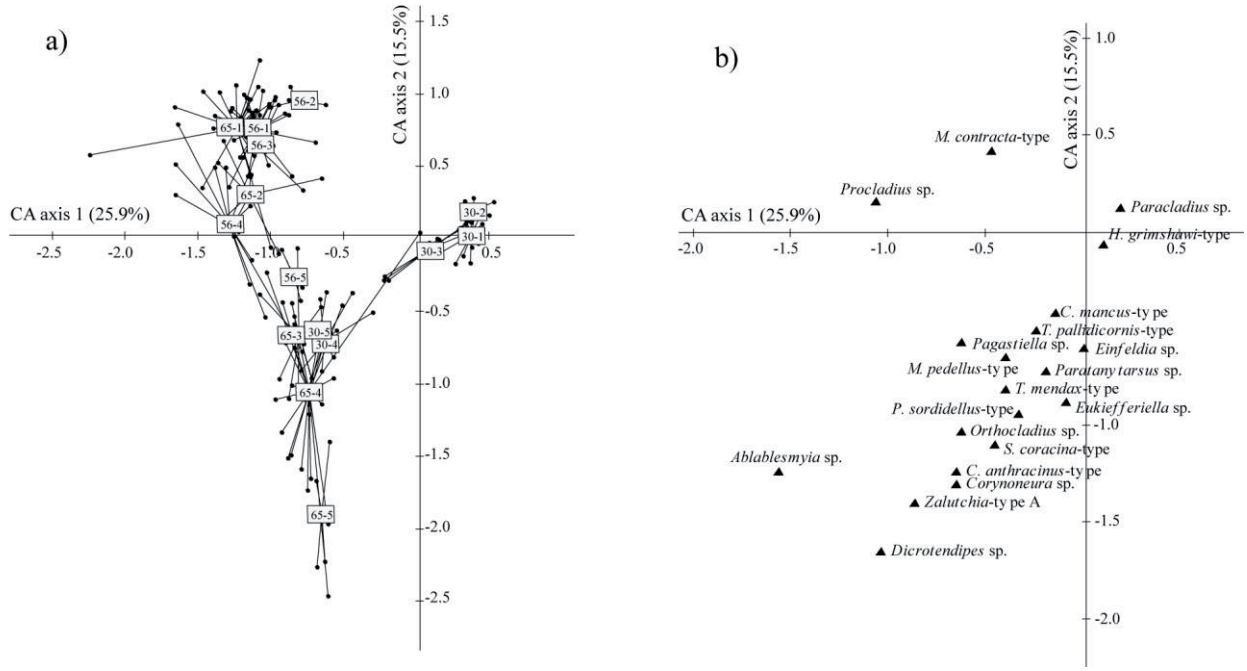
a)
CA axis 1 (25.9%)
CA axis 2 (15.5%)
56-2
65-1
56-1
56-3
65-2
56-4
30-2
30-1
30-3
56-5
65-3
30-5
30-4
65-4
65-5
b)
CA axis 1 (25.9%)
CA axis 2 (15.5%)
M. contracta-type
Procladius sp.
Paracladius sp.
H. grimshawi-type
C. mancus-type
T. pallidicornis-type
Pagastiella sp.
Einfeldia sp.
M. pedellus-type
Paratanytarsus sp.
T. mendax-type
P. sordidellus-type
Eukiefferiella sp.
Orthocladius sp.
S. coracina-type
Ablabesmyia sp.
C. anthracinus-type
Corynoneura sp.
Zalutchia-type A
Dicrotendipes sp.

Fig. 5

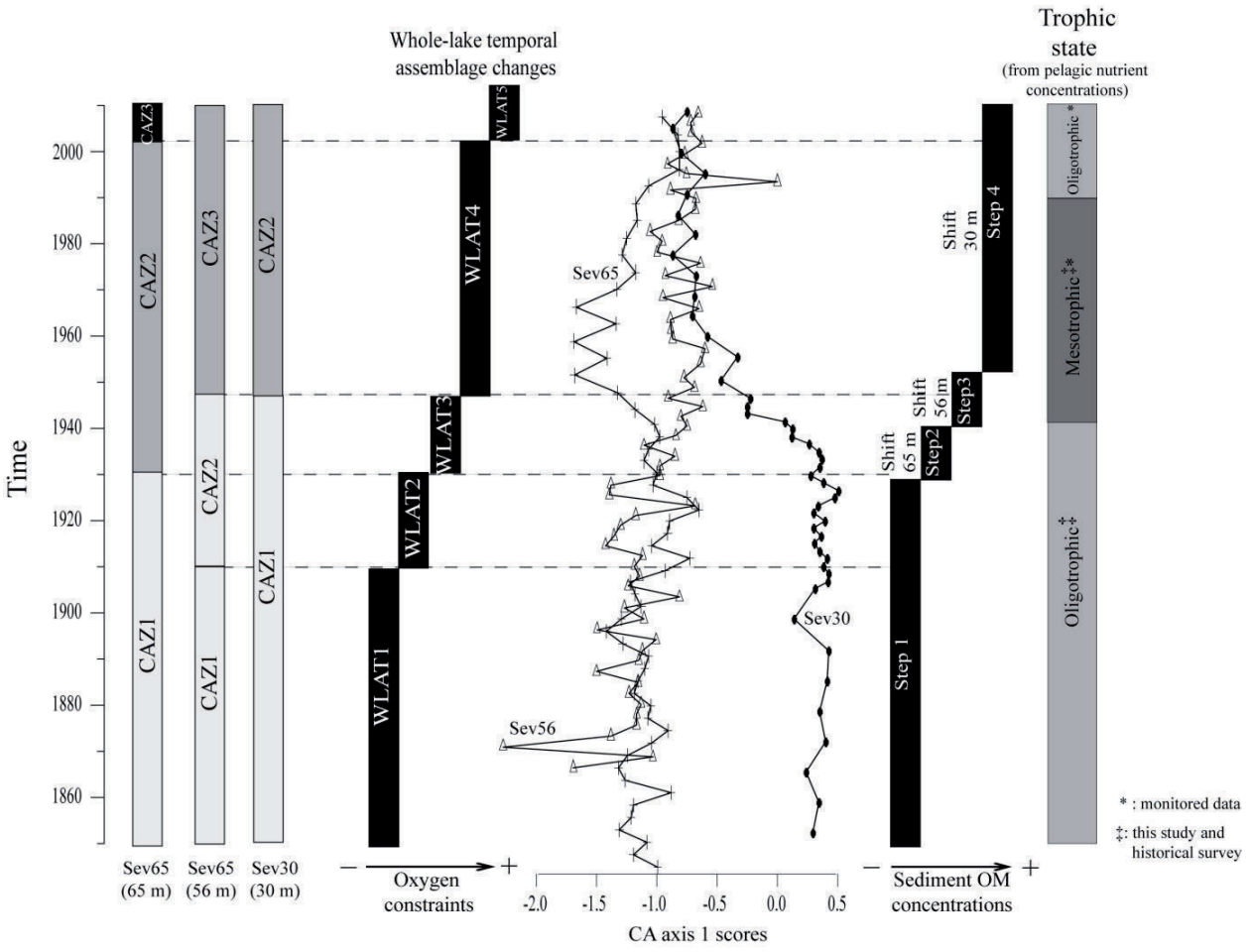
Whole-lake temporal assemblage changes
Trophic state
(from pelagic nutrient concentrations)
Time
2000
1980
1960
1940
1920
1900
1880
1860
CAZ3
CAZ2
CAZ1
WLAT5
WLAT4
WLAT3
WLAT2
WLAT1
Sev65
Sev56
Sev30
Shift 30 m
Step 4
Shift 56 m
Step3
Shift 65 m
Step2
Step 1
Oligotrophic *
Mesotrophic ‡*
Oligotrophic ‡
* : monitored data
‡: this study and historical survey
Sev65 (65 m)
Sev65 (56 m)
Sev30 (30 m)
Oxygen constraints
Sediment OM concentrations
-2.0
-1.5
-1.0
-0.5
0.0
0.5
CA axis 1 scores

Fig. 6

4. Implication des forçages locaux dans la vulnérabilité du réseau trophique de 3 grand lacs périalpins soumis au réchauffement climatique : une rétro-observation sur 150 ans à partir d'archives sédimentaires

❖ *Article : Local forcings affect food web vulnerability and responses to climate warming in deep temperate lakes*

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Local forcings affect lake zooplankton vulnerability and response to climate warming

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Keywords: climate change, lake, food web, human stressors, paleolimnology, time series modelling.

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Abstract

While considerable insights on the ecological consequences of climate change have been gained from studies conducted on remote lakes, little has been done on lakes under direct human exposure. Ecosystem vulnerability and responses to climate warming might yet largely depend on its ecological state and thus on local anthropogenic pressures. We tested this hypothesis through a paleolimnological approach on three temperate large lakes submitted to rather similar climate warming but varying intensities of analogous local forcings (changes in nutrient inputs and fisheries management practices). Changes in the structure of the cladoceran community were considered as revealing for alterations, over the time, of the pelagic food web. Trajectories of the cladoceran communities were compared between the three study lakes (Lakes Geneva, Bourget and Annecy) over the last 70-150 years. Generalized additive models were used to develop a hierarchical understanding of the respective roles of local stressors and climate warming in structuring cladoceran communities. The cladoceran communities were not equally affected by climate warming between lakes. In Lake Annecy, which is the most nutrient-limited, the cladoceran community was essentially controlled by local stressors, with very limited impact of climate. In contrast, the more eutrophicated Lakes Geneva and Bourget were more sensitive to climate warming, although the magnitude of their responses and the pathways under which climate warming affected the communities varied between the two lakes. Finally, our results demonstrated that lake vulnerability and responses to climate warming are modulated by lake trophic status but can also be altered by fisheries management practices through changes in fish predation pressure.

Keywords: climate change, cladoceran, nutrient, top-down control, paleo-ecology, sub-fossil remains

Introduction

While climate change is increasingly acknowledged as an important driver of lake ecosystems (George and Harris 1985, Adrian et al. 1995, IPCC 2001), our understanding of the mechanisms by which climate affects lakes is still patchy (Keller 2007). The complexity of the issue of lake responses to climate change arises from the fact that the various climatic components act on both lake physical, chemical and biological characteristics through many inter-connected pathways (Battarbee 2000, Leavitt et al. 2009). In addition, the relative importance and potential interactions of these different pathways might vary depending on the nature and on the magnitude of climate variability, but also according to lake characteristics (Pham et al. 2008, Cardille et al. 2009). Understanding and potentially forecasting the impact of climate variability on lakes therefore imply to hierarchize and scale up these different pathways (Leavitt et al. 2009). Achieving such an objective yet requires long-term datasets that can be obtained using paleolimnological records spanning time ranges long enough to encompass pre- and post-disturbance periods (Battarbee 2000).

In that aim, lots of efforts have been dedicated to reconstructing past changes in polar and alpine lakes over the last century (e.g., Battarbee et al. 2002, Quinlan et al. 2005, Smol et al. 2005) since (i) there are highly climate sensitive (ACIA 2004) and (ii) their remoteness allows obtaining a signal of their response to climate change that is not obscured by noise from local human influences (Battarbee et al. 2002). These studies have considerably improved our understanding of the ecological consequences of climate change on this specific category of lakes. Yet, the geographical non-uniformity in climate changes (that depend on the latitude, longitude and altitude, Blenckner and Hillebrand 2002, Livingstone et al. 2005) may limit the generalization of such observations to other climatic regions.

The impact of climate variability on temperate lakes has been quite less investigated and most of the knowledge was provided from the study of long-term data series, hence

restricting the implications of these results to the few lakes that have been subjected to long-term monitoring. Although the impacts of climate change are expected to be less severe in temperate than in polar or alpine regions (ACIA 2004), most of these studies provided strong evidences of climate-related trends in physical, chemical or biological components of temperate lakes (e.g., DeStasio et al. 1996, Straile et al. 2003, Winder and Schindler 2004). Their trajectories facing global changes may also be complicated by interactions between climate and local stressors, since combination of concomitant environmental forcings can amplify or hide their individual effects on the studied ecosystem (e.g., Matzinger et al. 2007, Pham et al. 2008). Surprisingly, paleolimnological records of the recent ecological trajectory of temperate lakes are still quite rare and very few have attempted to unravel or hierarchize the impact of various external forcings (e.g., Simpson and Anderson 2009, Bennion et al. 2012, Dong et al. 2012).

The purpose of this work was to compare, over the last century, the responses of the zooplankton community of three temperate, large and deep peri-alpine lakes submitted to rather similar climate variability but varying intensities of analogous local forcings (changes in nutrient inputs and fisheries management practices). The overarching aim was to hierarchize the role of local pressures versus climate warming on the responses of zooplankton over the past century. Our working assumption was that the zooplankton communities of these three lakes should show similar responses to climate warming, consistently with Straile and Adrian (2000)'s findings. The alternative hypothesis was that because these lakes have been under different anthropogenic local forcings, their response to climate warming should be distinct (Umbanhowar et al. 2011). For such purposes, long-term changes in the cladoceran community structure were reconstructed from their sub-fossil remains in all three lakes, using a paleolimnological approach. This study focused on cladocerans rather than on other paleo-proxies since they are highly sensitive and quickly

96 react to all three considered local and global forcings (lake nutrient levels, fisheries
97 management practices and climate). Indeed, zooplankton, and cladocerans in particular,
98 respond to changes in nutrient levels, which modify the quantity and quality of their food
99 resources (Reynolds 1998) through a bottom-up effect (McQueen et al. 1986). In addition,
100 changes in fish community and, potentially, in the nature and magnitude of size-selective
101 predation may induce changes in the size structure of cladoceran prey community by top-
102 down effects (Brooks and Dodson 1965). Last, climate warming may affect cladoceran
103 community, directly through its influence on biological processes (Moore et al. 1996) and
104 indirectly since species-specific differences in response to climate warming may affect the
105 phenological coupling of trophic relationships (Winder and Schindler 2004) leading to
106 cascading effects up or down in the food web (Moore et al. 1996, Jeppensen et al. 2010).
107 Hence, we considered that temporal alterations in the composition and size structure of the
108 cladoceran community should mirror the impacts of the considered forcings on the pelagic
109 food web.
110

111 ## Material and methods

112 ### *Study sites*

113 Lakes Geneva, Bourget and Annecy are all warm-monomictic lakes located on the northwest
114 edge of the French Alps. The surface water of these lakes is never frozen over. They are the
115 largest deep lakes in France with surface areas of 582, 42 and 27 km^2 and maximum depths of
116 309, 145 and 69 m for Lakes Geneva, Bourget and Annecy respectively. The geographical
117 vicinity of these lakes, with Lakes Bourget and Annecy situated within 50 and 70 km of Lake
118 Geneva, places them within a similar climatic context characterized mostly by a global
119 increase in temperature over the last 20 years (Auer et al. 2007). All three lakes are currently
120 submitted to monitoring surveys, started in 1957 for Lake Geneva (managed by the

International Commission for the Protection of Lake Geneva Waters and the French National Institute for Agronomical Research–INRA–), 1996 for Lake Annecy (managed by the Intercommunal Association of Lake Annecy and INRA) and 2004 for Lake Bourget (Lake Bourget water agency and INRA).

Supposingly oligotrophic by the late 19th century, Lakes Geneva, Bourget and Annecy underwent eutrophication, in the mid-20th century, due to industrial effluents and domestic sewage (Giguet-Covex et al. 2010, Perga et al. 2010). The magnitude of eutrophication, however, differed between lakes. Lakes Geneva and Bourget reached a eutrophic status by the end of the 1970's while Lake Annecy never got higher than oligo-mesotrophic (Perga et al. 2010). Phosphorus abatement measures have successfully reduced total phosphorus concentrations ([TP]) in the water column of the three lakes over the last 40-30 years. Based on their winter [TP], Lake Annecy is now oligotrophic while the other two are currently oligo-mesotrophic. In addition, fish communities have been modified through fisheries management practices, which are essentially centred on the zooplanktivorous whitefish, *Coregonus lavaretus*. In brief, whitefish stocking is a common practice to support the fishery recruitment (Gerdeaux and Anneville 2006). It was introduced in Lake Annecy by the end of the 19th century and stocked sporadically from 1900 to the 1930s and then annually from 1936 to 1997. In Lakes Geneva and Bourget, stocking has been performed to support local populations (since 1970 for Lake Geneva and every year during 1943-1965 and 1986-2008 for Lake Bourget, Champigneulle et al. 2001).

Paleolimnological reconstructions were conducted for Lakes Geneva and Bourget while data for Lake Annecy were available from a previous study (Perga et al. 2010).

Coring and chronology

145 Several short sediment cores (30 to 72 cm long) were collected in the lakes deepest points in
146 May 2010 (Lake Geneva) and February 2009 (Lake Bourget) using a quadruple gravity corer
147 (UWITEC, Mondsee, Austria). Sediment dating was performed from one reference core for
148 each lake using radiometric methods (^{210}Pb, ^{226}Ra, ^{137}Cs and ^{241}Am activities) and counting of
149 annual laminations, which provided accurate chronologies for high-resolution sampling in
150 both lakes (Appendix A). All working cores (those on which paleolimnological analyses were
151 performed) were sampled following annual laminations, with the same temporal precision
152 (annual or, at most, biennial resolution) and correlated to the reference core using lithological
153 tie points and lamina counting performed on both reference and working cores (Appendix A,
154 Zolitschka 2003). Such high-temporal resolution and accuracy were required in order to
155 integrate instrumental and paleo-data from multiple cores. Sampling and dating details of
156 Lake Annecy were described in Perga et al. (2010).
157

158 ***Subfossil cladocerans analysis***

159 Cladoceran remains (countings and measures) were analyzed as in Alric and Perga (2011).
160 *Daphnia* (*D. longispina* species complex) were identified at the generic level (*Daphnia* sp.).
161 Sididae, Cercopagidae, Leptodoridae and *Bosmina* were identified at the species level and
162 Chydoridae at the family level. Results were reported as number of subfossil remains per unit
163 weight of dry sediment [remains (g dry wt)$^{-1}$] and per net accumulation rate (remains cm^{-2}
164 year^{-1}) for each taxon. The length of *Daphnia* sp. post-abdominal claws was measured on a
165 hundred remains for each sediment sample in order to estimate changes in the size structure of
166 the *Daphnia* sp. population (Perga et al. 2010, Alric and Perga 2011). The length of *Daphnia*
167 sp. post-abdominal claws was further used as a proxy of the modification in fish predation
168 pressure on zooplankton for the three lakes (Perga et al. 2010), since predation by fish has a

169 structuring effect on cladoceran body size (size-efficiency hypothesis, Brooks and Dodson
170 1965).
171
172 ***Climatic data and proxy for changes in lake nutrient concentrations***
173 For each lake, datasets of annual, wintry and summery air temperature as well as
174 precipitations have been extracted from the gridded HISTALP data set
175 (www.zamg.ac.at/histalp/; Auer et al. 2007), according to lakes geographical coordinates. The
176 HISTALP database is based on monthly-homogenised long-term series of temperature,
177 precipitation and other meteorological variables for the so-called Greater Alpine Region (4-
178 19°E, 43-49°N, 0-3500 m asl). The air temperature and precipitation records extend both back
179 to <1800 and thus completely covers the time period of the sediment record of our three lakes.
180 Temporal changes in annual water [TP] of the three lakes were reconstructed using a diatom-
181 transfer function (Appendix B).
182
183 ***Statistical analyses***
184 Temporal trends in annual, winter and summer air temperature as well as precipitations were
185 tested through Mann Kendall's tau test. Chronological clustering analyses, computed from
186 Bray-Curtis dissimilarity indexes, were performed on biostratigraphic sequences of sediment
187 cores LEM10_P801 (Lake Geneva), LDB09_P301 (Lake Bourget) and 06-03 (Lake Annecy,
188 see Perga et al. 2010) to reveal the timing of major changes in the cladoceran communities for
189 all three lakes (CONISS in R package rioja, Juggins 2009). The number of statistically
190 significant biozones was assessed using the broken-stick approach (Bennett 1996). Significant
191 changes in the abundance of each taxon between adjacent zones were thereafter tested, using
192 non-parametric tests to avoid problems related to data heteroscedasticity and non-gaussian

193 distribution (Kruskal-Wallis tests accounting for Bonferroni's corrections: extensive results
194 are not shown in the Results section, for conciseness).
195 Patterns of cladoceran community change were summarised using principal
196 component analysis (PCA) of relative abundance data after Hellinger transformation
197 (Legendre and Gallagher 2001). The first two principal components (PC) were identified as
198 explaining significant proportions of the variance in the species data when compared with
199 those expected under the broken-stick distribution. The scores on the first two PC were
200 retained as dependent variables for subsequent modelling. A generalized additive model
201 (GAM, Hastie and Tibshirani 1990) was then used to investigate the relationships between the
202 trajectory of cladoceran community and external forcings as well as to separate and quantify
203 their influences. GAM is a semi-parametric regression technique with the main advantage of
204 not being tied to a particular functional relationship (i.e., linearity) and to be less restrictive in
205 assumptions about the underlying statistical distribution of the data. In GAM, predictors
206 (covariates) are assumed to affect the response variable, through additive sum of unspecified
207 smooth functions. GAM parameterization and adaptation to paleoecological data were
208 performed following Simpson and Anderson's (2009) technical recommendations. The
209 feature selection process was based on shrinkage method by a double penalty approach
210 instead of the more commonly used stepwise procedure (Marra and Wood 2011). In our
211 study, climate data (HISTALP data set), diatom-inferred TP and *Daphnia* sp. claws size (as a
212 proxy for changes in fish predation pressure) were introduced as the predictor variables in the
213 model while the changes in the scores of the first two PC were considered as the response
214 variables.
215 PCA and GAMs were performed on R2.11.0 statistical software (R Development Core
216 Team, 2009) using the vegan package (Oksanen et al. 2010) and mgcv package (Wood 2008,
217 Wood 2011) for R, respectively.

Results

External forcings

In all three lakes, mean annual air temperatures exhibited a common and significant increasing trend from the last 1980s. Between 1986 and 2009, the mean annual air temperature has increased by more than 1°C as compared with the prior period for all lakes (Fig. 1a-c). Increased annual air temperature essentially resulted from warmer winters (+ 1.6-1.7° C). Since no trend was observed for mean annual precipitations on the three lakes (Mann Kendall's tau test, $P>0.05$), only mean air temperatures were used in the following to account for climate change.

The reconstructed diatom-inferred TP (DI-TP) indicated that, for all three lakes, [TP] values were <10 µgP L^{-1} before the 1940s (pre-eutrophication period) when the first symptoms of eutrophication started (Fig. 1a-c). DI-TP increased first weakly from the 1940s (early eutrophication period), then dramatically from the 1960s (intense eutrophication period) in all three lakes. Maximum DI-TP were reached in the late 1960's for Lake Annecy (14 µgP L^{-1}), the early 1970s for Lake Geneva (70 µgP L^{-1}) and the mid 1970s for Lake Bourget (80 µgP L^{-1}). The progressive decreases in DI-TP, resulting in values of [TP]<20 µgP L^{-1} in the late 2000s for Lakes Geneva and Bourget and <10 µgP L^{-1} for Lake Annecy, were in agreement with monitored dynamics of annual water [TP] (Appendix B).

In all three lakes, the average size of *Daphnia* sp. post-abdominal claws was large during the pre-eutrophication period and started to decrease from and all over the eutrophication period (Fig. 1a-c). *Daphnia* sp. postabdominal claw got about 20% smaller as compared to the pre-eutrophication period in average, across all three lakes, hence suggesting that eutrophication triggered an increased fish predation pressure on zooplankton. Changes in *Daphnia* sp. size (and thus fish predation pressure on zooplankton) during the re-

oligotrophication period yet diverged between lakes: *Daphnia* sp. size kept on shrinking in Lake Annecy, while *Daphnia* sp. size remained low but stable in Lake Geneva. In contrast, *Daphnia* sp. size increased back in Lake Bourget during the re-oligotrophication period, suggesting of a released fish predation pressure on *Daphnia* sp.

Trajectories of the cladoceran communities in lakes

Overall, the cladoceran remains retrieved from the sediment cores originated from six pelagic taxa [*Daphnia* sp., *Bosmina longirostris* (O.F. Müller 1776), *Eubosmina coregoni* (Baird 1857), *Eubosmina longispina* (Leydig 1860), *Bythotrephes longimanus* (Leydig 1860) and *Leptodora kindti* (Focke 1844)] as well as one littoral taxon [*Sida crystallina* (O.F. Müller 1776)] and two littoral subfamily [Aloninae and Chydorinae] of the Chydoridae family. Because the number of remains for Aloninae and Chydorinae was low, they were pooled in a single group (i.e., Chydoridae). All the eight taxa were found in Lakes Geneva and Bourget but littoral and predatory cladocerans were not detected in Lake Annecy sediment records. Chronologically constrained cluster analyses identified five cladoceran assemblage zones for Lakes Geneva and Bourget and four zones for Lake Annecy (Fig. 2a-c), which matched the time-periods of major [TP] changes in all three lakes. Cladoceran assemblages in zones 1, 2 and 3 (corresponding to pre-, slow and intense eutrophication) showed changes in their structure that were similar between lakes. Thereafter, cladoceran trajectories diverged from the zone 4 corresponding to the beginning of gradual decrease in [TP].

In zone 1 (before the 1940s for all lakes, corresponding to the pre-eutrophication period), *Daphnia* sp., *E. longispina* and *S. crystallina* remains were found in low abundance but exhibited the greatest contribution to total assemblages. *Bosmina longirostris* and *E. coregoni* were rare and found only in Lakes Geneva and Bourget.

Zones 2 (before the 1960s for Lakes Geneva and Bourget and the mid 1950s for Lake Annecy: slow eutrophication) and 3 (during the 1970s for Lakes Geneva and Bourget and the 1960s for Lake Annecy: intense eutrophication) were characterised by (i) a significant increase of *Daphnia* sp. abundance (from six- to twelve-fold) and (ii) shifts in the composition of *Bosmina* remains towards *B. longirostris* dominance (Fig. 2a-c). *Bosmina* sp. (*E. longispina* in the three lakes plus *E. coregoni* in Lake Geneva and Bourget) abundances further increased during the eutrophication period. During this period also, *S. crystallina* abundance decreased steadily in the three lakes and chydoridae abundances increased in Lakes Geneva and Bourget (Fig. 2a,b).

Zone 4 (from the 1980s for Lakes Geneva and Bourget and the 1970s for Lake Annecy) corresponded to time-periods of decreasing [TP] for all lakes but also coincided to the beginning of significant changes in mean annual air temperature. At this stage, the responses of the cladoceran communities diverged between the three lakes. *Daphnia* sp. abundance started decreasing in both three lakes with yet different magnitudes (Fig. 2a-c). Lake Bourget exhibited the largest decrease (-74%), followed by Lake Geneva (-59%) and then Lake Annecy (-23%). The total abundance of *Bosmina* (*E. longispina* and *B. longirostris* in the three lakes plus *E. coregoni* in Lakes Geneva and Bourget) remained higher (from two- to ten-fold), even in zone 5, than in the pre-eutrophication period, but the dynamics of the different *Bosmina* species varied between lakes. *Eubosmina longispina* remained the dominant *Bosmina* species throughout this time period in Lakes Annecy (66% of the total *Bosmina* population) and Geneva (on average 89% of the total *Bosmina* population in zone 4 and 5) while *B. longirostris* made up a significant share of the *Bosmina* population in Lake Bourget. Moreover, the contributions of predatory cladocerans, *L. kindti* and *B. longimanus*, to total assemblages were much higher in Lakes Geneva and Bourget in zone 5 than at any other time zone. These species were not detected in Lake Annecy sediment core (Fig. 2a-c).

292

293 ***Additive models***

294 In preamble to the additive models, PCA were performed for each lake over the taxa
295 abundance datasets. The first two axes of PCA accounted for 66, 59 and 93% of the total
296 variance in cladoceran assemblages respectively in Lakes Geneva, Bourget and Annecy and
297 consistently separated samples according to zones determined by the previous cluster analyses
298 (results not shown). The relative contributions of different taxa to the formation of the first
299 and second principal components (PC1 and 2) are presented in table 1. Briefly, in Lakes
300 Geneva and Bourget, one PC (respectively PC2 and PC1) accounted for the correlated
301 changes in the contributions of *Bosmina* sp. and predatory cladoceran species while the other
302 PC (PC1 and PC2 respectively for Lakes Geneva and Bourget) essentially accounted for the
303 negatively correlated changes in *Daphnia* sp. contribution to total assemblages versus that of
304 littoral species. For Lake Annecy, PC1 represented changes in the composition of *E.*
305 *longispina* and *S. crystallina* while PC2 accounted for the negatively correlated changes in
306 *Daphnia* sp. contribution to total assemblage versus that of *B. longirostris*. Analyses of
307 biostratigraphic sequences, PCA and even GAMs were similar whatever the considered unit
308 for cladoceran remains (abundance in [remains (g dry wt)$^{-1}$] or fluxes [remains cm^{-2} year^{-1}]).

309 The final models selected external forcings as covariates that differed among lakes for
310 both PC and explained between 35-87% of the deviance of PC1 and 2 scores (Appendix C).
311 The fitted relationships between PC1 or 2 scores and the different predictors were either
312 nonlinear (edf > 1) or linear (edf = 1) according to the lake, PCs and predictors (Appendix C).

313 In the three lakes, all three covariates contributed significantly to fitted PC1 and 2
314 scores, although their respective importance changed between lakes and PCs (Fig. 3a-c;
315 Appendix C). First, the contribution of annual air temperature to fitted PC scores was always
316 higher in Lakes Geneva and Bourget than in Lake Annecy. Actually, fitted scores for Lake

317 Annecy were essentially determined by covariates related to local forcings ([TP] and
318 predation pressure) and the contribution of the climate component was rather low and only
319 significant on PC2 (driving negative PC2 scores, i.e., promoting *B. longirostris* over *Daphnia*
320 sp.). In both Lakes Geneva and Bourget, [TP] was one the major contributor to fitted PC
321 scores, acting mostly by favouring *Daphnia* sp. versus littoral species (PC1 and PC2 for
322 Lakes Geneva and Bourget respectively) (Fig. 3a,b; Appendix C). The second series of fitted
323 PC scores, corresponding to high abundances of *Bosmina* and predatory cladocerans (i.e.,
324 PC2 and PC1 for Lakes Geneva and Bourget respectively), were essentially driven by
325 predation pressure and mean annual air temperature in Lake Geneva while similar cladoceran
326 dynamics were driven by mean annual air temperature and [TP] in Lake Bourget (Fig. 3a,b;
327 Appendix C).
328 The relative magnitudes according to which each covariate contributed to fitted PC
329 scores differed between Lakes Geneva and Bourget but the temporal dynamics of their
330 contribution were similar: [TP] contributed for almost all over the study periods while the
331 influence of fish predation pressure was more temporally limited. In addition, in both lakes,
332 significant influence of mean annual air temperature occurred from the late 1980s.
333 Differences between the two lakes arose from the different covariate interactions. In Lake
334 Bourget, the contribution of predation pressure was coupled, over the whole study period, to
335 [TP], suggesting a correlation between these two forcings. In Lake Geneva, the contribution
336 of predation pressure was coupled with that of [TP] from the late 1950s to the mid 1980s, i.e.,
337 periods of drastic changes in [TP], and from the late 1980s with that of mean annual air
338 temperature. In Lake Annecy, the effect of predation pressure on changes of PC1 and 2 scores
339 was significant all over the study period with significant shift in the 1950s for PC1 scores as
340 well as in the 1960s and the mid 1980s for PC2 scores. In addition, the contribution of

predation pressure was coupled with that of [TP] from the 1960s to the late 1970s, while it was coupled with the contribution of mean annual air temperature from the mid 1980s.

Discussion

Our study intended to evaluate and hierarchize the effects of local forcings versus climate warming on the trajectory of cladoceran communities in three temperate, peri-alpine lakes submitted to similar climate change. Amongst the two local forcings considered herein, one acts essentially through bottom-up processes (nutrient concentrations) while the other one acts through top-down processes (fisheries management practices). Since effects of top-down controls are supposed to be strongest at the highest trophic levels and tend to fade out when going downward to lower trophic levels (and because the reciprocal is also true for bottom-up controls, McQueen et al. 1986, 1989), we figured that primary consumers might provide a better picture of controls on the pelagic food web than primary producers. Hence, a paleoecological approach based on cladoceran sub-fossil remains was favored over the most commonly used diatom frustule remains.

A first limit to paleo-ecological approaches lies in the representativity of fossil assemblages as compared to their source communities. Potential differences in the cladoceran trajectories observed between lakes and/or changes along cores in the composition of the cladoceran remains could result from varying taphonomical bias or archiving processes between lakes and/or over time, rather than from actual ecological changes in the source communities. A previous sediment trap experiment conducted in a nearby deep peri-alpine lake underpinned that the morphology of these deep, concave lakes favors a rather direct and faster sedimentation, and thus archiving, of cladoceran remains (Alric and Perga 2011). As a consequence, the cladoceran sediment records in these lakes exhibit high compositional fidelity. Yet, while all eight taxa were found in Lakes Geneva and Bourget, no remains for

366 littoral and predatory cladocerans were detected in Lake Annecy sediment records, although
367 some of them are present within the living community of this lake. The absence of such taxa
368 in the sediment record may be due to lower abundances of total remains for time periods
369 preceeding the 1940s (whole sediment samples were counted but total number of remains
370 were at some occasions <400, Perga et al. 2010). In addition, these taxa are generally less
371 represented in Lake Annecy as compared to the two other lakes (for instance, predatory
372 cladocerans made up <0.9% of the whole cladoceran abundance in Lake Annecy in 2011, but
373 2-4 % in Lakes Geneva and Bourget, M.-E. Perga personal data). Rare cladoceran species
374 (<1%) are typically no or poorly represented in fossil assemblages (Frey 1986, Kattel et al.
375 2007, Nykänen et al. 2009). So, overall differences in the cladoceran remains composition
376 between lakes essentially mirror actual differences in the relative abundance of species in
377 their living communities. Besides, further degradation of some of the most sensitive remains,
378 once buried in sediment, might create a tendency to higher abundances towards the uppermost
379 part of cores, artefactually interpreted as increased abundance of these taxa over the most
380 recent times. This should be evoked for *L. kindti*, which remains (mandible and caudal furca)
381 are known for being fragile and easily broken. Yet, the comparison of trends observed in the
382 sediment record with those monitored since 1974 in Lake Geneva, confirmed that the
383 abundance of this particular taxa in the water column has increased two- to three-fold over the
384 last 40 years (Molinero et al. 2007), as very accurately mirrored in the sediment record.
385 Hence, the sediment archives provided a trustful picture of the changes in the cladoceran
386 community structure over time and between lakes.
387 Except for climate for which direct data were available, our approach also required
388 paleolimnological reconstructions for the forcings considered herein. Within
389 paleolimnological approaches, past [TP] have been quite usually inferred from diatom
390 remains (Hall and Smol 1992). Because some long-term monitoring data were also available,

391 we were able to compared DI-TP to measured water [TP]. Both were globally consistent and
392 highly correlated (Appendix B). At some occasions, DI-TP were over-estimated but the
393 availability of monitored [TP] as well as the comparison with several other paleo-proxies
394 allowed correcting for these inconsistencies. Overall, the confidence in TP reconstructions is
395 relatively good. Actually, the challenge was essentially in the reconstruction of the third
396 forcing factor included within this study, i.e., a proxy related to fisheries management
397 practices. Unfortunately, neither long-term data for fish stocking, nor catch per unit effort
398 (CPUE) were available over the study periods. Temporal changes in fisheries practices and
399 regulations were then approached through changes in fish predation pressure on zooplankton,
400 and more specifically on *Daphnia* sp. This proxy relied indirectly on the size-efficiency
401 hypothesis (Brooks and Dodson 1965), i.e., that fish predation pressure exerts a strong
402 structuring impact on the body size of their preys (Kerfoot 1981, Gliwicz and Wrzosek 2008).
403 Indeed, such a relationship between fish stocks and prey size structure has already been used
404 to infer past abundances of zooplanktivorous fish from the size of *Daphnia* sp. ephippia in a
405 set of 52 shallow lakes from Denmark, Greenland and New Zealand (Jeppesen et al. 2002).
406 Such a relationships also holds for our three study lakes since we could show that the
407 temporal changes in *Daphnia* sp. body size were strongly, inversely correlated with fisheries
408 catches in the main zooplanktivorous species (Spearman's rank correlation test between
409 *Daphnia* sp. claws size and catches, Lake Geneva: $\rho = -0.511$, $P = 1.58 \times 10^{-6}$, Lake Bourget:
410 $\rho = -0.340$, $P = 0.009$, Lake Annecy: $\rho = -0.426$, $P = 0.008$, Appendix D), confirming that
411 even though other environmental conditions such as food quality/quantity or temperature
412 (Hart and Bychek 2011) and invertebrate predation (Branstrator 2005), can affect *Daphnia* sp.
413 body size, they may not be strong enough to compromise the relevancy of using *Daphnia* sp.
414 body size as a proxy for fish predation pressure. In addition, as evidenced by *Daphnia* sp.
415 responses in re-oligotrophication periods in all three lakes, changes in *Daphnia* sp. body size

were independent from those in their (absolute or relative) abundances, excluding any circularity in using *Daphnia* sp. body size to predict the whole cladoceran composition over time.

Hence, despite such limits, results presented here reveal that long-term changes in cladoceran communities of Lakes Geneva, Bourget and Annecy, have been driven by a set of complex interactions between climate, nutrient status and intensity of fish planktivory.

External forcings regulating cladoceran communities over the long term

The paleolimnological reconstructions showed that cladoceran communities of the three lakes exhibited similar trajectories until the 1980s, but then diverged. GAMs for their part showed that, depending on time periods, trajectories of cladoceran were driven by different combinations of forcings.

Before the 1980s, P was the main driver of the pelagic food web. Increasing [TP] from the 1940s triggered similar changes in the cladoceran community structure although maximum [TP] levels differed between lakes. Through a bottom-up effect, increasing [TP] allowed the increase of pelagic cladoceran abundance (McCauley and Kalff 1981), in particular *Daphnia* sp., and resulted in a drastic decline in species more typical for shallow habitats (*S. crystallina*) due to lower water transparency and reduction of littoral habitats. Consistent patterns were observed in other deep peri-alpine lakes (Ravera and Parise 1978, Manca et al. 2007). In all lakes, decreasing *Daphnia* sp. body size coincided with the onset of eutrophication, attesting that the intensity of top-down control by planktivorous fish increased with higher nutrient concentrations. Another striking pattern at this same time-period was the appearance of *B. longirostris* and sometimes dominance within the *Bosmina* population. Such transition was actually postulated to result from an indirect predation-mediated effect (i.e., reducing of the competitive interaction between *Bosmina* sp. and *Daphnia* sp. under a high

441 fish predation pressure) rather than from a direct effect of increased nutrient inputs on the
442 genus *Bosmina* (Brooks 1969, Kerfoot 1974). Our results support this hypothesis, because the
443 appearance of *B. longirostris* coincided with the beginning of *Daphnia* sp. size reduction in
444 the late 1940s. This interpretation is consistent with indirect facilitation processes previously
445 tested in mesocosms (Vanni 1987). Then, despite fish were regularly stocked in all lakes from
446 the 1920s, evidences for strong fish predation pressure appeared only from the 1940s when
447 lake nutrient concentration increased and could sustain an important fish population.
448 Therefore, although fish predation pressure exerted a strong structuring effect on the
449 cladoceran community structure before 1980, this top-down control was, *in fine*, allowed
450 because of increased lake productivity.
451 In contrast, from the 1980s, GAM results highlighted that decreasing nutrient
452 concentrations were not the only forcing explaining changes in cladoceran communities in all
453 three lakes. Actually, only *Daphnia* sp. abundance was driven by [TP] and hence responded
454 to reduced nutrient inputs. In contrast, the dynamics of all the other cladoceran taxa seemed to
455 be determined by other forcings. Indeed, *Bosmina* sp. abundance remained relatively high
456 although nutrient concentration dropped. In addition, the abundance of *L. kindti* and *B.*
457 *longimanus* increased in Lakes Geneva and Bourget. Consequently, decreasing [TP] in all
458 three lakes did not result in reverse trajectories for the cladoceran communities. Such a lack
459 of recovery to the pre-eutrophication community structure has been observed in other
460 European lakes for which the re-oligotrophication period coincided with a period of air
461 warming as a result from positive phase of North Atlantic Oscillation. In such lakes, these
462 trajectories have been attributed to climate change (e.g., Jeppesen et al. 2007, Søndergaard et
463 al. 2007). Yet, while all three studied lakes have also been undergoing similar warmer air
464 temperature during the last 25 years, results emphasized that they were not equally sensitive
465 to climate change, but also that their subsequent responses to warming differed.

To what extent can climate explain the lack of recovery to pre-eutrophication state?

GAM results underpinned a significant implication of warming to changes in cladoceran communities in the three lakes, but the climate contribution to the cladoceran dynamics was contrasted between lakes. In Lake Annecy, major changes in the cladoceran community structure after the 1980s occurred as changes in the relative proportions of *Bosmina* sp. and *Daphnia* sp., and these changes could not be related only to any obvious climate warming effect. These conclusions are strengthened by a recent study that showed, from monitoring data, that the cladoceran succession pattern in Lake Annecy was less sensitive to the 2003 heat wave in contrast to Lake Geneva where it was deeply affected (Anneville et al. 2010). The continuous shrinking of *Daphnia* over the last 60 years and the persistence of an abundant *Bosmina* population in Lake Annecy actually result from a still increasing fish predation pressure on zooplankton, artificially maintained through annual whitefish stocking until 1997. In this lake, changes in fish planktivory as a result of fisheries management practices might outweigh the impact of global warming. Then, in this lake, climate acted marginally on the cladoceran community, while it has been exerting a major structuring effect over the last 30 years in Lakes Geneva and Bourget. However, since part of Lakes Geneva and Bourget responses to climate occurred through predatory cladocerans, the lowest contribution of climate to the cladoceran trajectories observed in Lake Annecy might partly arises from the lack of recording of predatory cladocerans in its sediment archives. To test how much differences in lake vulnerability to climate were depending on predatory cladocerans, another series of GAMs, including only *Daphnia* sp. and *Bosmina* sp. in the response variable, were conducted. The ranked contributions of the forcings to the observed trajectories were very similar in these additional GAMs as in those performed on the whole

491 datasets (results not shown), showing that the differences in lake vulnerability to climate
492 warming is not artifactual and poorly depends on predatory cladocerans. This comparison also
493 underpinned that predatory cladocerans make up only a minor part of the cladoceran
494 responses to warming, which essentially occurs through an increased contribution of some
495 *Bosmina* species (*B. longirotris* and *E. coregoni*, for Lakes Geneva and Bourget) and a higher
496 *Bosmina* sp. to *Daphnia* sp. ratio (Lakes Geneva and Annecy).

497 Not only the contribution, but also the pathways according to which climate has been
498 impacting cladoceran communities partly differed between the three lakes. Air temperature
499 was, from the 1980s, a significant contributor to PCs scores in all the three lakes. The
500 direction of correlations suggested a positive effect of warmer temperatures on the abundance
501 of *Bosmina* species, *L. kindti* and more moderately on *B. longimanus* in Lakes Geneva and
502 Bourget, and, in a lesser extent and only on *B. longirostris* in Lake Annecy. These results
503 support the hypothesis of a direct effect of climate on cladoceran communities through
504 physiological processes. Indeed, *Bosmina* sp. generally presented a faster development and
505 growth than *Daphnia* sp. and warming is expected to even emphasize this gap (Vijverberg
506 1980). In addition, longer stratification periods should promote the thermophilic taxa as *L.*
507 *kindti* and *B. longimanus* (Wagner and Benndorf 2007, Manca and DeMott 2009). In Lake
508 Bourget, GAM did not highlight any clear interaction between climate and local forcings.
509 Hence, the latter might be the major pathway under which climate has been influencing the
510 cladoceran community structure. More indirect ones cannot be excluded though. Indeed, the
511 effect of increased air temperature could also involve trophic processes and competition
512 between *Bosmina* sp. and *Daphnia* sp., through alterations of the composition of primary
513 producers. For instance, climate warming is suspected to promote summer bloom of
514 filamentous algae (Paerl and Huisman 2008) and such blooms of the filamentous
515 cyanobacteria *Planktothryx rubescens* have been observed in Lake Bourget from the 1990s

516 (Jacquet et al. 2005). The filamentous morphology of cyanobacteria is though to be more
517 detrimental to food-collection mechanisms of larger filter-feeding species (Haney 1987),
518 hence conferring a trophic competitive advantage to *Bosmina* sp. over *Daphnia* sp. (Gliwicz
519 1990).

520 In Lake Geneva, but also marginally in Lake Annecy, the contribution of increased air
521 temperature to PC scores was shown to be coupled and positively related to fish predation
522 pressure from the late 1980s. Climate warming might, thus, also exert a more indirect
523 structuring effect on cladoceran communities, by increasing the top-down control in these
524 lakes. This was highlighted by the persistence of small *Daphnia* sp. during re-
525 oligotrophication in these two lakes, with subsequent implications for competition with
526 *Bosmina* sp. Hence, in Lakes Geneva and Annecy, and in contrast to Lake Bourget or to the
527 eutrophication period, the intensity of fish predation pressure was decoupled from lake
528 nutrient status from the late 1980s. Indeed, warming was shown to benefit to whitefish
529 recruitment (Eckmann and Rösch 1998) and thus to stocking (Gerdeaux and Anneville 2006).
530 However, the interaction between fish predation pressure and climate warming occurred only
531 in two of the lakes, although all three are stocked with whitefish. These were the lakes in
532 which whitefish is stocked every year as yolk-sac larvae. No such interaction was detected for
533 Lake Bourget in which stocking occur much later in the season, at juvenile stages
534 (Champigneulle et al. 2001). These differences suggest that the stocking practices may also
535 modulate cladoceran sensitivity and response to climate. Indeed, whitefish larvae stocked
536 early in the season would exhibit a higher growth rate and lower predation losses due to
537 warmer summers (Straile et al. 2007) and exert an even stronger predation pressure on the
538 cladoceran compartment at the season at which it is very sensitive to top-down regulation
539 (Cryer et al. 1986, Mehner and Thiel 1999) as compared to fish stocked at juvenile stages.
540

Local forcings affect lake sensitivity to climate change

In a number of ecosystems, climate change has been shown to interact with and to inflate deleterious consequences of local human stressors, resulting in a faster erosion of biodiversity (Benning et al. 2002), in a limited efficiency of restoration measures (Anneville et al. 2005, Jeppesen et al. 2007), in a distortion of the strength of trophic interactions (Hoekman 2010, Kratina et al. 2012) or in decreased ecosystem resilience (Buma and Wessman 2011). This issue though has rarely been considered the other way round, i.e., ecosystem sensitivity to climate might vary depending on existing local pressures (but see Palmer et al. 2008 for an example on river basins). In this study, we showed that three lakes subjected to similar climatic variability but different intensities of analogous local stressors show uneven susceptibility to climate warming. Cardille et al. (2009) archived similar conclusions when investigating carbon and water cycling in 7000 lakes under similar climate influence. In their study, varying lake sensitivity to climate was attributed to geomorphological or hydrological differences between systems, i.e., lake and watershed size, age, upstream and downstream connections. Indeed, geomorphological characteristics are though to modulate lake susceptibility to meteorological forcings, shallow lakes being more sensitive than deeper ones (George 2010). In our study, lakes were chosen in order to minimize geomorphological differences but it has to be acknowledged that their depth and size remain different (especially between Lake Geneva and the other two). Still, such size differences are not likely the main factor responsible for their uneven vulnerability to climate warming. Indeed, Lake Annecy is the smallest and the least deep of the study lakes. Since it is also rather sheltered from winds and poorly influenced by floods, Lake Annecy warms up quicker than the other two (Anneville et al. 2010). However, the cladoceran community structure, and by extension the pelagic food web, of Lake Annecy was shown to be poorly responding to climate warming. In Upper Lake Constance, another deep, temperate peri-alpine lake with a current oligotrophic

566 status, climate warming was considered as a major forcing of the phytoplankton productivity
567 changes since the mid 1980s. However, simulations predicted that the ongoing decline in [TP]
568 and further decreasing shall outweigh climate effects on phytoplankton development in the
569 foreseeable future (Stich and Brinker 2010). Consistently, such lack of sensitivity of the
570 cladoceran community in Lake Annecy so far might be the consequence of its low nutrient
571 concentration. In addition, the analysis of long-term data-series for phytoplankton of five
572 deep temperate peri-alpine lakes revealed that mesotrophic, as compared to oligotrophic,
573 lakes are those for which phytoplankton response (in terms of abundance) to climate is the
574 strongest (Anneville et al. 2005) and this pattern might have consequences further up in the
575 food chain. Indeed, the trajectories of cladoceran communities of the larger but more
576 eutrophic Lakes Geneva and Bourget were substantially driven by climate warming. Lake
577 nutrient status, inherited from its trophic history, is a local human stressor that is likely to
578 strongly modulate lake vulnerability to climate warming.
579 The contribution of climate warming to cladoceran trajectories was, yet, also different
580 amongst lakes of similar nutrient status, with a more marked influence on Lake Bourget than
581 on Lake Geneva but also varying pathways. GAM results suggested a rather direct influence
582 of climate warming on cladoceran community structure that was interpreted as a
583 physiological response to warmer waters in both lakes. The contribution of this direct
584 pathway was much stronger in Lake Bourget than in Lake Geneva but we believe these
585 differences depend on geomorphological characteristics rather than from local pressures.
586 Indeed, Lake Bourget warms up quicker than Lake Geneva every spring and reach higher
587 surface temperatures (Jacquet et al. 2005), partly because of its smaller depth and lower
588 hydrological influence of tributaries. Results however suggested that the impact of climate
589 warming on cladoceran communities in Lake Geneva and Annecy could also occur along an
590 indirect pathway, involving increased recruitment of zooplanktivorous fish following fisheries

management practices. More intensive stocking but also release of different development stages in Lake Geneva and Annecy, as compared to Lake Bourget, might explain the observed coupling between fish predation pressure and climate warming, hence highlighting that fisheries management is another local stressor that might modify pathways under which climate warming might impact ecosystems. To conclude, since local perturbations affect lake vulnerability and responses to climate warming, they should be accounted for when trying to predict future impacts of climate change.

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834 Table 1. Relative contributions of the different cladoceran taxa to the two first principal
835 components (PC1 and 2) for all three lakes.

Lake	Taxa	PC1	PC2
Geneva	*Daphnia* sp.	0.479	
	B. longirostris		0.458
	E. coregoni		-0.462
	E. longispina		-0.496
	B. longimanus		-0.387
	L. kindti	0.405	
	S. crystallina	-0.489	
	Chydoridae	-0.354	
Bourget	*Daphnia* sp.		0.610
	B. longirostris	-0.409	
	E. coregoni	-0.377	
	E. longispina	0.414	
	B. longimanus		-0.091
	L. kindti	-0.384	
	S. crystallina	0.528	
	Chydoridae		-0.452
Annecy	*Daphnia* sp.		0.628
	B. longirostris		-0.544
	E. longispina	-0.533	
	S. crystallina	-0.542	

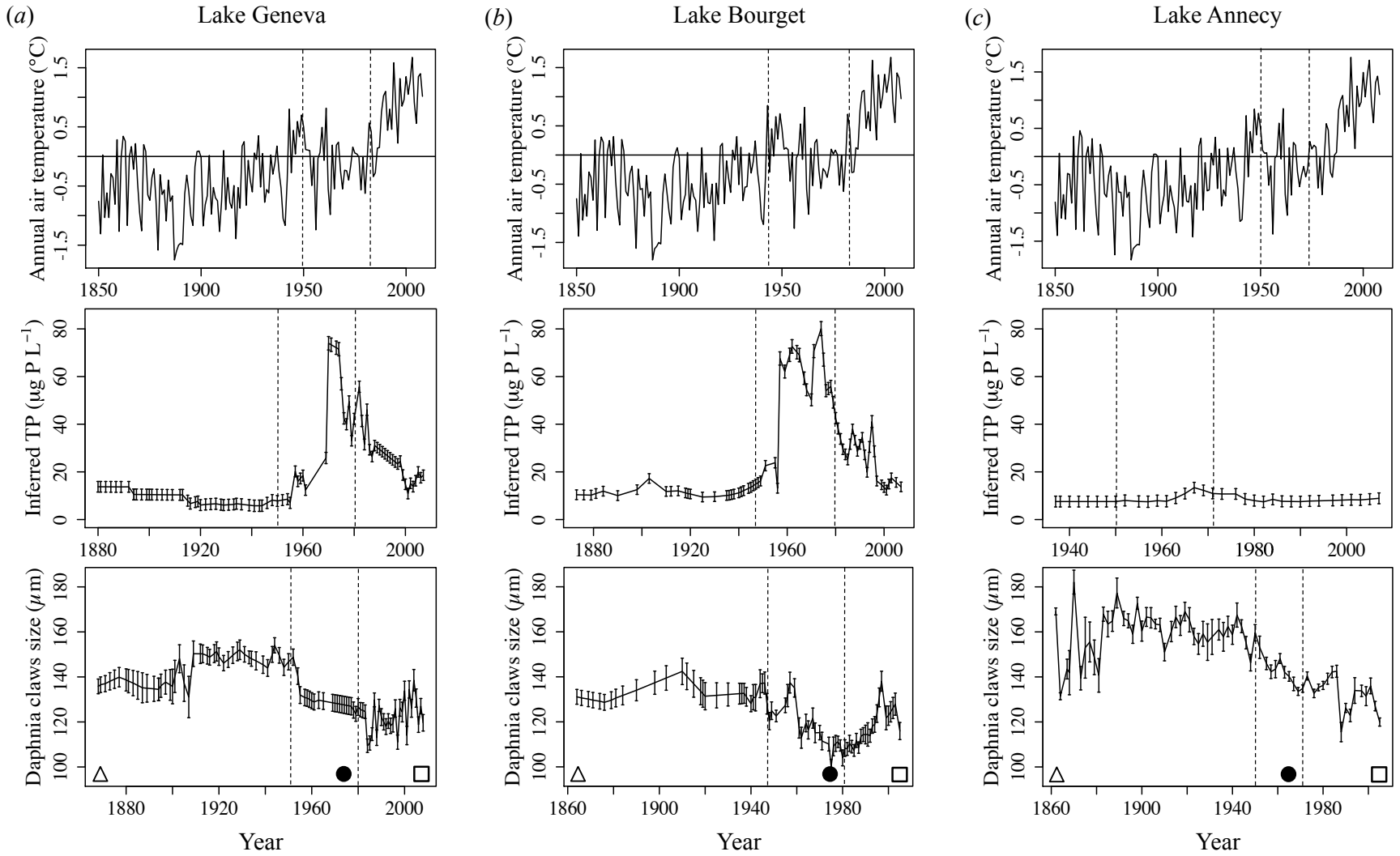
836

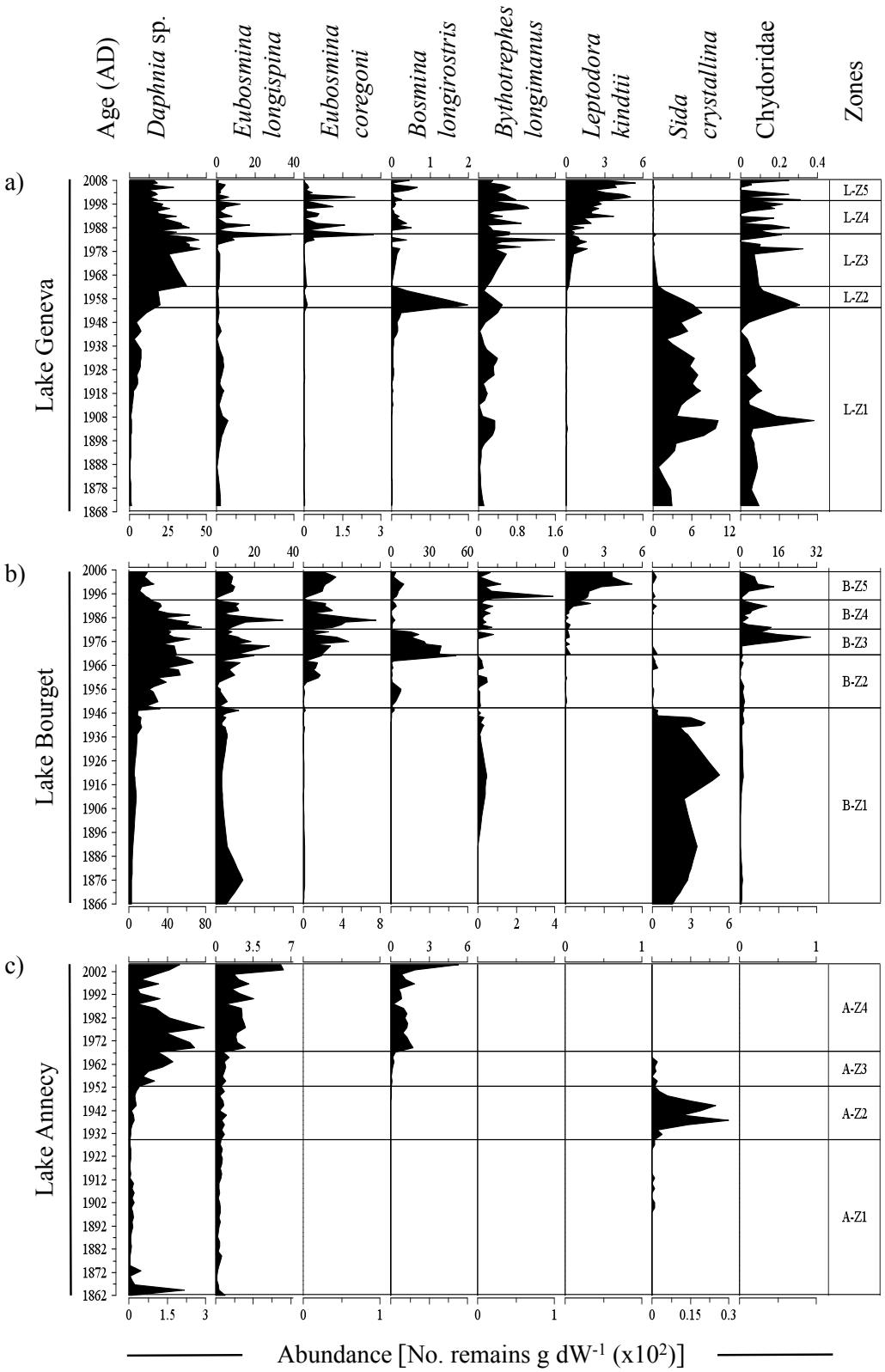
Figure captions

Figure 1. Temporal changes in average annual air temperature (anomalies from the 1901-2000 average), DI-TP, and the length of *Daphnia* claws in (a) Lake Geneva, (b) Lake Bourget and (c) Lake Annecy. Symbols correspond respectively to the periods of (open triangles) pre-eutrophication, (closed circles) eutrophication and (open squares) re-oligotrophication. These time periods are characterized by changes in intensity of the different external forcings.

Figure 2. Stratigraphic abundance of cladoceran remains in sediment cores (as absolute concentration per unit weight of dry sediment) of (a) Lake Geneva, (b) Lake Bourget and (c) Lake Annecy. Zones correspond to the time periods identified by chronological clustering on core LEM10_P8 for Lake Geneva and LDB09_P3 for Lake Bourget and 06-03 for Lake Annecy.

Figure 3. Temporal contribution of covariates selected in final GAMs fitted to cladoceran PC1 and 2 for (a) Lake Geneva, (b) Lake Bourget and (c) Lake Annecy. The dashed lines are approximate 95% confidence interval on the contribution. Where the dashed lines include the zero lines, the contribution of the covariate is not statistically significantly different from the intercept. The order of covariates for each lake corresponds to the importance of their contribution to explain the fitted cladoceran PCs scores (see Appendix C for more details). Symbols correspond to the periods of changes in intensity of different external forcings (see Fig. 1 for more details).

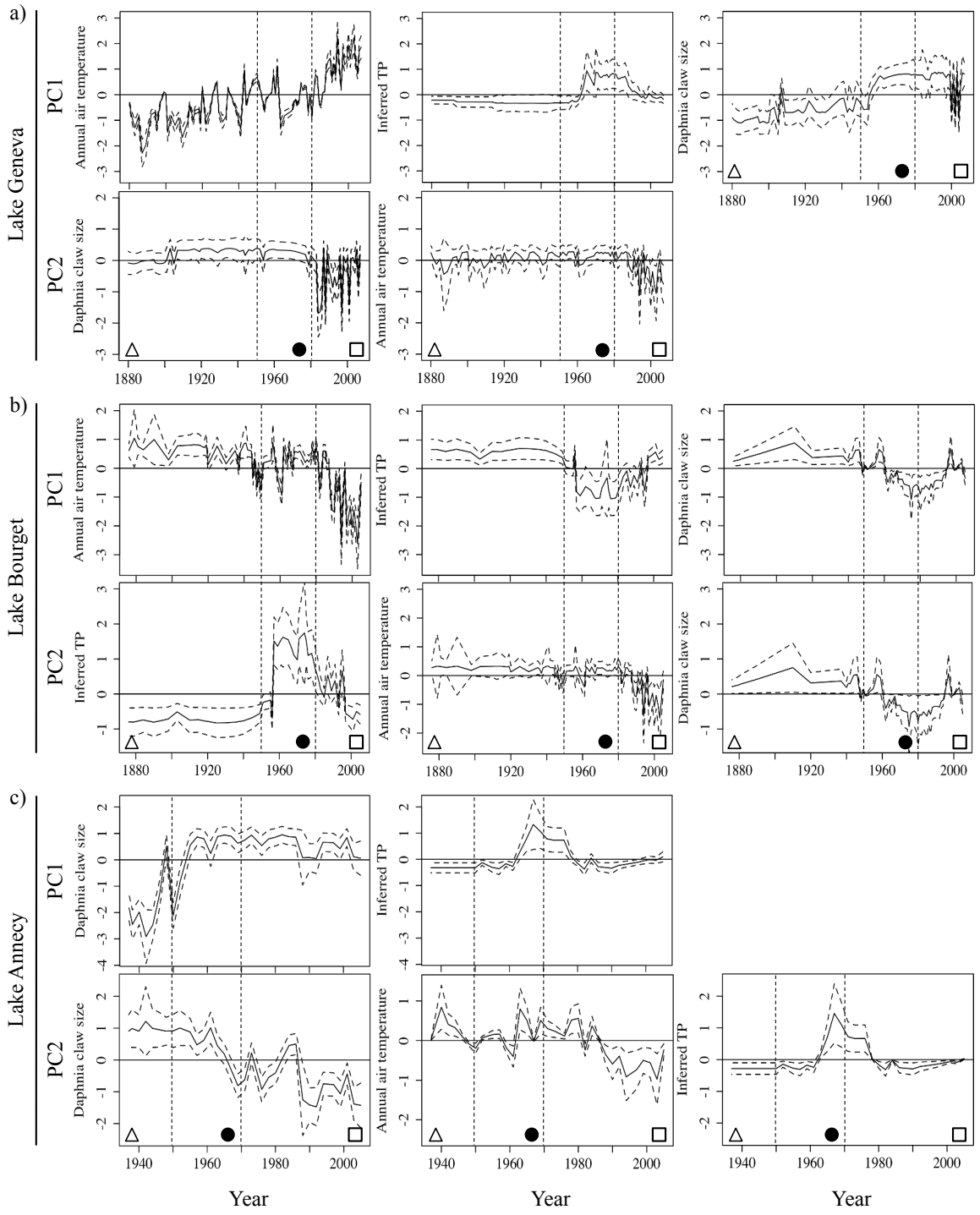

(a)
Lake Geneva
(b)
Lake Bourget
(c)
Lake Annecy
Annual air temperature (°C)
Inferred TP (µg P L⁻¹)
Daphnia claws size (µm)
Year


Age (AD)
Daphnia sp.
Eubosmina longispina
Eubosmina coregoni
Bosmina longirostris
Bythotrephes longimanus
Leptodora kindtii
Sida crystallina
Chydoridae
Zones
a)
Lake Geneva
L-Z5
L-Z4
L-Z3
L-Z2
L-Z1
b)
Lake Bourget
B-Z5
B-Z4
B-Z3
B-Z2
B-Z1
c)
Lake Annecy
A-Z4
A-Z3
A-Z2
A-Z1
Abundance [No. remains g dW-1 (x102)]

860

861

862


a)
Lake Geneva
PC1
PC2
Annual air temperature
Inferred TP
Daphnia claw size
b)
Lake Bourget
c)
Lake Annecy
Year

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5. Réponses des communautés de chironomes à des pressions anthropiques contrastées : une rétro-observation sur 150 ans à partir d'archives sédimentaires

❖ *Article : Depth-specific responses of the chironomid community to contrasting anthropogenic pressures: A paleolimnological perspective from the last 150 years.*

Soumis dans Freshwater Biology

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Depth-specific responses of the chironomid community to contrasting anthropogenic pressures: A paleolimnological perspective from the last 150 years.

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Keywords Chironomidae, eutrophication, climate change, benthic food web, fish introduction

Summary

1. The specific effects of three types of anthropogenic forcing (nutrient concentrations, climate change and fisheries management practices) on the benthic invertebrate community over the last 150 years in a re-oligotrophicated large, deep subalpine lake were investigated using chironomid remains. The structural changes in the chironomid assemblages in littoral and deep habitats were assessed based on sub-fossil remains retrieved from sediment cores sampled along a depth gradient (i.e., 30 m, 56 m and 65 m) and analyzed at a high temporal resolution (2 to 5 years).

2. Until the 1930s, the chironomid assemblages were strongly depth specific, but they were characterized by oxyphilous taxa at all of the studied depths. After the 1930s, the chironomid assemblages drifted towards common hypoxia-tolerant-dominated assemblages. The scores of the first axis of principal component analyses (PCA1 scores) for each studied depth were used as a proxy for the community structure. The specific influences of anthropogenic forcings on the PCA1 scores were assessed using general additive models (GAM). The temporal variability of the contribution of each covariate to our model estimates allowed their specific effects to be clarified. This study highlights the permanent recombination of stressors influencing chironomid assemblages over the last 150 years.

3. The sensitivity of the chironomid assemblages to the considered forcings was depth dependent. The profundal chironomid assemblages (65 m) responded mainly to nutrient enrichment, whereas the chironomid assemblages at shallower depths (56 m and 30 m) were mainly affected by the top-down effects exerted by the fish community on the pelagic food-web, triggering increasing pelagic losses from the pelagic to the benthic zone. Since the late 1980s, increased air temperatures have likely been responsible for the changes in the chironomid assemblages at all of the sampled depths, potentially through their effect on the strength and the length of thermal stratification, possibly coupled with changes in the mixing efficiency during winter.

4. Our results illustrate the strong linkages coupling the pelagic and the benthic food-webs and identify possible temporal substitutions of the anthropogenic forcings successively driving the chironomid assemblages. These findings provide additional evidence of the multiple environmental controls structuring chironomid assemblages and highlight the associated difficulties in predicting chironomid assemblage trajectories following mitigation efforts.

Introduction

Rapid changes in lake productivity, specifically eutrophication, generally result in negative effects on the lake's ecological status, such as toxic phytoplankton blooms (Jacquet *et al*., 2005; Dong-Kuyn *et al*., 2007), hypolimnetic anoxia or organoleptic properties in drinking water (Lange and Wittmeyer, 1996; Eby and Crowder, 2002; Müller and Stadelmann, 2004). In the past several decades, major advances have been realized in many lakes worldwide in controlling and reducing nutrient inputs (Eckrnann *et al*., 2007). This has led, in many cases, to spectacular abatement of nutrient inflows and concentrations in the water column (Carpenter and Cottingham, 1997; Schindler, 2006). However, recent lines of evidence have highlighted that this type of chemical recovery of a water mass does not necessarily imply recovery at the ecosystem scale (Carpenter and Lathrop, 1999; Span *et al*., 1994; Millet *et al*. 2010).

The ecological characterization of lakes has long been conducted through the study and characterization of conditions in the pelagic zone (Wetzel, 2001; Reynolds, 1992). The benthic foodwebs in lakes are increasingly being recognized as influencing the whole-lake function (Reynolds, 2008). The larvae of *Chironomidae* (Diptera) are a key component of the benthic food-web due to their high densities (Drake and Arias, 1995; Nyman and Korhola, 2005; Armitage *et al*., 1994) and their role in processing deposited organic matter (Nogaro *et al*., 2009). The oxygen supply has been shown to be one of the major constraints directly affecting chironomid communities (Verneaux and Aleya, 1998). In lakes, changes in chironomid assemblages are often reported to occur with the trophic state of the lake (Sæther, 1979; Wiederholm, 1980). Thus, the increase in phytoplankton production during eutrophication results in increased organic matter (OM) exports to the benthic food-web. These additional inputs of OM trigger increases in bacterial respiration and subsequent oxygen variability, ultimately impacting the chironomid community. This assumption has been directly tested in the eutrophic Lake Gijster (the Netherlands), which was artificially destratified during the summer (Heinis and Davids, 1993). In the profundal sediment of this lake, the chironomid assemblages are closer to a chironomid community typical of an oligo-mesotrophic lake than a eutrophic one, thus confirming that the relationship between the chironomid community structure and the lake trophic status is indeed mediated by the effects of the oxygen supply in the hypolimnion.

Many of the chemical, biological or physical factors acting in lakes impact the oxygen supply at the lake bottom, such as climate change (Jankowski *et al*., 2006), fish introductions (De Backer *et al*., 2012) and increased watershed organic matter (OM) inputs (Leavitt *et al*., 2009). Increases in temperature promote primary production as well as the length and duration of stratification, both of which are precursors of oxygen depletion (Danis *et al*., 2004). Additionally, changes in the zooplankton size structure following fish introductions can also trigger increased pelagic OM losses, without any change in primary production (a top-down effect) (Carpenter *et al*., 1985; Vanni, 1987).

Several anthropogenic forcings other than the inputs of anthropogenic nutrients can therefore affect the oxygen supply in benthic habitats. Hence, changes in chironomid assemblages in response to oxygen conditions are an ecological manifestation of the complex environmental interactions at both the intra- (e.g., nutrient enrichment, food-web efficiency) and supra-lake (climate) scales.

One of the significant ecological features of chironomids in lakes is their complex spatial distribution, which varies through several orders of magnitude (i.e., from dm to km, Sagova-Mareckova, 2002; Lobinske *et al*., 2002). In lakes, this spatial distribution principally varies with the depth gradient (Eggermont *et al*., 2008; Engels and Cwynar, 2011). Most chironomid species are able to grow in the littoral zone (i.e., above the euphotic limit, in the epilimnion), and littoral chironomid densities are usually high (i.e., several thousand per m², Drake and Arias, 1995; Lobinske *et al*., 2002). Furthermore, the high habitat diversity in the littoral zone promotes the coexistence of rich and functionally diverse chironomid assemblages (Langdon *et al*., 2010; Butkas *et al*., 2011). In contrast, only a few chironomid species are adapted to grow in the deepest zones (below the euphotic zone, in the hypolimnion) because of stronger ecological constraints (e.g., oxygen supply) as well as the lower habitat diversity in this zone compared to the littoral zone (Sæther, 1979; Wiederholm, 1980). Due to these differences in environmental conditions, the littoral and the deep chironomid assemblages are likely to respond differently to anthropogenic forcings (Langdon *et al*., 2006). Unfortunately, long-term monitoring data are seldom available, and littoral/deep characteristics are rarely precisely documented to assess the habitat-specific (i.e., littoral / deep) sensitivity to anthropogenic forcings at a whole-lake level.

This study focuses on the recent history of the ecological state of Lake Annecy, a large, deep peri-alpine lake, which has suffered during the last 150 years from fish introduction and management measures, eutrophication and climate change. This lake was of interest as a re-oligotrophication case study because hypoxic conditions still occur in the hypolimnion despite that a strong abatement of the nutrient concentrations in the water column was observed since the 1970s following a remediation plan.

The overarching aim of this study was to define how different types of anthropogenic forcing, i.e., the lake trophic status, changes in the pelagic food-web structure following fish introductions and management and climate change, have affected the benthic chironomid community. Several depths were taken into consideration to account for the bathymetric variability in the chironomid assemblages to allow an assessment of the extent of the depth-specific sensitivity of the chironomid assemblages to anthropogenic forcings.

Methods

Study site and local history

Lake Annecy is a deep (65 m) oligotrophic lake located at the western edge of the French Alps. This monomictic lake is the second largest lake in France, with a total area of 27.4 km². The water retention time in the lake is approximately 4 years, and the average water temperature is approximately 10°C (INRA). Since the beginning of the 20th century, Lake Annecy has suffered from two well-documented local anthropogenic disturbances.

In 1887, the whitefish *Coregonus lavaretus* was introduced to the lake (Leroux, 1908). The early young population was punctually supported until the 1930s. The whitefish standing stocks were subsequently reinforced every winter from 1936 to 1997 (INRA). Since then, in-lake reproduction has supported the whitefish standing stocks. Perga *et al*. (2010) reported that the whitefish have been exerting a strong top-down effect on *Daphnia* sp., illustrated by a 33% decrease in Daphnia body size over the last 60 years.

The trophic state of Lake Annecy increased beginning the early 1930s, reaching a mesotrophic state in the late 1960s, with an average of ca. 18 $\mu g.l^{-1}$ of soluble reactive phosphorus (SRP) in the water column (INRA). In 1957, a large collector was implemented to collect wastewater from localities around the shore of the lake, downstream in the lake outlet. This remediation plan triggered a decrease in the SRP concentration in the pelagic zone from the 1970s to the 1990s (Perga *et al*., 2010). Since the 1990s, Lake Annecy has returned to an oligotrophic state, with SRP concentrations < 10 µg l (data INRA/SILA)

From the 1850s and to the 2000s, the air temperature has significantly increased (Mann Kendall test, tau = 0.36, p < 0.001) (5E' 46N' site from the HISTALP database (http://www.zamg.ac.at/HISTALP/). The increase in air temperature has been especially great since the 1970s, with the mean annual air temperature increasing by ca. 1 °C (Histalp), followed by an increase in the lake water temperature of ca. 1°C between the late 19th century and late 20th century (Danis et al., 2004).

Sampling

In 2009, three sediment cores were retrieved at three depths (30 m, 50 m and 65 m) using a gravity corer (Uwitec, Austria). The cores were then cut in half. One half was dedicated to chironomid analysis, and the other half was dedicated to sediment chronology and age/depth modeling.

Chironomid analysis

Samples of 31.8 cm² with a 5 mm thickness were taken continuously along the three sediment cores. The samples were then exposed to successive 2 hour baths in HCl (10%) and KOH (10%) at room temperature to dissolve carbonates and deflocculate organic matter, respectively. The residues were then sieved through a 100 µm mesh-size filtercup. Chironomid head capsules (HC) were handpicked from the sieving residue under a stereomicroscope at a 40 × magnification. The HC were then

mounted on slides using Aquatex© mounting medium, and identification was performed under a compound microscope at a 200 to 1000 × magnification. Identification was conducted according to Brooks et al. (2007) and Wiederholm (1983). The HC counts were transformed into HC influxes (number of HC / year / 10 cm²) by dividing the number of HC found in a sample by the temporal integration of the sediment sample and by normalizing the sediment surface to 10 cm².

Dating

The deepest reference core, LDAsref (65 m), and two cores from shallower zones, Sev56 and Sev30 (56 m and 30 m, respectively), were dated. Short-lived radionuclides (^{210}Pb, ^{226}Ra, ^{137}Cs and ^{241}Am) were measured in LDAsref to provide an accurate chronology for recent sediments using high-efficiency, very low-background, well-type Ge detectors at the underground Modane laboratory (Reyss et al. 1995). The cores from shallower zones, Sev56 and Sev30, were correlated with the reference core using seven well-defined lithological time points and employing high-resolution spectrophotometry to identify the clay mineral enrichment layer when lithological markers were not visible (Damuth and Balsam, 2003). Details can be found in Frossard *et al*. (accepted with revisions).

Statistical analysis

A redundancy analysis (RDA) was performed to assess the structuring effects of time (i.e., sample dates) and habitats (i.e., sampling depths) on the chironomid HC influx. The significance of the variability in the chironomid HC influx constrained by these two variables was tested using a permutation test with 9.999 permutations.

To examine depth-specific changes in the chironomid assemblages, principal component analyses (PCA) of the chironomid HC influx were performed for each core. The significance of the PCA axes was assessed graphically using the broken stick model (Legendre and Legendre, 1998). The PCA axis 1 scores (PCA1) were considered as a proxy for the chironomid assemblage structure. The PCA1 were then used as a response variable in the modeling process.

The HC influxes were Hellinger transformed before both the RDA and the PCAs (Legendre and Gallagher, 2001), and rare taxa with less than 5 occurrences and a contribution of less than 5 % to the chironomid assemblage in at least one sample were dropped prior the multivariate analyses.

The δ^{15}N OM values obtained from bulk sediment (Perga *et al*., 2010) were thus used as a proxy for the lake's trophic state (nutrient concentration). The robustness of the OM δ^{15}N values from bulk sediment as a nutrient proxy has been validated in several studies (Wu *et al*., 2006; Bergfur *et al*., 2009) and was strengthened by Griffiths *et al*. (2010).

The size of *Daphnia* sp. claws (DC) (Perga *et al*., 2010) was considered as a proxy for the standing stocks of zooplanktivorous fish. The increases in the densities of zooplanktivorous fish, especially

regarding *Coregonus lavaretus* densities, during the 20th century due to fisheries management led to a decrease of ca. 30 % in DC size (Perga *et al*., 2010). This top-down effect altered the ability of zooplankton (*Daphnia* sp.) to control pelagic primary production, as small zooplankton exhibit a lower individual grazing efficiency than do larger zooplankton (Peters and Downing 1984), and it could potentially cascade to the benthic food-web and impact chironomid assemblages (Brooks and Dodson, 1965; Lazzaro et al., 2009). This top-down effect of zooplanktivorous fish therefore indirectly controls the amount of pelagic OM exported from the pelagic food-web to the benthic food-web, which is then further available for microbial respiration, leading to variability in oxygen supplies. Thus, this top-down proxy is related to underlying changes in pelagic functioning exerted by the fish community that propagate to the benthic food-web.

The climate proxies chosen for this study consisted of the reconstructed mean air temperature at the low elevation 5E' 46N' site from the HISTALP database (http://www.zamg.ac.at/HISTALP/) for winter (WINT: December, January, February) and summer (SUM: June, July, August), which were weakly correlated (i.e., $r^2_{adj} = 0.03$). The autumn (September, October, November) and spring (March, April, May) air temperatures were highly correlated with either the summer or winter temperatures and were therefore not integrated in further analyses. The air temperatures in some individual months of the year were also considered because chironomid assemblages have been shown to specifically respond to changes in air temperature of different months of the year (Heiri and Lotter, 2005). The reconstructed temperature for July (JUL), January (JAN) and December (DEC) were considered because they were (i) weakly inter-correlated (i.e., $r^2_{adj} < 0.01$) and (ii) highly correlated with the reconstructed air temperature of all other months of the year. The climate covariates finally consisted in a summer component (i.e., SUM, JUL) and a winter component (i.e., WINT, DEC), which were assumed to affect chironomid assemblages differently. The summer component addresses the effect of the climate on the duration and strength of stratification (i.e., the isolation of bottom water from the oxygen in the atmosphere), whereas the winter component affects the duration and the efficiency of lake mixing (i.e., oxygen loading before stratification), justifying the consideration of both. The temperature dataset consisted of a temperature anomaly time series expressed in 0.1°C increments from 1885 to 2005. Because the sediment samples covered several years (from two to five years), monthly and seasonal average temperatures were calculated according to the number of years covered by the samples (sample size correction).

The respective influences of three types of anthropogenic forcings, i.e., the trophic state, climate and pelagic functioning, on the chironomid assemblage structure (PCA1 scores) at the three different depths were assessed using generalized additive models (GAM, Wood, 2006). As the PCA1 scores did not significantly differ from Gaussian distributions (Kolmogorof-Smirnov tests; $p > 0.25$), the Gaussian distributions of the PCA1 scores were considered for the fitting of the model. GAMs were used because these models allow the consideration of non-linear relationships between an independent

variable and multiple predictors (Wood , 2006), which are a ubiquitous characteristic in ecology. Three types of covariates were considered in the GAM model.

The covariates were selected using a backward approach in which the least significant covariate was dropped from the model until only significant variables remained (which was then referred to as the final model). Changes in the ordination of the covariates did not affect the model selection. The model validation was conducted according to Züur *et al*. (2010). The temporal auto-correlation of the residuals in our final models, as assessed through auto-correlation functions (ACF), did not reveal any significant correlation.

A procedure similar to the one developed by Simpson and Anderson (2009) was used to assess the relative contributions of the tested covariates to the variability of the PCA1 scores. This procedure consisted of the extraction of the specific contribution of each covariate to the GAM estimates. Because our PCA1 scores were structured across time, the temporal variability of the contribution of each covariate to the GAM estimates reflected the temporal variability of the anthropogenic forcings on the chironomid assemblages. All statistical analyses and graphical displays were performed using R 2.15.0 (R Development Core Team, 2012) with the packages, "pgirmess" (Giraudoux, 2011) "vegan" (Oksanen *et al*., 2011), "mgcv" (Wood, 2006), "Kendall" (McLeod, 2011) and "FactoClass" (Pardo and DelCampo, 2007).

Results

The last 150 years of environmental changes

The environmental forcings considered in this study have exhibited different temporal patterns over the last 150 years. The δ^{15}N OM values clearly reflect the trophic changes in the lake, with an increase in δ^{15}N OM from the 1930s to the 1960s of 4-4.5 ‰ to ca. 5.5 ‰ being observed (Fig. 1). This increase in δ^{15}N OM corresponded to the period of eutrophication in the lake. Following the wastewater treatment plan implemented in the 1960s, the lake underwent re-oligotrophication, as identified by the decrease in δ^{15}N OM, which was recovered to pre-1930s values beginning in the 1990s. DC was stable until the 1950s at ca. 160 µm. From the 1950s to the 1990s, DC decreased sharply and then stabilized at ca. 130 µm (Fig. 1). From the 1900s to the 1930s, the two summer proxies identified cold summers, whereas different patterns in the winter proxies were depicted, with high December temperatures and no peculiar patterns being detected for the mean winter and mean January temperatures (Fig. 1). In the 1940s and 1950s, the different climate proxies indicated warm summers and cold winters. Since the 1960s, the summer and winter proxies have increased, especially the summer proxies during the last 20 years (Fig. 1).

Spatial and temporal changes in the chironomid assemblages

In Lake Annecy, the chironomid HC influx differed significantly both among habitats ($p < 0.001$) and over time ($p < 0.001$) according to the RDA (Fig. 2). Taken together, the two variables accounted for 39.1 % of the variability in the HC influx. The first two axes of the RDA explained a significant amount of the variability in the HC influx ($p = 0.005$). In the RDA triplot, RDA axis 1 segregated the assemblage types between the littoral and deep habitats (Fig. 2). The chironomid assemblages in the littoral habitat, i.e., in the 30 m core, mainly consisted of *Paracladius* sp., *H. grimshawi*-type and *Pentaneurini* species and were characterized by highly negative scores on RDA axis 1. In contrast, the chironomid assemblages in the deep habitats (56 m and 65 m) were mainly composed of *M. contracta*-type species and *Procladius* sp. Further differences in the chironomid assemblages between the two deep habitats were related to the highest relative abundance in the chironomid assemblages in the 56 m core corresponding to *M. contracta*-type species, *Procladius* sp. and *Paracladopelma* sp., while the chironomid assemblages in the 65 m core also integrated many littoral taxa, such as *Dicrotendipes* sp., *M. pedellus*-type and *C. mancus*-type species. The temporal effect (i.e., the sampling dates) was mainly supported by RDA axis 2 (Fig. 2). The temporal changes in all of the assemblages were overwhelmingly related to an increased proportion of *S. coracina*-type species in the chironomid assemblages at all depths.

Covariates influencing PCA1 scores at 30 m

PCA axis1 (PCA1) accounted for 21.7% of the assemblage variability and was the only axis providing significant information according to the broken stick model. Over time, the HC samples shifted from presenting positive PCA1 scores, associated with oxyphilous taxa (*H. grimshawi*-type, *Paracladopelma* sp. and *Paracladius* sp. contributing to 19.4 % of the PCA1), to negative CA F1 scores, associated with hypoxia-tolerant taxa (*S. coracina*-type and *C. anthracinus*-type contributing to 10.4 % of the PCA1). The changes in the PCA1 scores of the HC samples were therefore related to increasing hypoxic stress, with a major shift being recorded from ca. 1940-1950 (Fig. 3).

DC and SUM explained 86.1 % of the overall deviance of the PCA1 scores at 30 m. DC was the major covariate driving the PCA1 scores, through a non-linear relationship. The contribution of DC to the PCA1 scores was stable and positive until the early 1950s, indicating that large *Daphnia* sp. coincided with the presence of oxyphilous taxa at 30 m (Fig. 3 and Table 1). Around the 1950s, the contribution of DC abruptly shifted to presenting significant negative scores and then remained stable until the most recent time period (Fig. 3). The shift from an oxyphilous to a hypoxia-tolerant chironomid community was therefore correlated with smaller individuals of *Daphnia* sp.

SUM was linearly related to the PCA1 scores and explained a smaller portion of the variability in PCA1 scores than DC (Table 1). Its influence was variable over time (Fig. 3). SUM showed positive or null contributions to the PCA1 score estimates between the 1910s to the 1930s. Temporary negative contributions appeared in the 1950s, and since the 1980s, there has been a clear trend toward negative

contributions. The negative contribution of the SUM temperature to the modeled PCA1 scores indicates that the increase in the SUM air temperature was associated with hypoxia-tolerant taxa.

Covariates influencing PCA1 scores at 56 m

At 56 m, PCA1 accounted for 9.8 % of the overall variability in the chironomid assemblages. The four first PCA axes provided significant information according to the broken stick model. However, only the first axis exhibited significant temporal changes (MannKendall test; Tau = 0.56; $p < 0.001$). Therefore, the temporal changes PCA1 were specifically used to describe the changes chironomid assemblages. *Paracladopelma* sp. and *M. contracta*-type species showed positive scores, accounting for 20.8% of PCA1. Conversely, *S. coracina*-type and *T. mendax*-type presented negative scores, accounting for 21.6% of PCA1. Over time, the PCA1 scores shifted to displaying negative values, therefore indicating an increase in the oxygen constraint, which was associated with the replacement of oxyphilous taxa by hypoxia-tolerant ones.

The final GAM explaining the changes in the chironomid assemblage at 56 m was highly similar to that at 30 m. Indeed, both DC and SUM were again the only significant covariates in the final model (Table 2). Together, these two covariates explained 82.3% of the overall deviance of the PCA1 scores. SUM showed a linear relationship with the PCA1 scores, whereas DC was non-linearly related to the PCA1 scores (Table 2). DC was the main significant covariate driving the changes in CA F1 scores. The decrease in DC size was associated with a progressive dominance of hypoxia-tolerant taxa, which was especially marked in the 1940-1950s; this pattern has remained stable to the present (Fig. 4).

Three distinct time periods of changes in the SUM contributions to PCA1 can be distinguished. Before the 1940s, the contributions were positive, while in the 1950s, they were negative, and since the 1980s they have increasingly become more negative.

Covariates influencing PCA1 scores at 65 m

At 65 m, PCA1 accounted for 29.5 % of the overall variability of each of the chironomid assemblages and was the only significant axis according to the broken stick model. *S. coracina*-type and *M. contracta*-type were the two main taxa contributing to the PCA1 scores, accounting for 31 % and 24.5 % of the scores, respectively. *S. coracina*-type species were associated with high values of PCA1 scores and *M. contracta*-type with low PCA1 scores. The temporal increase in the scores of the samples along PCA1 therefore also suggests an increase in the oxygen constraint at this depth.

Four significant covariates explained the variability in PCA1 scores for the -65 m core, accounting for 83.5 % of the overall deviance. All four of these covariates showed a non-linear relationship with the PCA1 scores (Table 3). The trophic state (δ^{15}NOM) displayed the strongest explanatory power related to the changes in the chironomid assemblages (Table 3). There was a progressive increase in the

contributions of δ^{15}NOM to the GAM estimates starting in the 1930s, and the decrease occurring after the 1980s clearly identified this time span (i.e., the 1930s to the 1970s) as the period when the chironomid assemblages were the most impacted by eutrophication effects.

The influence of DC size on the chironomid community did not vary from the 1880s to the 1940s. After the 1940s, the contributions of DC to the PCA1 scores became increasingly positive, indicating that the temporal decrease in DC size was related to a progressive shift in the chironomid assemblage in favor of hypoxia-tolerant taxa. The temporal contributions of the two climate covariates (JAN JUL) did not display any clear trends during the studied time period and largely varied on either side close to zero values at a high temporal resolution (i.e., 2 sample intervals). However, their contributions have increased since the 1990s as temperatures have risen. As JUL and JAN were significantly and positively correlated with the PCA1 scores (ANOVA, $p < 0.01$), the increase in air temperature promoted a hypoxia-tolerant assemblage type at 65 m.

Discussion

Forcings and bathymetric approach

This study focuses on the three most widespread anthropogenic forcings reported in the literature, i.e., changes in pelagic function triggered by the top-down effect of zooplanktovorous fishes (mainly coregonids), nutrient enrichment (δ^{15}N OM in bulk sediment) and the climate (air temperature). The considered variables were fairly independent regarding their processes, and they therefore depict human forcings at lake, watershed and regional scales. The changes in chironomid assemblages were mainly related to changes in oxygen constraints, showing a shift from oxyphilous taxa before the 1930s to hypoxia-tolerant taxa since the 2000s, as demonstrated by Frossard *et al*. (accepted with revisions) using other analytical approaches. The changes in the oxygen constraints can be produced by the three forcings considered here.

The PCA1 scores were considered relevant to describe the sensibility of chironomids to anthropogenic forcings at a community level. Indeed, at 30 m and 65 m, PCA1 was the only axis that accounted for a significant amount of chironomid variability, whereas at 56 m, it was the only axis to exhibit significant temporal changes. Therefore, even though a large part of the variability in the chironomid assemblages was not considered in the analysis, this descriptor appeared to capture the most dominant change in the chironomid assemblage structures that was common to the three studied depths.

The differences in the identities of the chironomid assemblages and the observed depth-specific temporal changes provide evidence of depth-specific sensitivity to anthropogenic forcings. This finding indicates that the multiple-depth approach developed in the present study effectively increases our ability to judge of the extent of the ecological changes in chironomid assemblages in this large, deep peri-alpine lake.

Combinations of forcings

At 30 m and 56 m, the changes in the chironomid assemblages were mainly related to changes in pelagic functioning (zooplanktivorous fish, top-down), rather than the trophic state, as is usually reported (Wiederholm, 1980; Kansanen, 1986; Meriläinen *et al*., 2000). The chironomid assemblages at 30 m and 56 m were strongly linked to pelagic functioning. In contrast, at 65 m, the chironomid assemblages were strongly influenced by the variability in the trophic state, even though changes in pelagic functioning were also involved, though to a lesser extent. These habitat-specific responses specify habitat-specific vulnerability to anthropogenic forcings (Solimini *et al*., 2006; Free *et al*., 2009). For instance, an increase in nutrient concentrations can lead to macrophyte development in the littoral zone and support flourishing chironomid assemblages (Langdon et al., 2010), whereas in deep zones, it usually triggers decreases in oxygen levels and can lead to the collapse of chironomid fauna (Kansanen, 1986).

Interestingly, our final models all considered different climate components. Changes in air temperature can affect chironomids inhabiting the littoral zone through causing changes in water temperature, whereas deep-living species would instead be affected by the indirect consequences of altered mixing properties (Eggermont and Heiri, 2011). The summer climate component (SUM or JUL) significantly explains the changes in the chironomid assemblages at the three studied depths. However, the increase in the summer water temperature is unlikely to directly induce changes in the littoral chironomid fauna according to the thermal tolerance of chironomid species (Eggermont and Heiri, 2011). The increase in temperature facilitates the appearance and extends the duration of stratification and therefore promotes the spatial expansion of the hypoxic layer upward to a sub-littoral (30 m) depth, at least for a restricted period of time. The effects of JAN possibly reflect an alteration of the efficiency of mixing in Lake Annecy, even though the lake is holomictic. Indeed, when lakes experience mixing, the speed and duration of the mixing period have a strong influence on the hypolimnetic oxygen conditions in the following year (Jankowski *et al*., 2006). Therefore, the increase in JAN over time is likely to facilitate adverse, hypoxic conditions as soon as the beginning of the summer. The analysis of the temporal variability in the contributions of the different covariates to PCA1 scores was developed to clarify their specific effects on the chironomid assemblages.

Specific responses to forcings

Our results indicated that the impact of nutrient enrichment only significantly influenced the deepest part of the lake (65 m), beginning as early as in the 1930s. This result confirms the higher sensitivity of the deep-living chironomids to nutrient enrichment compared to those living in shallower zones (Wiederholm, 1980). In the 1960s, when the lake experienced the peak of eutrophication (mesotrophic), the waste-water treatment plan that was implemented led to a progressive restoration of water quality. Since 1980s, the influence of nutrients on the chironomid assemblages at 65 m has

recovered to the same level as in the 1850s. Therefore, since 1980s at 65 m, nutrient enrichment is no more responsible for the ongoing changes in the chironomid assemblages.

The onset of the decrease in the size of *Daphnia* sp. was concomitant with the first signs of eutrophication recorded in chironomids in the 1930s (Frossard *et al*., accepted with revisions). Eutrophication itself can trigger decreases in the size of *Daphnia* sp. (Vijverberg and Boersma, 1997). For instance, Smakulska and Gorniak (2004) found that the length of *D. cucullata* had a tendency to decrease with the TP gradient in the Siemianowka Reservoir. Therefore, prior to the decrease in nutrient concentrations beginning in the 1970s, the decrease of *Daphnia* sp. size cannot be exclusively attributed to the top-down effect of zooplanktivorous fish, which appears to have been a major driver since the late 1980s (Alric *et al*., accepted with revisions).

The non-linear relationship observed between the PCA1 scores and DC, especially at 30 m and 56 m, suggests the appearance of a threshold effect in the size of *Daphnia* sp. to control phytoplankton, in association with possible size refugee for algae (Chase et al., 2002), resulting in changes in pelagic trophic efficiency (Carpenter *et al*., 1985). A new stable state seems to have appeared after in the 1950s, with a new stable influence of the size of *Daphnia* sp. on the chironomid assemblages being detected. This stability suggests that changes in *Daphnia* sp. size induced a new relationship between pelagic primary production and the zooplankton exportation ability, resulting in a new stable pelagic functioning efficiency. Since the 1970s, with the return to decreased nutrients concentrations, a top-down effect of zooplanctivorous fishes, especially coregonids, has been the main driver of *Daphnia* sp. size (Perga et al., 2010). During this time period, the alteration of the benthic food-web in Lake Annecy can therefore be viewed as a *domino* effect of the effect of coregonids and other zooplanktivorous on the benthic food-web through (1) altering DC, (2) promoting OM losses from the pelagic to the benthic zone and (3) indirectly promoting the recurrence of hypoxic conditions in an oligotrophic context. The temporal perspective of our analysis therefore revealed a shift from nutrient to pelagic control.

Climate constraints related to temperature increases appear to have increasingly affected the chironomid assemblages since the 1980s. The influence of climate has increased since the 1980s. Furthermore, the increase in the temperature in the deep zone (Danis *et al*., 2004) of Lake Annecy has indirectly promoted oxygen depletion by enhancing heterotrophic activities (Charlton, 1980).

Winter temperatures only appear to have a significant effect on the deep chironomid assemblages (65 m), suggesting that changes in mixing behavior particularly impact deep-living chironomids. Our results therefore highlight different sensibilities of the chironomid assemblages at different depths to climate change. Furthermore, as both the winter and the summer climate components significantly explain the changes in the chironomid assemblages in the deep zone (i.e., 65 m), the chironomid assemblages respond independently to these different climate forcings, at least in part. This notion is

in line with the complex relationship between chironomid assemblages in deep lakes and the climate (Eggermont and Heiri, 2011). Therefore, the effects of this large-scale anthropogenic forcing on chironomid assemblages differ at a local (depth) scale.

Finally, our models further appeared to be sensitive to time-restricted changes in temperatures, such as warming in the 1950s, suggesting that chironomids respond rapidly to climate change, with higher temperatures being associated with hypoxia-tolerant assemblages.

Concluding remarks

The present study was able to depict the temporal partitioning of three anthropogenic forcings that can produce the same effects on chironomid assemblages (Velle *et al*., 2010; Brodersen and Quinlan, 2006). The high-resolution (subdecadal) analysis reported here highlighted the depth-specific sensibility of each of the anthropogenic forcings considered. Our results therefore contribute new insights to our understanding of why lakes that experience efficient reductions of nutrient loading do not recover their ecological identity (Jeppesen *et al*., 2005). The continuous substitutions and recombinations of stressors reported here could explain the decoupled responses of the benthic and pelagic food-webs following nutrient restoration (Little *et al*., 2000; Marchetto *et al*., 2004).

The obtained detailed chronology of the influences of the different anthropogenic forcings on chironomids at different depths provides further information on the extent of forcing substitutions. Since the 1990s, the climate component appears to have been a significant driver of the chironomid assemblages, and its influence is expected to increase in the coming century (IPCC, 2007). If the climate component cannot be addressed by local populations, coregonid stocks can be managed. At this point, the definition of a stressor can be very subjective, as large coregonid stocks provide economic benefits through fisheries. On the other hand, their associated top-down effects are likely to promote hypoxic conditions in the hypolimnion, preventing the recovery of chironomid assemblages. In the context of the Water Framework Directive (WFD, 2000), by reaching a re-oligotrophicated state, Lake Annecy has achieved a “good ecological state”. Based on our results, because the chironomid structure appears to be still strongly altered and because the current forcings are not related to the trophic state (i.e., top-down effect and climate), nutrient restoration appears insufficient to accurately predict the trajectories of the chironomid assemblages in this lake.

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Figure legends

Fig. 1 Temporal trends of the covariates considered to model the PCA1 scores of the chironomid assemblages at three depths in Lake Annecy.

Fig. 2 RDA triplot depicting the relationships between the chironomid assemblages and the habitats (i.e., sampling depths) and time (sampling dates). Both covariates have a significant effect on the chironomid assemblages (see text). A total of 50 taxa were used to perform the RDA, but only the 20 taxa with the highest RDA axis 1 scores and / or high RDA axis 2 scores are represented to facilitate ecological interpretations.

Fig. 3 Temporal dynamics of the contribution of significant covariates to the 30 m PCA1 score estimates. Solid lines refer to the contribution to the F1 score estimates, and dashed lines indicate the standard errors of the contributions. The top label of the boxes refers to the anthropogenic pressure, and the bottom labels of the boxes refer to the proxy used.

Fig. 4 Temporal dynamics of the contribution of significant covariates to the 50 m PCA1 score estimates. Solid lines refer to the contribution to the F1 score estimates, and dashed lines indicate the standard errors of the contributions. The top label of the boxes refers to the anthropogenic pressure, and the bottom labels of the boxes refer to the proxy used.

Fig. 5 Temporal dynamics of the contribution of significant covariates to the 65 m PCA1 score estimates. Solid lines refer to the contribution to the F1 score estimates, and dashed lines indicate the standard errors of the contributions. The top label of the boxes refers to the anthropogenic pressure, and the bottom labels of the boxes refer to the proxy used.

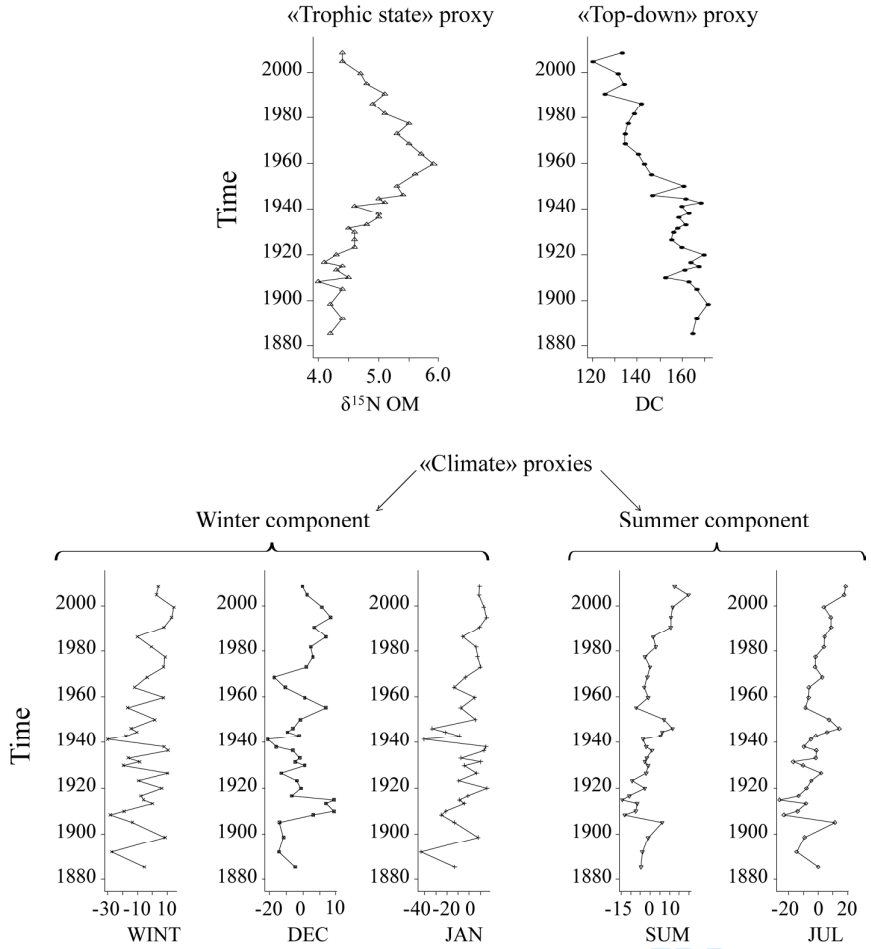

«Trophic state» proxy
«Top-down» proxy
Time
2000
1980
1960
1940
1920
1900
1880
4.0 5.0 6.0
δ15N OM
120 140 160
DC
«Climate» proxies
Winter component
Summer component
-30 -10 10
WINT
-20 0 10
DEC
-40 -20 0
JAN
-15 0 10
SUM
-20 0 20
JUL

Fig. 1

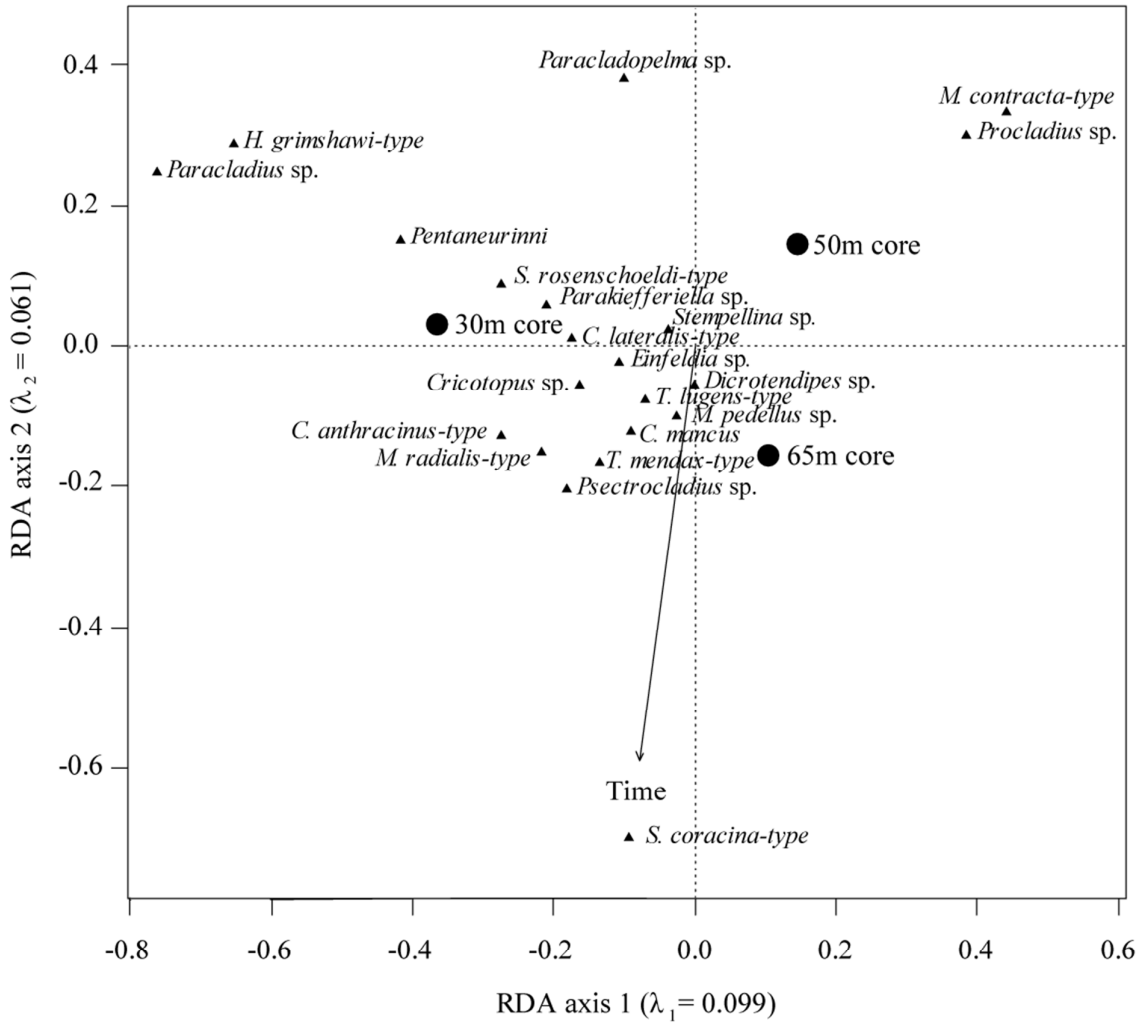

Paracladopelma sp.
M. contracta-type
Procladius sp.
H. grimshawi-type
Paracladius sp.
Pentaneurinni
50m core
S. rosenschoeldi-type
Parakiefferiella sp.
Stempellina sp.
30m core
C. lateralis-type
Einfeldia sp.
Cricotopus sp.
Dicrotendipes sp.
T. lugens-type
M. pedellus sp.
C. anthracinus-type
C. mancus
M. radialis-type
T. mendax-type
65m core
Psectrocladius sp.
Time
S. coracina-type
RDA axis 2 (λ2 = 0.061)
RDA axis 1 (λ1 = 0.099)
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-0.6
-0.4
-0.2
0.0
0.2
0.4
0.6

Fig. 2

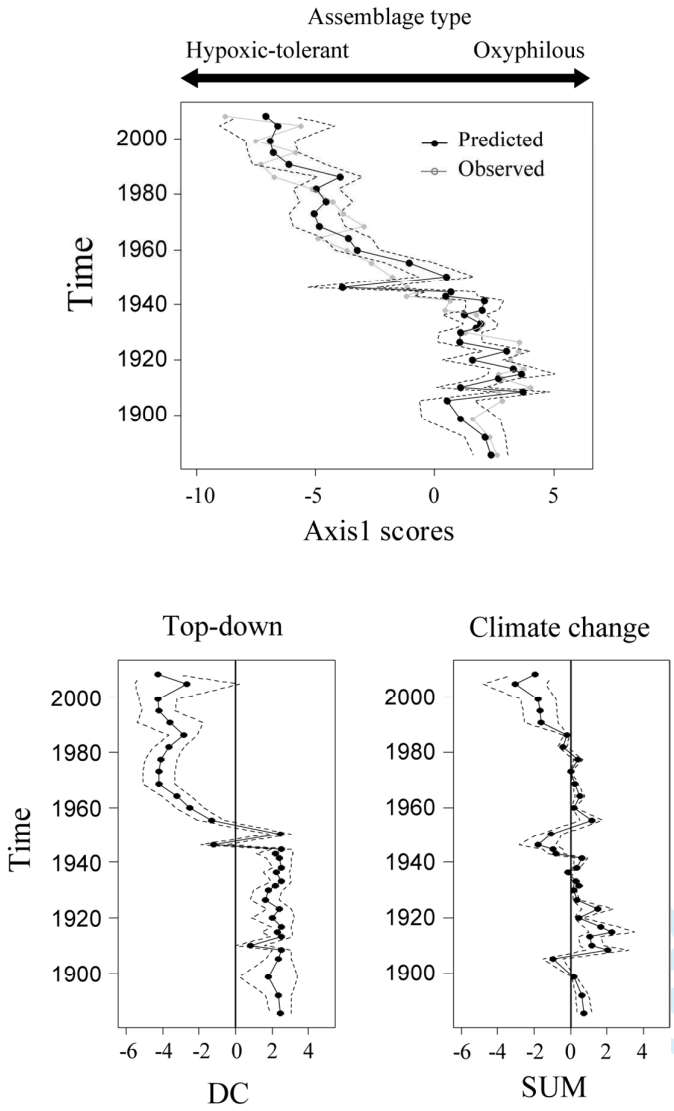
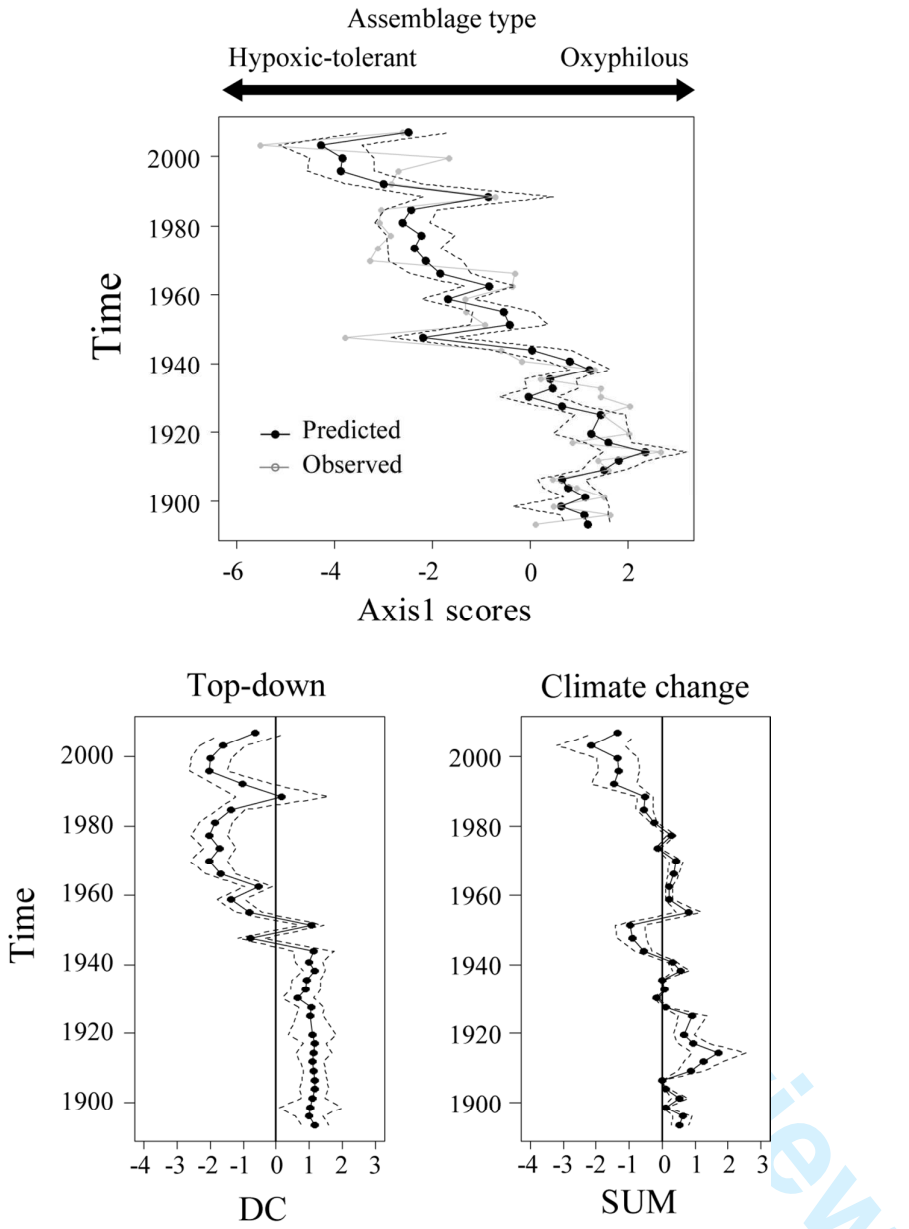

Assemblage type
Hypoxic-tolerant
Oxyphilous
Predicted
Observed
Time
2000
1980
1960
1940
1920
1900
-10
-5
0
5
Axis1 scores
Top-down
Climate change
Time
DC
SUM
-6
-4
-2
0
2
4

Fig. 3


Assemblage type
Hypoxic-tolerant
Oxyphilous
Time
2000
1980
1960
1940
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Predicted
Observed
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Axis1 scores
Top-down
Climate change
Time
DC
SUM
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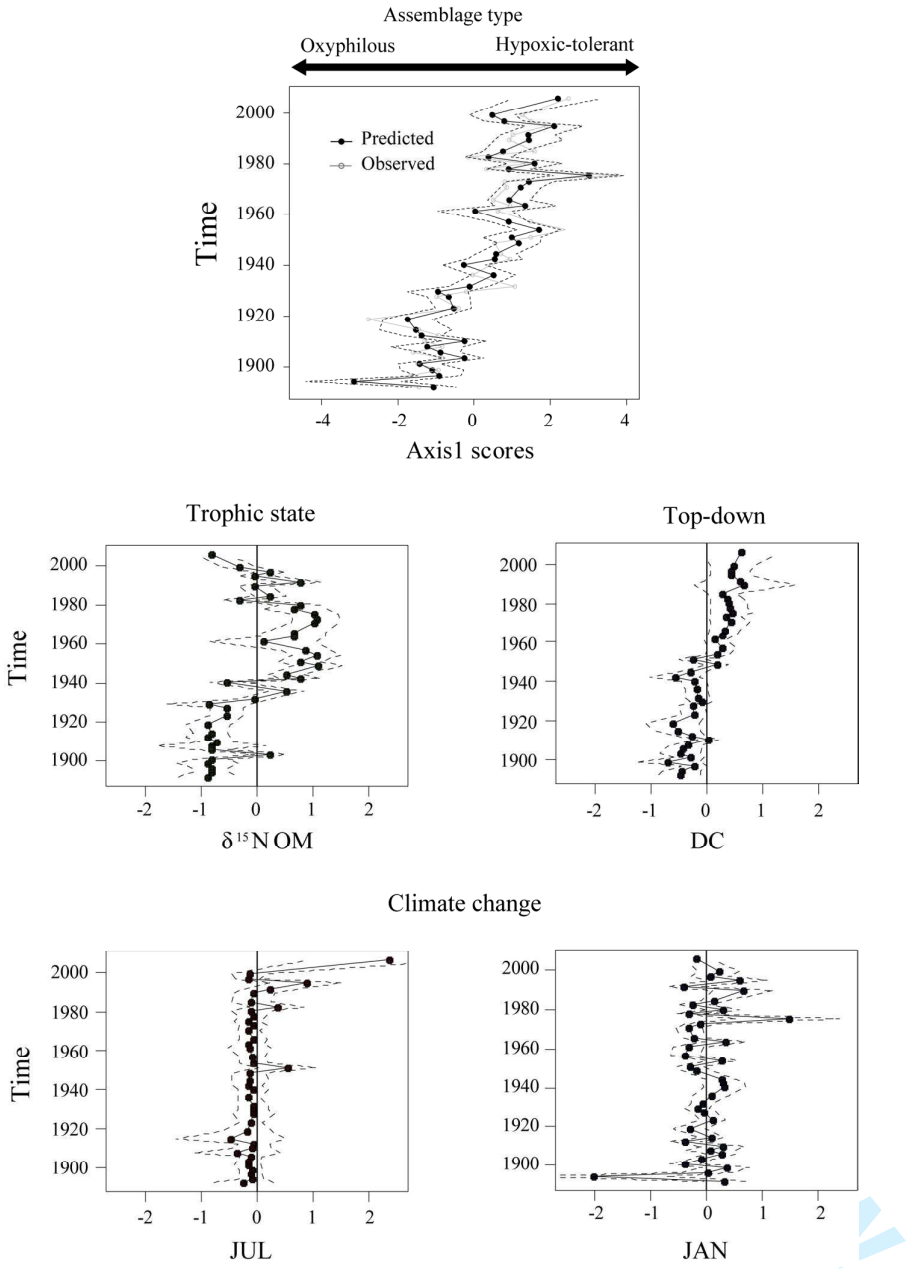
Fig. 4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Oxyphilous
Hypoxic-tolerant
Predicted
Observed
Time
Axis1 scores
Trophic state
δ15N OM
Top-down
DC
Climate change
JUL
JAN

Fig. 5

Tables

Table 1: Statistics for the final model explaining the variability of the 30 m core PCA1 scores.

Final model: PCA axis1 scores ~ s(DC)+SUM				
		Estimates	t	p
Parametric terms	Intercept	0.88±0.26	3.39	0.002
	SUM	-0.16±0.04	-3.464	0.002
		edf	F	p
Smooth terms:	s(DC)	2.91	29.07	< 0.001

Table 2: Statistics for the final model explaining the variability of the 56 m core PCA1 scores.

Final model: PCA axis1 scores ~ s(DC)+SUM				
		Estimates	t	p
Parametric terms	Intercept	0.52±0.15	-3.37	0.002
	SUM	-0.11±0.03	-4.14	< 0.001
		edf	F	p
Smooth terms:	s(DC)	2.99	18.33	< 0.001

Table 3: Statistics of the final model explaining the variability of the 65 m core PCA1 scores.

Final model: PCA axis1 scores ~ s(δ^{15}N OM)+s(JAN)+s(JUL)+s(DC)				
		Estimates	t	p
Parametric terms	Intercept	0.22±0.11	2.07	0.05
		edf	F	p
Smooth terms:	s(δ^{15}N OM)	2.85	8.61	< 0.001
	s(JAN)	2.93	6.29	0.002
	s(JUL)	2.73	4.41	0.01
	s(DC)	1.52	3.98	0.03

6. Evolution des sources du carbone dans le lac d'Annecy depuis 150 ans : reconstitution à partir des isotopes (δ13C) stables extraits des archives sédimentaires

❖ *Article : Reconstructing long-term changes (150 years) in the metabolism of a clear water lake using the stable carbon isotope composition (δ13C) of chironomid and cladoceran subfossil remains*

Soumis dans Limnology and Oceanography

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Reconstructing long-term changes (150 years) in the metabolism of a clear water lake using the stable carbon isotope composition ($\delta^{13}C$) of chironomid and cladoceran subfossil remains

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Running head

Changes in clear water lake metabolism

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Abstract

Long-term (150 years) trends in the $\delta^{13}C$ of the head capsules (HC) of three chironomid taxa and pelagic cladoceran exoskeletons are reported for a clear water re-oligotrophicated deep lake using a multi-depth approach. Trends were taxa-specific, and Bayesian analyses defined three temporal sequences for homogenous HC $\delta^{13}C$ values. From the 1850s to 1930s, HC $\delta^{13}C$ values were stable and similar between the littoral and the deep zones, suggesting littoral and deep chironomids relied on similar carbon sources. HC and cladoceran $\delta^{13}C$ values were around -32 ‰, providing no evidence of organic carbon reworking by microbial mineralization. From the 1930s to the 1950s, littoral HC and cladoceran $\delta^{13}C$ values decreased by 2 ‰. This was likely related to an increase in the respiration processes in the epilimnion following an increase of the lake trophic state. Deep HC $\delta^{13}C$ values remained stable during this period, indicating that most of the additional primary production due to eutrophication was transferred. Since the 1950s, littoral HC and cladoceran $\delta^{13}C$ values remained steady, whereas values for deep HC decreased drastically (- 4 ‰) despite the restoration of the oligotrophic conditions in the open water. This pattern attests of organic carbon accumulation and microbial mineralization at the lake bottom. These processes were attributed to a lower trophic efficiency within the pelagic food web. Our results suggest that before the 1930s, the metabolism of this lake was characterized by low heterotrophic activities, and since the 1990s, the increase in heterotrophic activities was supported by autochthonous organic carbon recycling.

45 # Introduction

46 Lake metabolic processes strongly influence the contribution of lakes to the global carbon
47 budget (Tranvik et al 2009). Lakes can act either as a carbon sink or as a source of carbon to
48 the atmosphere, and net CO2 fluxes are, in most cases, the result of balancing internal
49 autotrophic and heterotrophic processes (del Giorgio and Peters, 1994; Staehr et al., 2012).
50 Lake metabolism is affected by the lake's trophic state. Oligotrophic lakes tend to be net
51 heterotrophic as a consequence of respiring terrestrial carbon inputs (France et al., 1997; del
52 Giorgio et al., 1999; Karlsson et al., 2007). In contrast, increasing lake nutrient
53 concentrations, which increases primary production, promotes autotrophy and the uptake of
54 atmospheric carbon (Schindler et al, 1997). As a consequence, and due to their
55 disproportionate rates of carbon processing relative to their integral surface area (Cole et al.,
56 2007; Prairie, 2008), changes in lake metabolism can have significant implications on, at
57 least, the regional carbon budget (Yvon-Durocher et al., 2010).

58 In addition to these geochemical properties, lake metabolism is influenced by the trophic
59 relationships within its food web. Indeed, the structure and the dynamics of lake food webs
60 directly affect lake metabolism (Otto et al., 2007; O'Connor et al., 2009). The trophic
61 efficiency concept of Lindeman (1942), which is the ratio comparing total organic matter
62 produced at one trophic level with that of the consecutive trophic level, influences lake
63 metabolism by defining the amount of organic matter transferred, respired or lost at each
64 trophic level. At a whole food web scale, the sum of the trophic efficiencies of all of the
65 different trophic levels can be defined as the "functioning efficiency of a lake" (Fig. 1).
66 Partialling out the system-specific properties (e.g. trophic state, external carbon supplies,
67 morphology), a lake with a high functioning efficiency should have minimal organic matter
68 loss, and therefore minimal microbial heterotrophic activities. Further, the concept of

functioning efficiency supports the notion of an ecosystem response to stress, as proposed by Odum (1985), i.e., ecosystem responses to stress should involve increased ecosystem respiration by promoting microbial heterotrophic activities (Fig. 1).

Carbon isotope ratios ($\delta^{13}C$) are robust descriptors that can be used to assess the carbon recycling and respiration that directly relate to lake metabolism (del Giorgio and France, 1996; Perga and Gerdeaux, 2004; McCallister and del Giorgio, 2008; van Breugel et al., 2005; Perga et al., 2010). The higher the respiration is, the lower the $\delta^{13}C$ values are for consumers. This is a result of the carbon recycling involved in photosynthetic, trophic and metabolic fractionation (Vander Zanden and Rasmussen, 2001; van Breugel et al., 2005).

Chironomids (Diptera) usually constitute a large fraction of a lake's benthic fauna under various environmental conditions (Armitage et al., 1994). Their use in carbon isotope measurements provide valuable insights regarding the carbon cycle in lakes (Deines and Grey, 2006; Jones et al., 2008; Jones and Grey, 2001). A progressive ^{13}C depletion in chironomid larvae with depth (e.g. Kiyashko et al., 2001; Borderelle et al., 2008) has been correlated with increased internal carbon recycling processes through high heterotrophic activities. Borderelle et al. (2008) suggested that the $\delta^{13}C$ differences of macroinvertebrates between the littoral and the deep zones ($\Delta\delta^{13}C_{litt\text{-}deep}$) could be used to quantify the extent of the heterotrophic processes within the lake system. The $\Delta\delta^{13}C_{litt\text{-}deep}$ describes a lake's tendency to promote heterotrophy due to its food web structure and is therefore linked to both functioning efficiency and ecosystem stress (Fig. 1). Technical improvements in isotope-ratio mass spectrometry now enable researchers to obtain $\delta^{13}C$ measurements at low material masses and thus measure $\delta^{13}C$ for subfossil chironomid head capsules (HC) or cladoceran exoskeletons recovered from lake sediments (van Hardenbroek et al., 2010, Perga 2010, 2011). Recent studies relied on subfossil invertebrate consumers' $\delta^{13}C$ values to infer changes in the carbon cycle of lakes on a plurimillenial scale (van Hardenbroek et al. 2012; Wooller et al. 2012).

In this study, we used a paleolimnological approach to report long-term (150 years) changes in the metabolism of a clear water deep lake, Lake Annecy (France), that experienced eutrophication (1930-1970) and subsequent re-oligotrophication (1980-today) (Fig. 2). We focused specifically on a clear water lake because the metabolic response of such lakes to changing nutrient concentrations are likely to differ from the response of colored lakes, which are less influenced by inputs of terrestrially derived allochthonous carbon (Pace et al., 2007). Carbon isotope analysis was conducted for chironomid head capsules (HC) and pelagic cladoceran remains found preserved in the sediment. In order to assess changes in the functioning efficiency and in depth-specific mechanisms involved in the changes of the carbon cycle, two different depths (i.e. littoral and deep zones) were investigated for chironomids. Due to possible different trophic niches among chironomid species, the study considered the three dominant taxa present in the lake during the last 150 years (i.e. *Microspectra contracta*-type, *Sergentia coracina*-type and *Tanypodinae*). Comparing chironomid HC carbon isotope trends with those from pelagic cladoceran remains allowed for specific observations in the lake's benthic and pelagic zones. This led to a better understanding of metabolism changes at a whole-lake level. Four specific questions were addressed:

- Did Lake Annecy present any evidence of high microbial heterotrophic activities, even before the first signs of eutrophication in the 1930s?
- How did the balance between autotrophy / heterotrophy change during eutrophication?
- Did the subsequent reappearance of an oligotrophic state trigger a return to the pre-1930s carbon cycle?
- Has Lake Annecy's functioning efficiency changed during the last 150 years, and were these changes correlated with its trophic state?

Methods

Study site - Lake Annecy (45°48' N; 6° 8'–14' E) is a deep monomictic lake located in the French Alps at about 600 m above sea level. With a surface area of 27.4 km^2 and a maximal depth of 69 m, Lake Annecy is the second largest natural lake in France. The lake's water retention time is 4 years. The lake consists of two basins lying in a tectonic depression that was shaped by glaciers. The catchment area of 254 km^2 is drained by seven streams that shape marine marl formations (Jurassic to Low Miocene) and glaciolacustrine formations (Quaternary). The catchment is mainly covered by forests (47 %), rocky areas (22 %), and meadows (15 %), with a lower proportion of agricultural and urban areas (3 % and13 %, respectively) (data Syndicat Intercommunal du lac d'Annecy: SILA). The first human activities in the lake watershed occurred at approximately 1,700 B.P., following Roman colonization (Noël *et al*., 2001). However, following the development of the city of Annecy, human activities in the near shore areas substantially intensified following the late 19th century. The population of the city has grown from 10,000 inhabitants in 1870 to 86,000 inhabitants in 2007 (National Institute for Statistics and Economic Studies; www.insee.fr).

Historical data – Before the late 1960s, very few historical data were available about the biotic and abiotic characteristics of Lake Annecy. Nevertheless, in the late 1930s, Lake Annecy showed the first signs of eutrophication (Hubault, 1943). In 1957, a wastewater treatment plan was engaged to protect the lake, which was then at an early stage of eutrophication. Since the late 1960s, the pelagic areas of Lake Annecy have been monitored monthly or bi-monthly by the SILA. The SILA survey shows that Lake Annecy was mesotrophic between 1970 and 1980 (mean value of soluble reactive phosphorus SRP = 12 µg P l^{-1}) and that it returned to an oligotrophic state in the late 1980s (SRP < 10 µg P l^{-1}). From

142 this period to the time of this study, the water content of SRP has progressively decreased to
143 the current value of 3 - 4 µg P l^{-1} (Fig. 2).

144 The natural fish community of Lake Annecy was enriched with arctic char and whitefish
145 introductions at the end of the 19th century (Le Roux 1908).

146 *Sampling* - For chironomid analyses, two sediment cores were collected in November 2009 at
147 depths of 30 m and 65 m in the northern deepest basin of Lake Annecy (Fig. 2). Cores were
148 cut transversally. Each half-core was sub-sampled at 0.5 cm intervals. The first half-core was
149 devoted to chironomid HC stable isotope analysis, and the other half-core was used for dating
150 and chironomid assemblage reconstructions (Frossard In press). These assemblage
151 reconstructions revealed significant differences in the assemblage composition at depths of 30
152 m and 65 m. The ecology of the main taxa indicates that the 65 m depth core kept the HC of
153 the past profundal chironomid community, whereas the 30 m depth core kept the HC of the
154 littoral chironomid community (Frossard In press).

155 Cladoceran remains were collected from an additional core retrieved from the deepest part of
156 the lake (Fig.2). The core was sub-sampled following annual laminations, each sample
157 representing 3-4 years.

158 All cores were sampled using a gravity corer (UWITEC, Mondsee, Austria)

159 *Laboratory analysis* - Chironomid HC samples were treated at room temperature for 2 hours
160 with hydrochloric acid (10%) and potassium chloride (10%), successively, to dissolve
161 carbonates and deflocculate organic matter prior to sieving through a 100 µm-mesh filter cup.
162 According to van Hardenbroek et al (2010), this treatment procedure does not significantly
163 affect HC $\delta^{13}C$ values. Chironomid remains for the main taxa present in the assemblage
164 reconstructions were handpicked out from the residue under a stereomicroscope at 40 ×
165 magnification and thoroughly rinsed with demineralized water to prevent exogenous carbon

166 contamination. Almost all HC considered in this study were HC coming from 4th instar larvae
167 molts. When not enough HC were present in a sample, adjacent samples were pooled to reach
168 a suitable sample weight (20 µg, Van Hardenbroek et al 2011). Samples were then dried
169 (40°C for 3 days) and stored prior to carbon stable isotope analysis. The temporal position of
170 each resulting sample was determined as the date at the top of the sample minus the half of
171 the temporal interval covered by the sample. Identification of the HC of *Microspectra*
172 *contracta*-type was possible under low magnification because of their dark yellowish color
173 and the presence of a visible spur on the antennal pedestal for the HC of the 4th instars. HC of
174 Tanypodinae and *Sergentia coracina*-type were identified according to the general shape of
175 HC, the mentum shape, and teeth features following standard nomenclature (Brooks et al.,
176 2007).

177 Cladoceran remains were treated as described in Perga et al (2010, 2011). Briefly, sediment
178 samples were boiled in 10% KOH for 30 min to remove organic matter, and 10% HCl was
179 used to remove exogenous carbonates that could coat the remains. Samples were rinsed, re-
180 suspended in distilled water and sieved on a 150 mesh-size filter cup to remove pollen grains
181 and small particles. Cladoceran exoskeletons were sorted using repeated centrifugation at
182 2000 rpm for 2 min. After each centrifugation, the supernatant was collected and examined
183 under a stereomicroscope to make sure there were no particles other than those from
184 cladoceran remains. In Lake Annecy, cladoceran remains retrieved for sediment samples are
185 almost exclusively composed of pelagic species (mainly *Daphnia* and *Bosmina* sp., Perga et
186 al, 2010). Pellets were re-suspended in distilled water and centrifuged again until no remains
187 could be found in the supernatant. Pooled supernatants were filtered onto 25 mm Whatman®
188 precombusted GF/F filters and dried overnight at 60°C. When not enough remains were
189 present in a sample, adjacent samples were pooled to reach a suitable sample weight (0.3 mg).
190 Previous experiments performed on cladoceran subfossil remains showed that this chemical

191 treatment and taphonomic processes have very minor effects on the $\delta^{13}C$ values of the remains
192 (Perga 2011).

193 *Core dating*

194 A sediment chronology was obtained from one reference core (LDAref) sampled in the
195 deepest part of the lake (65m), using radiometric methods (^{210}Pb, ^{226}Ra, ^{137}Cs and ^{241}Am
196 activities) and counting of annual laminations, which provided an accurate chronology (see
197 Alric et al. 2012 for details). The chronologies for the studied cores (65 m and 30 m) were
198 obtained by correlation with LDAref. Core correlations involved i) seven lithological tie
199 points, ii) lamina counting and iii) high-resolution spectrophotometry allowing the
200 identification of clay mineral enrichment layers when lithological markers and laminas were
201 not visible (see Frossard In press for details).

202 *Carbon isotope analysis* – Stable carbon isotopic ratios are expressed as $\delta^{13}C=$
203 $[(Rsample/Rstandard) -1] \times 1000$ with $R= {}^{13}C/{}^{12}C$ and the Pee Dee Belemnite as standard.
204 The chironomid HC isotopic analyses were undertaken at the CRPG laboratory of Nancy
205 (France) using an Isoprime mass spectrometer coupled to a Eurovector EA3000 elemental
206 analyzer. The cladoceran isotope analyses were performed at the SINLAB, New Brunswick,
207 Canada (http://www.unb.ca/cri/sinlab), on a Finnigan Delta Plus mass spectrometer interfaced
208 via a Conflo II to a NC2500 Elemental Analyzer. The internal precision for HC analyses was
209 tested on sugar with a known constant $\delta^{13}C$ (i.e. -26 ‰) and was 1sd=0.16 ‰ (n = 67). The
210 internal precision for cladoceran analyses was tested on acetanilide with a known constant
211 $\delta^{13}C$ (i.e. -27.6 ‰) and was 1sd=0.14 ‰ (n = 22).

212 *Statistical analysis* - The contributions of time, depth (littoral versus deep zones) and taxa to
213 the variability of HC $\delta^{13}C$ were estimated through a three way ANOVA.

214 Change points in the temporal dynamics of $\delta^{13}C$ values for both chironomid HC and
215 cladoceran remains were detected using a Bayesian change point analysis using the R package
216 "bcp" (Erdman & Emerson, 2007). This analysis partitioned the temporal dynamics of the
217 $\delta^{13}C$ values of cladoceran remains and chironomid HC into contiguous sequences such that
218 the mean was constant within each sequence. The Bayesian analysis was configured with
219 1000 Markov-Chain Monte-Carlo (MCMC) iterations associated with a burn-in period of 100
220 iterations while the prior variance was fixed as the variance of the overall tested datasets. The
221 outputs of the analysis associated with each value a posterior probability to be a change point.
222 Change points were considered when the posterior probabilities were higher than 0.6, as most
223 of the other probabilities were very close to zero. As the temporal dynamics of the HC $\delta^{13}C$ of
224 *S. coracina*-type followed linear trends both in the littoral and in the deep zones, no change
225 point could be defined. Three homogenous sequences were then defined for the whole-
226 chironomid dataset based on the change point analysis from each taxon. These three
227 homogenous sequences capture the global behavior of HC $\delta^{13}C$ trends. In these sequences, the
228 differences in the $\delta^{13}C$ between the littoral and the deep zones were estimated for each taxon
229 ($\Delta\delta^{13}C$ = HC $\delta^{13}C_{litt}$ –HC $\delta^{13}C_{deep}$) and their significances were tested using T tests. For *S.*
230 *coracina*-type, the difference in the HC $\delta^{13}C$ trend between the littoral and deep zones was
231 estimated through a slope comparison test using the R package "smart". HC $\delta^{13}C$ temporal
232 trends of the chironomid taxa were modeled using general additive models (GAM) following
233 the procedure developed by Wood (2006) using the R package "mgcv". Due to the lack of
234 data for Tanypodinae HC $\delta^{13}C$ in the deep zone from the 1950s until the 2000s, GAM
235 estimations were not reported for this time period. Model validations were conducted
236 according to Züur et al (2010) for both three ways ANOVA and GAM. Statistical analysis and
237 graphical display were performed with R 2.13.1 (R Development Core Team, 2008).

238

Results

Daphnia $\delta^{13}C$ trend - The $\delta^{13}C$ values for cladoceran remains were obtained as single values for 13 dates ranging from 1875 to 2002 with a time-interval of ca. 10 yrs (Fig. 3). The $\delta^{13}C$ for *Daphnia* remains ranged from -27.7 ‰ to -32.5 ‰. One change point was identified at ca. 1940, defining two homogenous temporal sequences in the $\delta^{13}C$ values of *Daphnia* remains (Fig. 3). Before the 1940s, the $\delta^{13}C$ of *Daphnia* remains were stable and significantly higher (i.e. - 28.4 ± 0.4 ‰) than after the 1950s (i.e. - 31.9 ± 0.5 ‰; t test, t = 14.61, df = 9.55, $p <$ 0.01). The overall temporal ^{13}C depletion in *Daphnia* remains was therefore slightly higher than 3 ‰.

Chironomid HC $\delta^{13}C$ trends - Chironomid HC $\delta^{13}C$ values were obtained for the years 1850 through 2005 with a time resolution of ca. 10 years. Among the 188 samples analyzed, 63% were triplicates and 24% were duplicates. Each of the three taxa showed an overall decrease in their HC $\delta^{13}C$ values over time, at the two depths (Fig. 3). In the littoral zone, the overall decrease was between -2.2 ‰ (for *S. coracina*-type) and -2.8 ‰ (for *M. contracta*-type). In the deep zone, the overall decrease was more than -4 ‰ for the two burrowing and collector-feeder taxa (*S. coracina*-type and *M. contracta*-type), but in the same order of magnitude as that of the littoral zone for the walking predator Tanypodinae (-2.6 ‰). A significant 81 % of the variability in the HC $\delta^{13}C$ values was explained by chironomid taxa, time (i.e. sample ages) and depth (i.e. deep / littoral) along with interaction between zones × time and zones × taxa (Table 1). The significance of these interactions indicates, first, that for each depth the temporal trend of HC $\delta^{13}C$ values varies significantly between taxa, and second, that each taxon has different trends between depths (Fig. 3).

Despite the specificity of the HC $\delta^{13}C$ trends with zones and taxa, two main periods of HC $\delta^{13}C$ decrease emerged from the change points analysis, i.e. the 1930s and the 1950s (Fig 3).

These periods showed common significant change points for at least two taxa (*M. contracta*-type and Tanypodinae) and/or the two depths. This led to the definition of three main temporal sequences, i.e., 1850-1930 (S1), 1930-1950 (S2) and 1950-2005 (S3), where each correspond to specific HC $\delta^{13}C$ values for the chironomid assemblages of Lake Annecy during the last 150 years (Fig. 3, Table 2). An additional significant change point appeared in the 1970s, but only for the HC $\delta^{13}C$ of *M. contracta*-type from the deep zone. For *S. coracina*-type, no change point can be defined because their HC $\delta^{13}C$ linearly decreased with time at the two depths. In summary, the three sequences showed the following characteristics:

- The S1 sequence (1850-1930), was characterized by the highest HC $\delta^{13}C$ values for all taxa in both the littoral (lowest value = -32.2 ‰ for *S. coracina*-type) and deep zones (lowest value = -34.6 ‰ *S. coracina*-type). Within S1, the differences in the HC $\delta^{13}C$ of each taxon between the littoral and the deep zones ($\Delta\delta^{13}C = \delta^{13}C_{litt} - \delta^{13}C_{deep}$) were small (0.9 ‰ and 1.5 ‰ for *M. contracta*-type and Tanypodinae, respectively), but significantly different from 0 (Table 2). The higher value of $\Delta\delta^{13}C$ for *S. coracina*-type (2.5 ‰) should be noted with caution because of the unique value obtained from the deep zone. During S1, the HC $\delta^{13}C$ values were similar among the taxa and stable for *M. contracta*-type and the Tanypodinae.
- The S2 sequence (1930-1950) was characterized by a significant decrease in the HC $\delta^{13}C$ values of all taxa (-2.2 ‰ for the littoral *M. contracta*-type, -1.6 ‰ and -1.5 ‰ for the Tanypodinae in the littoral and deep zones, respectively). For *S. coracina*-type, the $\delta^{13}C$ decrease was lower than for the two other taxa in the littoral zone (0.6 ‰), but was higher in the deep zone (1.95 ‰). For this taxon, the linear ^{13}C depletion over the whole survey time-period was significantly higher in the deep zone than in the littoral zone (slope comparison test, $p < 0.01$), with respective slopes of -0.04 ± -0.02 ‰ and -0.02 ± -0.03 ‰ year^{-1}. The $\Delta\delta^{13}C$ for *S. coracina*-type thus increased over time. During this time period,

288 there was no significant $\Delta\delta^{13}C$ for any taxon (Table 2). At a whole lake level, there was a
289 decrease in the $\delta^{13}C$ values common to both chironomids and zooplankton (Fig 3).
290 • The S3 sequence (1950-2005) refers to a period of greater heterogeneity in the HC
291 $\delta^{13}C$ trends of the different taxa (Fig 2). For the Tanypodinae, the second change point
292 was related to a stabilization of the HC $\delta^{13}C$ values that did not show any significant
293 decrease over the entire S3 sequence and were very similar at both depths (Table 2). For
294 the two burrowing taxa, the HC $\delta^{13}C$ from the deep zone still decreased during S3, with
295 mean depletions of 2.2 ‰ for *S. coracina*-type and 4 ‰ for *M. contracta*-type. In the
296 littoral zone, the HC $\delta^{13}C$ were stable at their lowest values for *M. contracta*-type and
297 slightly decreased for *S. coracina*-type. For these burrowing taxa, the heterogeneity in the
298 HC $\delta^{13}C$ also appeared spatially and led to high and significant values for their $\Delta\delta^{13}C$ (3.1
299 ‰ and 4.5 ‰ for *M. contracta*-type and *S. coracina*-type, respectively). This result
300 showed, for these two taxa, an important $\delta^{13}C$ -depletion in the deep chironomid HC as
301 compared to the littoral ones.

Discussion

This study revealed significant changes in the $\delta^{13}C$ values for HC and cladoceran remains over the last 150 years. The chemical composition of HC and cladoceran remains have been shown to be weakly affected by diagenetic processes (Verbruggen et al., 2010; Perga, 2011), and because their $\delta^{13}C$ values closely reflect those of the whole organism (Frossard In Press, Perga, 2010), these biological remains are likely to provide robust material for a study of the past $\delta^{13}C$ composition of these organisms. Recently, Wooller et al (2012) and van Hardenbroek et al (2012) showed non-monotonic trends in HC $\delta^{13}C$ values over plurimillennial time periods, thus supporting the reliability of $\delta^{13}C$ values from biological remains as valuable proxies for inferring changes in the carbon cycle and subsequently in lake metabolism. We extended this approach using two different depths and considering different chironomid taxa. The comparison of HC $\delta^{13}C$ values between littoral and deep zones allowed detailed changes in $\Delta\delta^{13}C$ to highlight a change in functioning efficiency. In addition, depth-specific mechanisms involved in the changes of the carbon cycle were investigated for chironomids. The taxa-specific patterns in $\delta^{13}C$ values strengthened the notion that chironomid taxa exploited different trophic niches in association with differences in foraging behavior (Kelly et al. 2004; Vallenduuk and Moller Pillot, 2007; Moller Pillot, 2009). Further, most dates were replicated to strengthen the robustness of the depicted trends and the associated interpretations.

Pre-eutrophication functioning – The S1 sequence (1850s-1930s) was characterized by the highest and most similar HC $\delta^{13}C$ values for the three chironomid taxa. The high $\delta^{13}C$ values are in line with low primary production and photosynthetic use of an inorganic carbon supply from atmospheric origin (C_{atm}) (Fig. 4). The period also showed the smallest depth-related ^{13}C depletion (mean $\Delta\delta^{13}C < 1$ ‰), indicating a great homogeneity (both spatial and inter-taxa) in

326 the carbon sources assimilated by benthic consumers such as chironomids. Following the
327 $\Delta\delta^{13}C$ interpretation proposed by Borderelle et al (2008), the low depth-related chironomid
328 HC ^{13}C depletion observed during the S1 was a sign of efficient trophic functioning.

329 These interpretations of the HC $\delta^{13}C$ were also supported by the cladoceran remains' $\delta^{13}C$
330 values (mean $\delta^{13}C$ = -28.4±0.4 ‰), which were the highest during S1 and which were
331 consistent with a carbon source mixture of atmospheric dissolved organic carbon (DIC) fixed
332 phytoplankton ($\delta^{13}C$ range: -28 ‰ to -31 ‰; France, 1995) and maybe terrestrial organic
333 matter OM ($\delta^{13}C$ near -27 ‰; Deines, 1980). Compared to the $\delta^{13}C$ of the cladoceran remains,
334 the chironomid HC $\delta^{13}C$ values were depleted by ca 3 ‰ and 4 ‰ in the littoral and in the
335 deep zones, respectively. Two hypotheses can explain these differences. First, they could be
336 related to the assimilation of bacteria by chironomids, especially ^{13}C-depleted methane-
337 oxidizing bacteria (MOB, Deines & Grey, 2006; Eller *et al*., 2005) following methane
338 production that can occur in the sediment layers even if oxic conditions prevailed in the open
339 water (Jones & Grey, 2011; Costello et al, 2002; Pester et al, 2004).Second, the lower
340 chironomid $\delta^{13}C$ values compared to the cladoceran ones could be related to a difference in
341 the temporal integration of the food sources. Cladoceran remains found in lake sediment
342 mainly came from high cladoceran production in the summer, subsidized by ^{13}C enriched
343 summer phytoplanktonic production (Perga 2011). During this season, depleted DIC
344 originating from respiration processes (DIC_{resp}) can be scavenged in the hypolimnion (van
345 Breugel et al, 2005). By contrast, the chironomids fed on phytoplankton of different seasons,
346 including spring, fueled by a higher proportion of DIC_{resp} provided to the epilimnion after the
347 spring turn-over. Further, as benthic metabolic processes have a lower temporal variability as
348 compared to pelagic compartments (Rooney & McCann, 2012), the food sources available to
349 chironomids are likely to represent a mean-year food mixture rather than seasonal pulses.
350 Whatever the involved process, the depleted values of HC $\delta^{13}C$, as compared to those of

cladocerans, was a result of the larger assimilation by chironomids of OM supported by heterotrophic processes (i.e. C_{resp} or methane-derived). The low difference in the $\delta^{13}C$ between cladocerans and chironomids, and the elevated $\delta^{13}C$ values of these consumers during S1, argues that the level of heterotrophic activity in Lake Annecy was low until the 1930s. This result contrasts with the dominance of heterotrophy in small oligotrophic lakes subsidized by allochthonous sources of OM (France et al, 1997; Cole et al, 2000). In larger lakes, such as Lake Annecy, oligotrophy was not accompanied by high heterotrophy because these lakes are much less influenced by allochthonous organic matter inputs (Perga & Gerdeaux, 2004; Pace et al, 2007). Until the 1930s, Lake Annecy seemed to be dominated by autotrophy, despite its oligotrophic status (Fig 4a) and high trophic transfer efficiency of the produced organic matter.

Response to eutrophication - The decrease in HC $\delta^{13}C$ values of ca 1.5-2 ‰ during the S2 sequence for all chironomid taxa from the littoral and deep zones occurred concomitantly with the first signs of eutrophication (Hubault, 1943). This ^{13}C depletion affected littoral benthic consumers to a greater degree than deeper consumers (except for *S. coracina*-type) and also affected pelagic consumers with a greater intensity (^{13}C depletion of more than 3 ‰) than the benthic ones (Fig 3). This decrease in the $\delta^{13}C$ values of benthic and pelagic consumers could be attributed to an increase in the proportion of phytoplankton in the chironomid and *Daphnia* food mixture, as this food source is often reported to be ^{13}C depleted compared to OM_{allo} and benthic algae (Karlsson et al, 2007; Karlsson & Säwström, 2009). This assertion is further in line with the increase in the Hydrogen Index in the sediment profiles since the 1930s (Frossard In press). However, change in productivity alone cannot explain the ^{13}C depletion of consumers during S2 because $\delta^{13}C$ values of both DIC and phytoplankton have been shown to increase as lake productivity intensified (France et al, 1997). As previously shown in Lake Mendota, USA (Hollander & Smith, 2001), our results offer evidence that a source of ^{13}C-

depleted organic matter influenced both pelagic and benthic consumers during eutrophication. Because *Daphnia* and littoral chironomids showed greater ^{13}C depletion during S2 than the deep chironomids, this source of ^{13}C depleted organic matter seemed to originate from the euphotic zone, so that a photoautotrophic source was very plausible. The greater decrease in the cladoceran remains' $\delta^{13}C$ values than in the littoral chironomid ones, and the difference in temporal $\delta^{13}C$ integration between these consumers, argues for a higher importance of this depleted source during summer than during the rest of the year.

Considered together, these observations lead to the conclusion that, during eutrophication, the additional phytoplankton production enhanced the decomposition of the autochthonous organic matter by heterotrophic activities in the epilimnion during lake stratification (Fig 4). Subsequently, this led to a ^{13}C depletion of the DIC assimilated by the summer phytoplankton. The high rate of organic matter decomposition in the epilimnion implies a moderate sedimentation of OM to the bottom of the lake during summer. This can explain the less important ^{13}C depletion of deep chironomids during S2 and the low and non-significant values of $\Delta\delta^{13}C$ values shown by chironomid taxa during S2. According to Borderelle et al (2008), the low $\Delta\delta^{13}C$ values indicate that during eutrophication, Lake Annecy was able to maintain a high functioning efficiency, so that pelagic organic matter (OM) was efficiently transferred within the pelagic food-web with limited losses to the benthic. The increasing abundance of both chironomids (Frossard 2013) and cladocerans (Perga 2010) at that time further corroborates this assertion. Therefore, during S2, Lake Annecy underwent an enhancement of its primary production that did not exceed its assimilation capacity.

Unexpected response during re-oligotrophication - Since the 1960s, the lake restoration program has led to a significant decrease in nutrient concentrations in open water (Fig. 2), while hypoxic conditions have appeared at the bottom of the lake. Since the 1990s, Lake Annecy has re-oligotrophicated with low primary production (Balvay et al, 2003).

401 Surprisingly, no recovery in the HC and cladoceran remains' $\delta^{13}C$ values has been observed
402 for any of the studied consumers or at any depth. In the littoral zone and in the epilimnion, the
403 consumer $\delta^{13}C$ values have been rather stable from the early 1950s until the 2000s. Therefore,
404 in the euphotic zone, the proportion of depleted summer phytoplankton assimilated by
405 chironomids and cladocerans has remained constant since the end of the S2. By contrast, in
406 the deep zone, there were ongoing decreasing trends in the HC $\delta^{13}C$ values covering the re-
407 oligotrophication period. Thus, the S3 sequence was marked by the assimilation of another
408 ^{13}C-depleted source of carbon assimilated by deep chironomids; the proportion of this carbon
409 increased throughout S3. This led to an increase in the $\Delta\delta^{13}C$ of the burrowing chironomids,
410 with mean values of 3.1 ‰ for *M. contracta*-type and 4.5 ‰ for *S. coracina*-type. The current
411 divergence in the HC $\delta^{13}C$ values between the littoral and the deep zones echoes the often
412 reported depth-related $\delta^{13}C$ decrease for chironomids, induced by methane-derived carbon
413 support from methane-oxidizing bacteria (MOB) consumption in the deep zones of lakes
414 (Kiyashko et al, 2001; Jones & Grey, 2004; Jones et al, 2008). MOB assimilation was highly
415 plausible because the current summer hypoxic conditions (data INRA/SILA) and the
416 increasing OM content within the sediment (Frossard, 2012 In Press) both provide suitable
417 conditions for MOB growth (Jones & Grey, 2011). The high phytoplanktonic export to the
418 deep zone despite re-oligotrophication can be explained by a decrease in the ability of
419 zooplankton to control phytoplankton biomasses due to the decrease in *Daphnia* size from the
420 1930s to the current time period, following changes in fish assemblages (Perga et al, 2010)
421 and climate change (Frossard). Hence, despite re-oligotrophication, a large amount of
422 sedimented phytoplankton is maintained in the littoral and pelagic zones and is growing in the
423 deep zone (Fig. 4).

424 *Concluding remarks:* This study reports trends for chironomid HC $\delta^{13}C$ for the last 150 years
425 and underlines the informative nature, in paleolimnological studies, of considering both

426 littoral and deep $\delta^{13}C$ for chironomids - especially when comparing them to the $\delta^{13}C$ values of
427 cladoceran remains. This type of approach allowed for the evaluation of the functioning
428 efficiency of the lake through the determination of the extent of $\Delta\delta^{13}C$ values. When applied
429 to Lake Annecy, this new functioning descriptor showed that the increase in the lake's
430 primary production during the eutrophication period had a lower impact on the lake's trophic
431 functioning efficiency than the top-bottom control of the pelagic food chain, which occurred
432 simultaneously with the lake re-oligotrophisation. This conclusion is also supported by
433 previous results obtained from the chironomid community's reconstruction in Lake Annecy
434 (Frossard, in press). It could potentially affect the suitability of considering nutrient
435 concentrations or trophic levels as the main controlling factors for benthic communities
436 (Saether, 1979; Widerholm, 1980). Currently in Lake Annecy, the benthic food-web seems to
437 be strongly coupled to the pelagic food-web. Therefore, in paleoenvironmental
438 reconstructions, inferences of past phosphorus concentrations or trophic levels from subfossil
439 chironomid assemblages should consider trophic efficiency to avoid possible
440 misinterpretations due to additional anthropogenic impacts.
441 The stratigraphic trends of chironomid and cladoceran $\delta^{13}C$ obtained in Lake Annecy led to
442 the proposal of three successive schemes for the lake's functioning for the three key periods
443 of the lake history. The oligotrophic functioning of Lake Annecy and its response to
444 eutrophication showed a few particularities when compared to other functioning models
445 (Hollander & Smith, 2001) or $\delta^{13}C$ stratigraphic trends of bulk sediments and zooplankton
446 studied in other great lakes during eutrophication (Brown et al, 2012), Before the
447 eutrophication period, the low primary productivity was not supplied by terrestrially-derived
448 carbon, which would have made the consumer $\delta^{13}C$ values much lower than those observed
449 during S1 (Kaufman et al, 2012). The specificity of Lake Annecy's response to eutrophication
450 is that the ^{13}C depleted DIC originated from both the epilimnion and the hypolimnion. In

451 recent times, Lake Annecy has moved toward heterotrophy in an oligotrophic context, but
452 interestingly, heterotrophy appears to be supported more by autochthonous than by
453 allochthonous organic matter. These results provide new insights for the various expressions
454 of benthic-pelagic linkages in lakes, as changes in the pelagic food-web structure cascade to
455 impact the carbon cycle among the benthic food-web.

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658 *Tables*

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661 Table 1. Statistical summary of the three-way-ANOVA performed on the HC $\delta^{13}C$ values

662 with response variable and time (from 1850 to 2005), zones (littoral and deep zones) and taxa

663 (*Microspectra contracta*-type, *Sergentia coracina*-type and *Tanypodinae*) as covariates

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Variables	df	Sum of squares	Mean squares	*F*	*p*
Time	1	400.92	400.92	537.49	<0.001
Taxa	2	69.79	34.89	47.78	<0.001
zones	1	69.70	69.70	93.44	<0.001
Time × zones	1	39.22	39.22	52.58	<0.001
Taxa × zones	2	20.52	10.26	13.76	<0.001
Residuals	153	135.01	0.75		

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Table 2. Mean HC $\delta^{13}C$ values for three chironomid taxa during the three temporal sequences. $\Delta\delta^{13}C$ = HC $\delta^{13}C$ litt - HC $\delta^{13}C$ deep. * = $p<0.001$, t-test.

Taxa	Sequences	mean $\delta^{13}C$ (littoral zone)	mean $\delta^{13}C$ (deep zone)	Mean $\Delta\delta^{13}C$
M. contracta-type	S1: 1850-1930	-31.3±0.7 n = 29	-32.2±0.3 n = 17	0.9*
	S2: 1930-1950	-33.5±0.6 n = 5	-32.8±1.0 n = 8	-0.7
	S3: 1950-2005	-33.6±0.4 n = 11	-36.9±1.0 n = 9	3.1*
Tanypodinae	S1: 1850-1930	-31.4±0.6 n = 26	-32.5±0.6 n = 18	1.1*
	S2: 1930-1950	-33.0±0.6 n = 11	-33.9±1.1 n = 7	0.9
	S3: 1950-2005	-34.2±0.4 n = 11	-35.0±1.9 n = 4	0.8
S.coracina-type	S1: 1850-1930	-32.2±0.5 n = 8	-34.6 n = 1	2.5
	S2: 1930-1950	-32.7±1.0 n = 2	-36.6±1.3 n = 2	3.8
	S3: 1950-2005	-34.3±0.9 n = 8	-38.8±1.0 n = 14	4.5*

692 *Figure legends*

693

694 Fig. 1 Conceptual framework for biological control of lake metabolism. The functioning
695 efficiency defined as the sum of the Lindeman's trophic efficiencies between each trophic
696 level indicates the tendency of the system to promote heterotrophy. High functioning
697 efficiency relates to high trophic efficiencies and tends to minimize organic matter losses. As
698 a result, heterotrophic activities (i.e. respiration) are minimized, as are $\Delta\delta^{13}C$ values of
699 benthic invertebrates (Chironomidae in this study) between littoral and deep zones in
700 accordance with Borderelle et al (2008). $\Delta\delta^{13}C$ values of benthic invertebrates hence appeared
701 as a metric for functioning efficiency that ultimately relates to the extent of ecosystem stress
702 according to Odum (1985).

703 Fig. 2 a) Geographic location of Lake Annecy. b) Black circles represent sampling locations
704 for the sediment cores of chironomid head capsules at 30 m and 65 m. The black triangle
705 indicates sampling location for cladoceran remains. The temporal dynamic for the soluble
706 reactive phosphorus (SRP) concentrations were modeled using GAM (data INRA/SILA). The
707 oligotrophic state was defined for SRP concentrations below 10 µg/l according to Wetzel
708 (2001).

709 Fig. 3 Compilation of temporal dynamics of the HC $\delta^{13}C$ values for the main chironomid taxa
710 of Lake Annecy. Solid line indicates fitted values of the GAM, and dashed lines indicate
711 confident intervals of the GAM. The black models refer to samples from the deep zone (65
712 m), and grey models refer to samples from the littoral zone (30 m). The horizontal lines
713 represent the location of the change points identify by the Bayesian change point analysis
714 with a threshold posterior probability fixed at 0.6.

Fig. 4 Schematic changes in lake functioning along the three temporally defined sequences. S1 is characterized by an atmospheric carbon source and high trophic efficiency leading to few OM remaining for bacterial respiration. S2 covers the eutrophication period, where the increase in primary production triggered an increase of heterotrophy in the pelagic and littoral zones. The deep zone was weakly affected because of the still high trophic efficiency in the pelagial area, as indicated by low $\Delta\delta^{13}C$ values. S3 encompasses the re-oligotrophication period, where changes in the pelagic functioning maintained respiration processes and enhanced OM pelagic losses to the deep zones of the lake, promoting the assimilation of methane-derived carbon by chironomids in the deep zone.

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738 *Figures*

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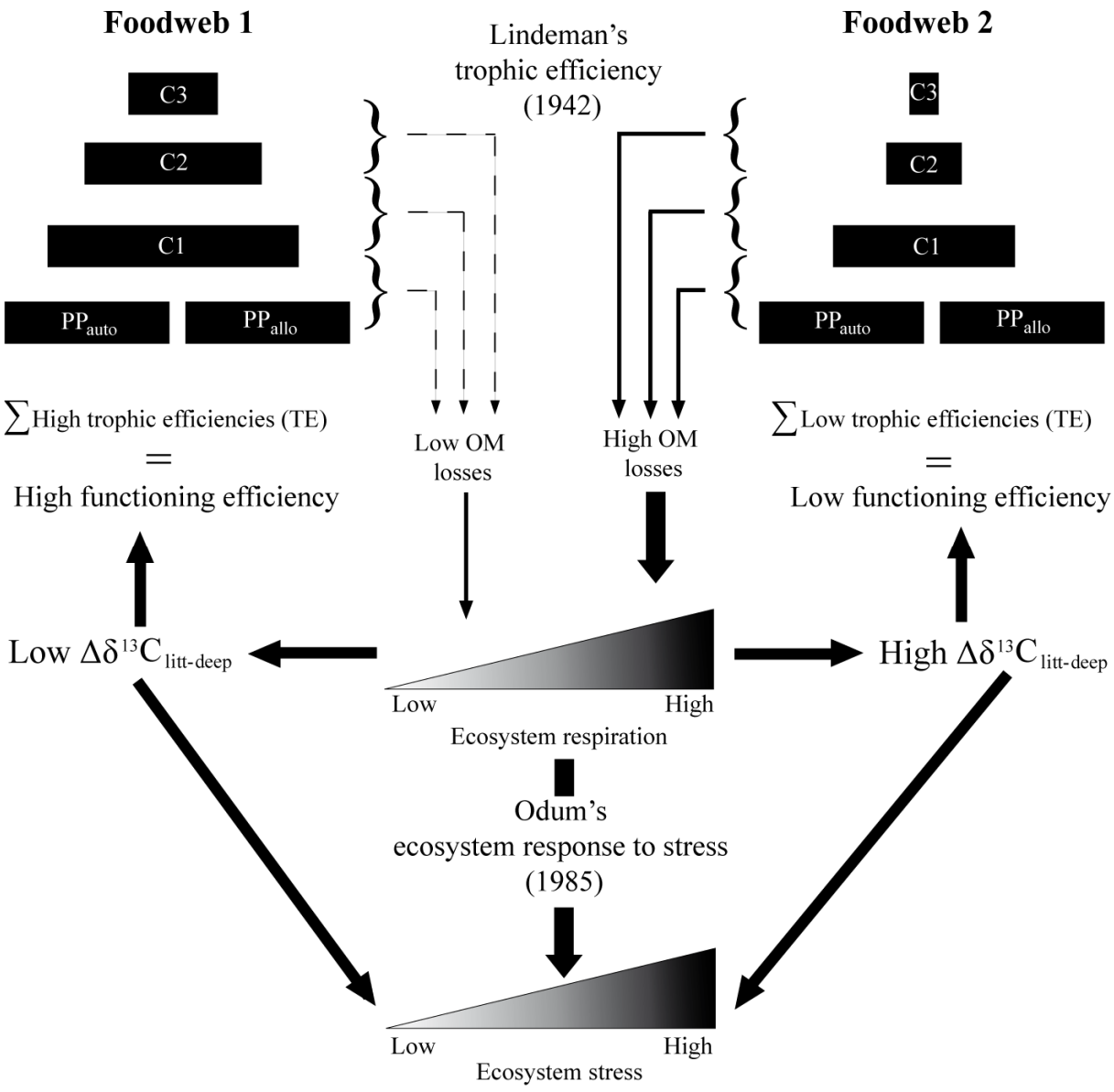
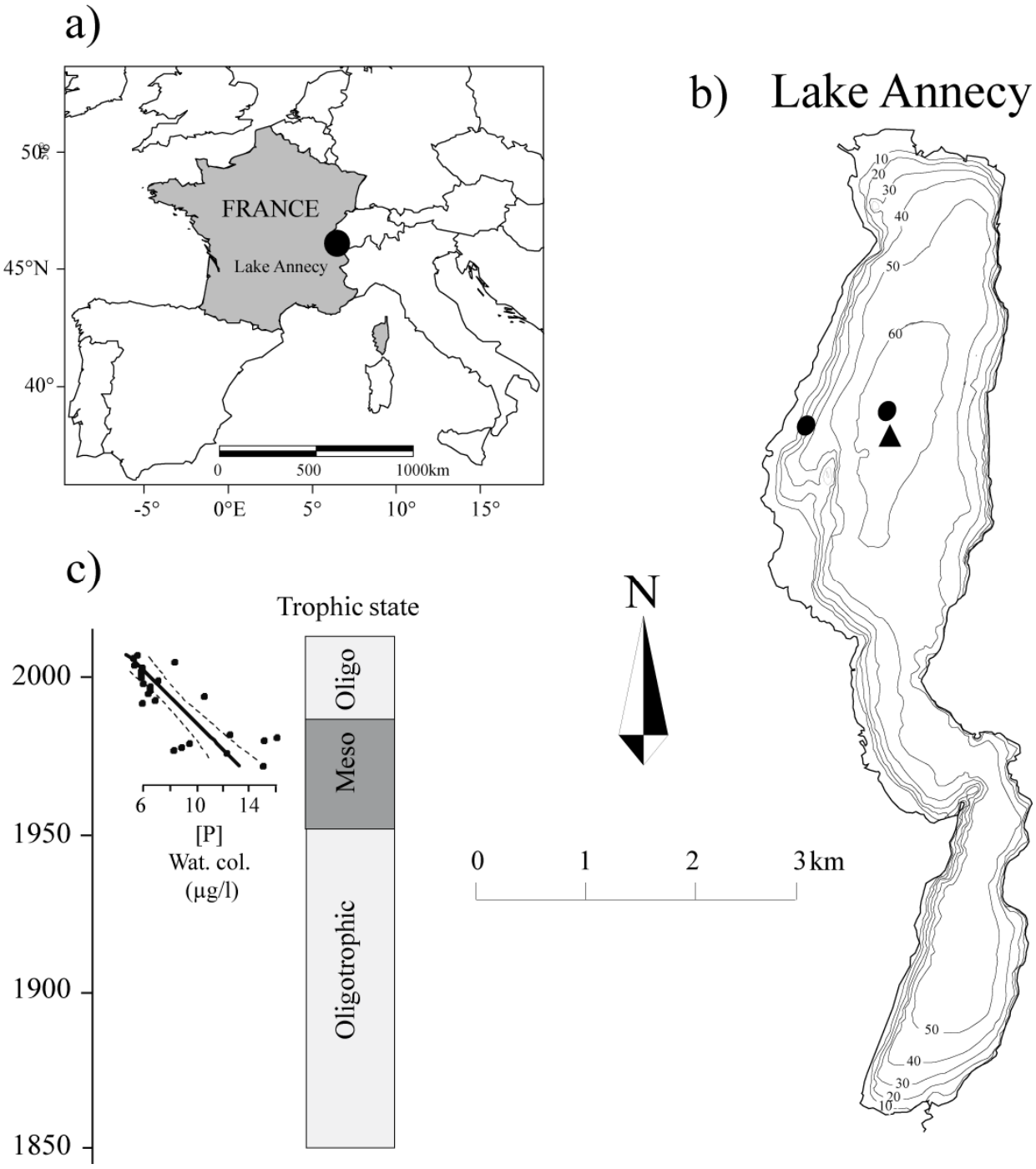


742

743 Fig. 1


a)
50°
45°N
40°
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Lake Annecy
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b) Lake Annecy
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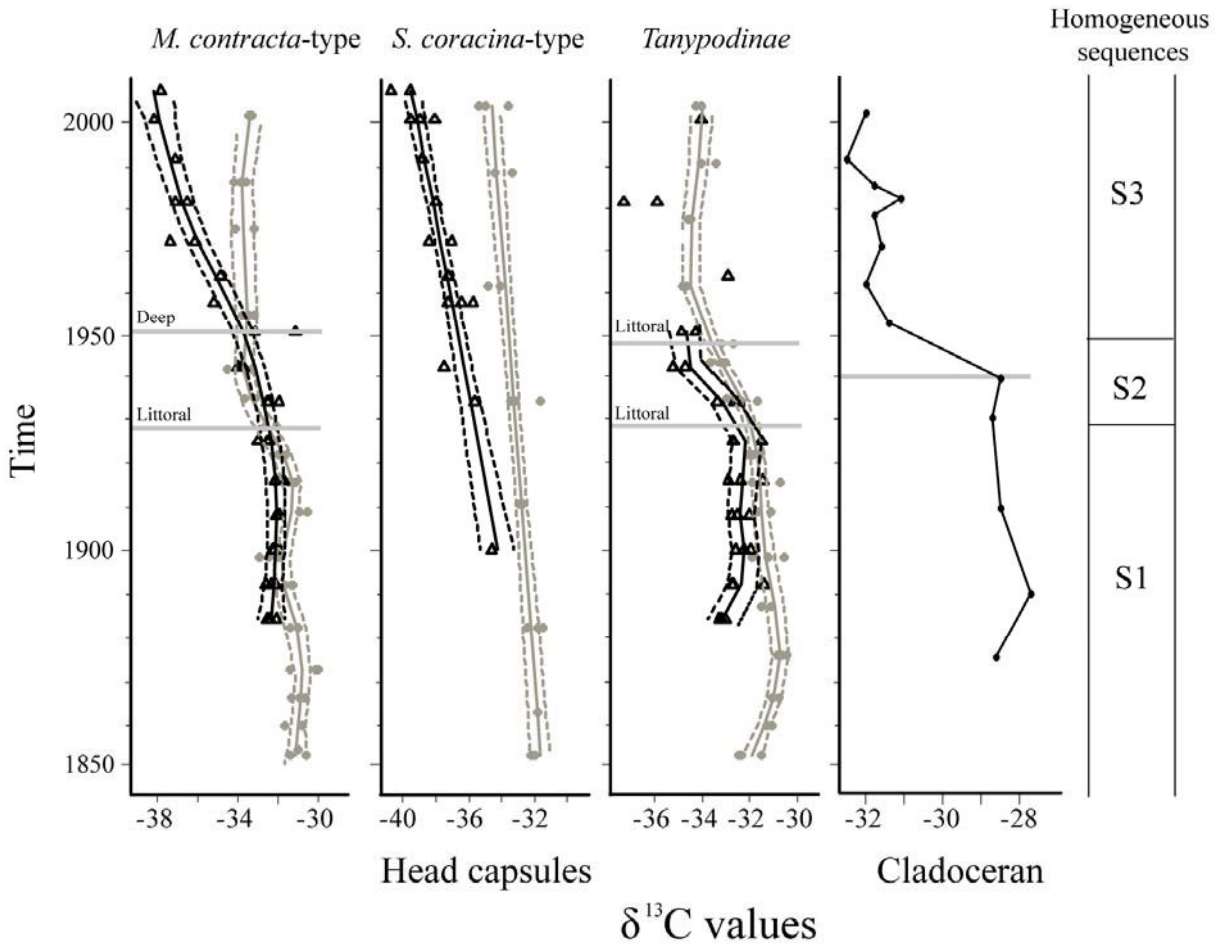
Fig. 2


M. contracta-type
S. coracina-type
Tanypodinae
Homogeneous sequences
Time
2000
1950
1900
1850
Deep
Littoral
Littoral
Littoral
S3
S2
S1
-38
-34
-30
-40
-36
-32
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-32
-30
-32
-30
-28
Head capsules
Cladoceran
δ13C values

Fig. 3

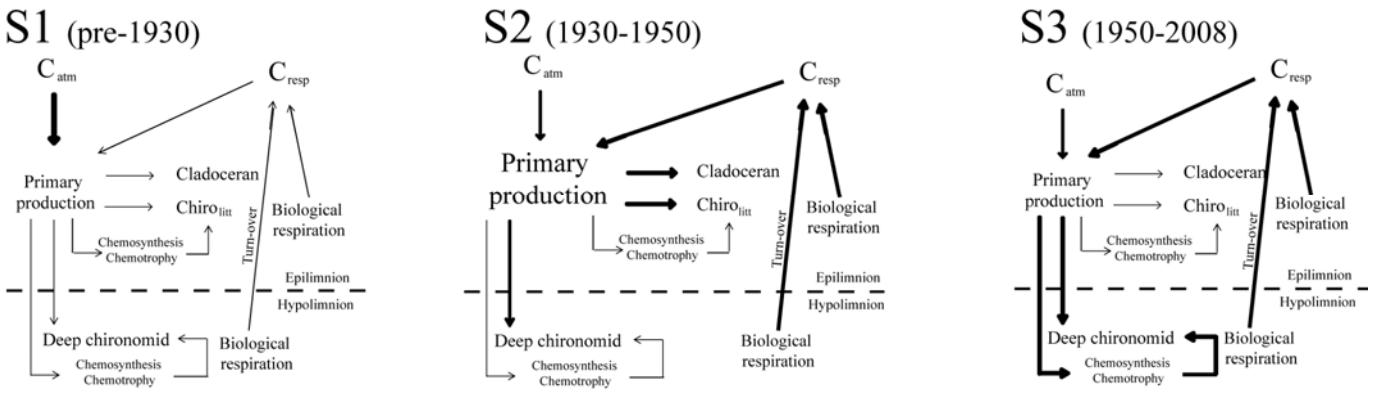
S1 (pre-1930)
C atm
C resp
Primary production
Cladoceran
Chiro litt
Chemosynthesis Chemotrophy
Turn-over
Biological respiration
Epilimnion
Hypolimnion
Deep chironomid
Chemosynthesis Chemotrophy
Biological respiration
S2 (1930-1950)
C atm
C resp
Primary production
Cladoceran
Chiro litt
Chemosynthesis Chemotrophy
Turn-over
Biological respiration
Epilimnion
Hypolimnion
Deep chironomid
Chemosynthesis Chemotrophy
Biological respiration
S3 (1950-2008)
C atm
C resp
Primary production
Cladoceran
Chiro litt
Chemosynthesis Chemotrophy
Turn-over
Biological respiration
Epilimnion
Hypolimnion
Deep chironomid
Chemosynthesis Chemotrophy
Biological respiration

Fig. 4

7. Evolution du niveau trophique dans 3 lacs périalpins depuis 150 ans : reconstitutions à partir des assemblages de diatomées extrait des archives sédimentaires

❖ *Article : Trophic history of French sub-alpine lakes over the last 150 years: phosphorus reconstruction and assessment of taphonomic biases.*

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Trophic history of French sub-alpine lakes over the last 150 years: phosphorus reconstruction and assessment of taphonomic biases.

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Condensed running head: Reconstructed trophic states for French sub-alpine lakes over the last century

Number of tables: 2

Number of figures: 4

ABSTRACT

Like many lakes worldwide, French sub-alpine lakes (Lake Annecy, Lake Bourget and Lake Geneva) have suffered from eutrophication in the XXth century. Although restoration has been undertaken and resulted in significant reductions in phosphorus (P) inputs and decreasing water concentrations over the last 30 years, the limnological monitoring does not extend back far enough to establish the reference conditions for P concentrations. Reference conditions are defined by the European Water Framework Directive as those associated with no, or only very minor, anthropogenic impact. The over-arching aim of this work was to reconstruct, using a paleolimnological approach, the pre-eutrophication P concentrations and the changing trophic status of these three lakes, from diatom-based transfer functions.

The objectives were three-fold: (i) to test whether fossil diatoms archived in deep sediment cores adequately reflect past changes in the planktonic diatom communities for these deep sub-alpine lakes based on data from Lake Geneva, (ii) to examine changes in the diatom communities over the last 150 years in all three lakes, and (iii) to infer the past Total P (TP) concentrations of the lakes from a diatom based transfer function.

Annual paleolimnological and neolimnological diatom countings for Lake Geneva were strongly correlated over the last 30 years. The most notable difference between the two datasets was the underestimation of the relative abundance of thin skeleton species such as *Asterionella formosa* and *Diatoma tenuis* using paleolimnological methods. The fossil diatom records revealed marked? shifts in the communities in the three lakes over time, most of which were changes typically associated with nutrient enrichment. Indeed, in all three lakes, the proportion of *Cyclotella* sp. was very high before the 1950's, but these species were then replaced by more eutrophic taxa, such as *Stephanodiscus* sp. From the 1980's, diatom species typical of re-oligotrophicated conditions appeared in all three lakes, although TP may no

longer be the only environmental factor driving the changes in the diatom communities. TP concentrations inferred from weighted averaging with classical deshrinking were very close to the values measured since the monitoring began. The annual TP concentrations in the three studied lakes were similar before eutrophication at < 10 µg L^{-1}. Restoration targets have been achieved at Lake Annecy (current TP is 6 µg L^{-1}), while further efforts are still required for the other two lakes (current TPs are 15 µg L^{-1} in Lake Bourget and 17 µg L^{-1} in Lake Geneva - mean calculated for the 10 last years).

1. INTRODUCTION

The European Water Framework Directive (WFD) (European Union 2000) has highlighted a crucial need for the ecological assessment of lakes. One of the key issues faced by managers concerns the establishment of reference conditions, defined by the WFD as those associated with no, or only very minor, anthropogenic impact (Anonymous 2003). These baselines are required to evaluate how much the lake under consideration has been impacted by human activities and to set the restoration objectives. The combination of spatial surveys, modelling, expert judgement and temporally based methods using historical data or palaeoreconstruction has successfully resulted into the establishment of reference conditions that are specific to some lake typologies (Wolfram et al. 2009). However many lakes lack typologic analogs for the type-specific definition of reference-conditions may not be accurate enough to assess ecological status. This is particularly true for the very large, deep European lakes, which have unique system dynamics due to their size (Loga et al. 2004). For these lakes, reference conditions need to be site-specific, and, in the absence of sufficiently long-term monitoring data, ecological and chemical reference conditions and deviation from the reference state may be defined through paleoecological approaches (Bennion and Battarbee 2007).

This study focused on three French deep sub-alpine lakes (Lakes Geneva, Annecy and Bourget) that are essential elements of social and economic activities in this region. All three lakes exhibited some symptoms of eutrophication in the mid-1970s (Anneville 2002; Millet et al. 2010; Perga et al. 2010). Total phosphorus (TP) concentrations measured during water mixing, either routinely such as for Lake Geneva or sporadically as for the two other lakes in the 1970s, confirmed that Lakes Geneva and Bourget had reached a eutrophic status (with respectively 90 and 120 µgP L^{-1}) while Lake Annecy was oligo-mesotrophic (17 µg L^{-1}) (SOERE GLACPE long-term database). P abatement measures were subsequently

78 successfully taken, leading to substantial decrease in P concentrations in the water column
79 over the last 30 years. Lake Geneva and Bourget are now mesotrophic with winter TP < 20 µg
80 L^{-1} while Lake Annecy is now oligotrophic (TP=6 µg L^{-1}, SOERE GLACPE long-term
81 database). Although all lakes are now under routine monitoring (begun in 1957 for Lake
82 Geneva, 2004 for Lake Bourget and 1996 for Lake Annecy), these datasets do not extend
83 back far enough to establish lake reference conditions.
84 This study aimed to use an existing diatom based transfer function to reconstruct the past
85 trophic status of the three French sub-alpine lakes, using a similar approach to that previously
86 employed in Lake Maggiore (Marchetto et al. 2004). Before applying the transfer function,
87 we tested that the diatom fossil communities archived in the sediment mirrored adequately the
88 changes in the pelagic diatom communities of the lakes over time. There have been, so far,
89 only a handful of such attempts on sub-alpine lakes. Bennion et al. (1995) and Wessels et al.
90 (1999) performed comparisons based on long term datasets for Lake Mondsee and for Lake
91 Constance, respectively, and Marchetto and Musazzi (2001) conducted a comparative study
92 on Lake Maggiore using the relative abundance of six selected planktonic species. Lake
93 Geneva provided a unique opportunity to conduct such a calibration analysis since (i) long-
94 term monitoring data, covering the last 30 years, were available, and (ii) its annually
95 laminated sediment could be dated at very high-resolution.
96
97 ## 2. METHODS
98 ### 2.1. Study sites
99 Lakes Geneva, Bourget and Annecy are all warm-monomictic lakes located on the northwest
100 edge of the French Alps. The surface water of these lakes is never frozen over. They are the
101 largest deep lakes in France with surface areas of 582, 42 and 27 km^2 and maximum depths of
102 309, 145 and 69 m for Lake Geneva, Bourget and Annecy, respectively.

103 The Lake Geneva monitoring survey (managed by the International Commission for the
104 Protection of Lake Geneva Waters and the INRA, French National Institute for Agricultural
105 Research) started in 1957. Sampling for the long-term monitoring is performed at the deepest
106 point of the lake (SHL2). Until 1980, sampling occurred once a month, and since 1981 has
107 been biweekly during the stratified season (March-November). Water samples dedicated to
108 phytoplankton analysis were collected in the 0-10 m depth water layer from 19XX to 1999,
109 which was extended down to 0-20 m depth from 2000. The microscopic analyses follows the
110 Utermohl method (Utermohl 1958), which is now a European Standard EN 15204 (AFNOR
111 2006). The chemical concentration of TP was measured using the acid molybdate method
112 (AFNOR 1982). Annual averages were calculated for the 0-20 m depth water layer, i.e.
113 phytoplankton sampling depth.
114 Long-term monitoring surveys started in 1996 for Lake Annecy (managed by the
115 Intercommunal Association of Lake Annecy and INRA) and 2004 for Lake Bourget (Lake
116 Bourget water agency and INRA). In addition, some data for lakes Annecy and Bourget P
117 concentrations dating back from the late 1960s-1970s were available from the 'French Alpine
118 Lakes" Long-Term Observatory Database (Database SOERE- INRA of Thonon-les-Bains –
119 France).
120 Need to add some further detail on known causes, nature and timing of enrichment based on
121 the monitoring data (ie what do we know/not know) and restoration measures taken for each
122 lake. Otherwise the Discussion that follows has no context.
123
124 2.2. Lake coring and sediment dating
125 Several short sediment cores were collected from the deepest point of each lake between 2004
126 and February 2009 using a quadruple gravity corer (UWITEC, Mondsee, Austria). Sediment
127 dating was performed from one master core for each lake using radiometric methods (^{210}Pb,

128 ^{226}Ra, ^{137}Cs and ^{241}Am activities) and counting of annual laminations, which provided
129 accurate chronologies for high-resolution sampling in both lakes. All working cores (those on
130 which paleolimnological analyses were performed) were sampled according to the annual
131 laminations, with the same temporal precision (annual or, at most, triennial resolution) and
132 correlated to the master core using lithological tie points and lamina counting performed on
133 both master and working cores (Zolitschka 2003). Such high-temporal resolution and
134 accuracy were required in order to integrate instrumental and paleo-data from multiple cores.
135
136 2.3. Fossil diatom preparation and counting
137 Diatom counts were performed on (how many?) subsamples from (which cores?) . This sub-
138 sample was weighed before and after drying (60°C during 48 h). A classical H_2O_2-HCl
139 digestion was used (Renberg 1990). After digestion, diatoms were mounted in Naphrax on
140 optical microscope slide. One slide corresponded to one core sample. On each slide, at least
141 400 valves were counted and identified by light microscopy using phase contrast or
142 differential interference contrast with 1000x magnification. In order to keep a homogenous
143 determination in the different samples, several pictures were taken for each species (100 x
144 1.6). The identifications and counts followed the European standard method (AFNOR 2004).
145 Determinations were carried out using the Krammer & Lange-Bertalot floras (1986, 1988,
146 1991a, b) and other specialized bibliographical data when needed.
147
148 2.4 Data Analyses
149 2.4.1 Diatom stratigraphy
150 Temporal changes in the diatom communities of the three lakes were summarized by those of
151 the main dominant species (sum of relative abundance > 80%) and represented using C2
152 (version 1.7.2, Juggins 2007). To reveal the timing of major changes in the communities,

153 chronological clustering analyses, computed from Bray-Curtis dissimilarity indexes, were
154 performed (CONISS in R package rioja, Juggins 2009). The broken-stick approach (Bennett
155 2006) was used to assess the number of statistically significant biozones.
156
157 2.4.2 Comparison of neo- and paleolimnological diatom records
158 For Lake Geneva, changes over time in the diatom taxonomic compositions (expressed as
159 species relative abundances per year) were compared between the neo- and paleo-
160 limnological records over a 30-year period (1977-2007). Correlations between the two
161 datasets were detected using a non-parametric Mantel test (Mantel 1967) performed on two
162 Bray-Curtis distance matrices (Ginkgo software, De Caceres et al. 2007). The Mantel test was
163 performed using xlstat (http://www.xlstat.com, Addinsoft)
164 To explore the differences between paleolimnological and limnological countings, the Indval
165 Method (Dufrene and Legendre 1997) was performed using PC-Ord 5 (McCune and Mefford
166 2006) to define which species characterized the phytoplankton? samples and which ones
167 characterized the core samples. It has been supposed that some differences could be explained
168 by the presence of benthic taxa in the paleolimnological dataset. Benthic taxa found in
169 sediments collected from deep basins can arrive by sediment focusing (Blais and Kalff 1995;
170 Likens and Davis 1975). Hence, ecological groups enabling discrimination between benthic
171 and planktonic species, as designed by Berthon et al. (2011) and Rimet and Bouchez (2012b),
172 were used to interpret the Indval Method results.
173
174 2.4.3 Transfer functions
175 Annual mean TP concentrations were reconstructed from a diatom inference model based on
176 86 surface sediment samples collected from sub-alpine lakes in the Alps region (Wunsam and
177 Schmidt 1995). The calibration dataset covered a large trophic gradient, with mean annual TP

ranging from 2-266 µg L^{-1}. The selected model was then applied to a diatom biostratigraphy of each lake. DI-TP reconstructions were performed using program C2 - version 1.7.2, (Juggins 2007). Several models were tested: weighted averaging with classical deshrinking (WA_{cla} regression), weighted averaging with inverse deshrinking (WA_{inv} regression) (ter Braak and van Dam 1989), weighted averaging partial least squares regression (WAPLS) (ter Braak and Juggins 1993), modern analogue technique (MAT) with squared chord distance and five analogues (Overpeck et al. 1985); and their relative performances were estimated using r^2 and the root mean square error of prediction (RMSEP) calculated using a cross validation method (bootstrapping, 500 permutations) on the calibration dataset.

The diatom-inferred TP reconstructions (DI-TP) were compared to TP monitoring data, and also to paleolimnologically reconstructed changes in *Daphnia* abundances (Alric 2012) since these are known to be good indicators of TP dynamics (DeMott and Gulati 1999).

3 RESULTS

3.4 Reconstructed changes in the relative abundance of diatoms

Fossils from 145, 239 and 203 diatom species for, respectively, Lakes Annecy, Bourget and Geneva, were recovered from the cores. Temporal changes of their relative abundances in the fossil diatom communities of all three lakes are presented in Fig. 1. The lowermost samples of all? cores were rich in *Cyclotella* species, with contributions and diversity of this group decreasing over time. This trend was partly reversed in the most recent samples. Another common feature was a generally increasing contribution of *Fragilaria crotonensis* (Kitton) over time.

In Lake Annecy, six biozones (a1-a6) were identified by chronological clustering. Major shifts in the fossil diatom communities were identified in 1941-1942, 1950-1951, 1963-1964, 1967-1968 and 1976-1977. *Cyclotella costei* (Druart & Straub) and *Fragilaria crotonensis*

were the dominant species, making, on average, up to 70% of the fossil remains. In the lowermost part of the core (a1 and a2), the fossil diatom community was the most diverse, with notable amounts of *Cyclotella distinguenda* var. *distinguenda* Hustedt, *Cyclotella comensis* Grunow in Van Heurck and *Stephanodiscus minutulus* (Kutzing) Cleve & Moller. *Cyclotella costei* and *Fragilaria crotonensis* were dominant in all counts from the 1950s (a3 to a6). In the 1950s-60s (a3), the relative abundance of *Cyclotella costei* exceeded 60% but the relative abundances of *Fragilaria crotonensis* increased up to 45% from the 1960s, so that the relative abundance of these two species were comparable within the biozones a4-a5. Increasing abundances of *Fragilaria crotonensis* in a4-5 occurred with increased amounts of *Stephanodicus parvus* Stoermer et Håkansson. The diatom composition in the most contemporaneous samples (a6) strongly resembled that of a3.

Changes in community structure were more striking in Lake Bourget. Five biozones were identified (b1-b5), with transitions occurring in 1926, 1956, 1986, and 1991. In biozone b1, the community was dominated by *Cyclotella costei, C. comensis* and *C. distinguenda* var. *distinguenda*. In biozone b2 (1930s-1950s), *C. distinguenda* var. *distinguenda* disappeared while the contributions of *Stephanodiscus minutulus* and *Fragilaria crotonensis* increased. Biozone b3 (late 1950-late 1980s) was almost exclusively represented by these two species.

Biozones b4 and 5 saw the reappearance of some of the *Cyclotella species* that were previously detected in biozones b1-2 such as decreasing abundances of *Stephanodiscus minutulus* (except for a peak in the 1990s). However, the contributions of species that were detected only at very low levels before, i.e. *Asterionella formosa* Hassall, *Aulacoseira islandica* (O.Muller) Simonsen ssp. *helvetica* (O.M.) Simonsen and *Stephanodiscus parvus*, substantially increased in biozones b4-5. Hence, the diatom composition in the most contemporaneous samples of b5 was not similar to any of those observed in the preceding biozones.

In Lake Geneva, six biozones were identified (g1-g6), with transitions in 1910, 1930, 1955, 1968 and 1994. *Cyclotella costei* and *Cyclotella comensis* were very abundant in biozones g1 and g2. The main difference between the two biozones was due to an increased contribution of *Tabellaria flocculosa* (Roth) Kutzing, *F. crotonensis* and *S. alpinus* for g2. The community then shifted in the early 1930s to dominance by *Tabellaria floculosa*, along with the appearance of *Fragilaria crotonensis*, *Aulacoseira islandica* ssp. *helvetica* and *Stephanodiscus minutulus* (g3). g4, between the mid 1950s and the early 1970s, was characterized by dominance of *S. minutulus*, *S. alpinus* and *F. crotonensis*. *Stephanodiscus* species were very abundant by the end of the 1980s (middle of the period g5) making up to 60% of the total community. In biozone g6, some *Cyclotella* species (*Cyclotella costei*) that had disappeared in the previous biozones, became more abundant. The community was, however, dominated by *F. crotonensis* (40%) and *Diatoma tenuis* Kutzing (20%), which had been so far, rare. *Tabellaria floculosa* represented 20% of the community in 2000. Because of their richness in the three latter species, the most contemporaneous samples of g6 were very different from the rest of the core.

3.5 Comparison of limnological and paleolimnological data in Lake Geneva

The temporal changes in the relative abundances of the most commonly occurring species over the 1977-2007 time-period in Lake Geneva are presented in Fig. 2. A comparison of the species relative abundances in the limnological data and paleolimnological data showed that the latter tend sometimes to under-estimate or over-estimate abundance of certain species in relation to the phytoplankton data, principally under-estimation of *Asterionella formosa*, *Cyclotella costei* and *Diatoma tenuis*, and over-estimation of *Stephanodiscus binderanus* and *Stephanodiscus minutulus* (between 1989 and 1996).

A total of eighty taxa were present in the limnological dataset and 114 in the paleolimnological one. The Mantel test showed that the two datasets were significantly correlated ($p < 0.0001$) but the strength of the relationship was low ($r = 0.498$). Here, only 52 taxa, which had a relative abundance >2%, were kept for the Indval method. Among these taxa, 38 species were typical for only one of the two datasets (Tab. 1). These 38 species were separated into benthic and planktonic groups according to the Berthon et al. (2011) criteria. Among the benthic taxa, the majority were only present in the paleolimnological dataset (Tab. 1 - group 1) and only one (*Achnanthidium minutissimum* (Kutzing) Czarnecki) was present in both datasets (group 2) but with a higher relative abundance in the paleolimnological data. Three taxa were detected only in the limnological dataset (*Fragilaria capucina* var. *radians* which is principally recognizable in living samples using the star shape of the colonies, *Navicula* sp. and *Nitzschia* sp. - group 3). Among the planktonic taxa, again, the majority belonged to group 1, seven belonged to group 3 and only two were present in the two datasets and in higher abundance in the limnological dataset (*Asterionella formosa* and *Diatoma tenuis* – group 4).

3.6 Transfer functions

In comparison with the WA and WAPLS models, MAT models performed poorly. There were no significant? differences between WA and WAPLS model performances. There were also no significant differences between the RMSEP calculated for the entire calibration dataset and that calculated on the core basis (Tab. 2). According to the calculated performances, all models had high confidence. Results presented in Fig. 3 are those obtained using the WA_{cla} regression for Lake Annecy and Lake Geneva and using the WA_{inv} regression for Lake Bourget. In the three lakes, for each sample, the proportion of taxa present in the calibration dataset was high: 90% in lake Annecy, 91% in lake Bourget, and lower in Lake

277 Geneva: 83% (average value). The bootstrapped average bias was low: 0.003 log units for
278 Lake Annecy and Lake Geneva, and 0.006 log units for Lake Bourget.
279
280 In Lake Annecy, before 1945, the DI-TP concentration varied between 10 and 15 µg L^{-1}. DI-
281 TP were transiently lower (around 7 µg L^{-1}) during the following 14 years but increased back
282 to 11 µg L^{-1} between 1963 and 1976. DI-TP values decreased back after this light
283 mesotrophic episode up to the top of the core (around 8 µg L^{-1}). DI-TP were consistent with
284 instrumental data and adequately tracked the decrease in P concentrations over the last 30
285 years. The temporal dynamics in *Daphnia* abundance generally matched that of DI-TP, with
286 maxima reached between the mid 1960's and 1970s, except for the period before the 1940s
287 for which very low *Daphnia* abundance did not support the relatively high DI-TP.
288 In Lake Bourget, before 1952, DI-TP concentrations were low (10-20 µg L^{-1}). DI-TP values
289 dramatically increased from the early 1950s to reach 68 µg L^{-1} in 1957. DI-TP transiently
290 decreased and fluctuated between 36-73 µgP L^{-1} to reach a maximum of 80 µg L^{-1} in 1973.
291 Between 1973 and 2007, DI-TP dropped to 14 µg L^{-1} in 2007, consistent with monitored data.
292 *Daphnia* abundance was generally consistent with that of DI-TP. *Daphnia* maxima matched
293 those in DI-TP. However, it seems that *Daphnia* abundance increased from the 1940s: i.e. a
294 decade earlier than the first detected increase in DI-TP.
295 In Lake Geneva, DI- TP concentration was low and fluctuated between 6 and 14 µg L^{-1} before
296 1955. In 1957, values started to increase, to reach 120 µg L^{-1} in 1974. The general dynamics
297 of DI-TP during eutrophication mirrored that of the monitored data, except that DI-TP tended
298 to over-estimate the P maximum. As for Lake Bourget, the general dynamics of *Daphnia*
299 abundances were consistent with DI-TP, except that they started to increase a decade earlier.
300 DI-TP values in Lake Geneva decreased from this peak, to reach 19 µg L^{-1} in 2007, except for

a period of very high DI-TP in the 1990s that was not corroborated by monitored TP data, nor by reconstructed changes in *Daphnia* abundance.

4 DISCUSSION

Fidelity of fossil records

Comparisons of paleolimnological counting to limnological ones are important to evaluate the accuracy and relevancy of paleoenvironmental inferences (Marchetto and Musazzi 2001). These comparisons enabled us an assessment of the fidelity of fossil records and the potential bias arising from taphonomic processes occurring before and after the remains are archived in the sediment. In deep and large sub-alpine lakes, representation of living diatom communities by deep-water sedimentary diatom assemblages has been already studied by Marchetto and Musazzi (2001), who compared relative abundance of six selected planktonic species in plankton counts and sediment samples in Lake Maggiore between 1981 and 1998, and by Wessels et al. (1999), who used historical phytoplankton data to check whether sediment data reflect planktonic diatom community in Lake Constance. A different approach was possible herein because the Lake Geneva has been heavily monitored, and also because its annually-laminated sediment can be dated with very high accuracy. These kinds of diachronic comparison performed on long time periods for a single lake are rare because limnological monitoring often contains temporal gaps. The Mantel test showed that the paleolimnological and limnological datasets were highly correlated, hence highlighting a high compositional fidelity of the sediment archive in Lake Geneva. Good preservation has also been shown for Cladoceran subfossils in these deep subalpine lakes and is attributed to rapid and direct sedimentation processes in an environment relatively undisturbed by sediment resuspension and transportation (Alric and Perga 2011). Nevertheless, diversity was greater in the

326 paleolimnological dataset than in the planktonic dataset of Lake Geneva. Four explanations
327 follow. First, presence of benthic species in the core samples most likely arises because of
328 lateral transport of diatom remains from littoral areas (sediment focusing - Blais and Kalff
329 1995; Likens and Davis 1975). It should be noted that the proportion of benthic species is
330 very low and did not exceed 9% in Lake Annecy and Lake Bourget and 6% in Lake Geneva.
331 In the case of these deep and concave lakes, this bias is kept low because of their morphology
332 with rather restricted littoral areas. Second, some species were found only in the limnological
333 data, which could be explained by modification of used material (microscope precision) and
334 by modification in taxonomic literature (Rimet and Bouchez 2012a); it could also be
335 explained by operator determination skills since both *Fragilaria* and *Aulacoseira* genera
336 encompass several morphologically similar species that can be confused. Third, two species
337 were under-estimated in the paleolimnological dataset relative to the planktonic dataset:
338 *Asterionella formosa* and *Diatoma tenuis* have a very thin siliceous skeleton and might be
339 more sensitive to taphonomic processes during sedimentation. Their preservation might be
340 lower than for other species with thicker frustules, which might explain why valves detected
341 on the slides were broken. Such underestimations of the abundance of *Asterionella formosa*
342 and *Diatoma tenuis* in profundal core samples were also observed by Marchetto and Musazzi
343 (2001). Broken frustules could be also explained by the centrifugation step of the slide
344 preparation even if it has been used with caution. Four???
345
346 Lake trophic histories
347 The three study lakes underwent a similar trophic development to that reported for Lake
348 Maggiore and Lake Constance, two large and deep sub-alpine lakes located at the opposite
349 edges of the Alps. The trophic status of Lake Maggiore and Lake Constance increased
350 between the 1950's and the 1980's, then decreased through the application of restoration

351 measures such as the construction of wastewater treatment plants and the ban of detergents
352 containing P compounds (Marchetto et al. 2004). The species succession in the planktonic
353 diatom communities over the last century in these two lakes is very comparable to the
354 succession observed here in Lake Annecy, Lake Bourget and Lake Geneva (Marchetto et al.
355 2004; Wessels et al. 1999). Lake Annecy did not reach a high level of eutrophication
356 (maximum TP concentration 15 µg L^{-1} in 1966 and 1970 – winter mean, limnological data)
357 but we observed a slight decrease in *Cyclotella costei* replaced by more eutrophic species,
358 namely *Stephanodiscus parvus*. The increase in TP concentration was more marked in Lake
359 Bourget and Lake Geneva with major shifts in the fossil diatom communities, most notably
360 the replacement of *Cyclotella* species by *Stephanodiscus* species. This pattern was reversed
361 following lake restoration. In Lake Geneva the transition between the oligotrophic and
362 eutrophic phases seemed to be slower than in Lake Bourget, with periods during which
363 mesotrophic species as *Tabellaria floculosa* and *Diatoma tenuis* developed (Rimet et al.
364 2009). Similarly to Lake Maggiore and Lake Constance (Marchetto et al. 2004), the trophic
365 status of Lake Bourget and Lake Geneva is still higher than before the eutrophication period.
366 The composition of the diatom assemblages in these two lakes today is markedly different
367 from that of the pre-eutrophication period with particularly lower abundance of *Cyclotella*
368 *comensis* in the recent sediment and presence of *Asterionella formosa* and *Diatoma tenuis* in
369 Lake Geneva. Re-oligotrophication is more advanced in Lake Maggiore where *Cyclotella*
370 *comensis* is currently abundant (reference?).
371
372 Total phosphorus reconstructions
373 WA_{cla} reconstruction models gave TP values closest to the monitoring data and the most
374 realistic in relation to the known history of Lake Annecy and Lake Geneva during the last
375 century (monitoring data + *Daphnia* abundance reconstruction). In contrast, the most

adequate model was the WA_{inv} for Lake Bourget. WAPLS did not provide satisfactory reconstructions. For instance, in Lake Bourget, during 1860-1940, the WAPLS reconstruction lead to marked fluctuations between 12 and 90 $\mu g.L^{-1}$ which were not supported? by others proxies such as Cladocerans (Alric 2012).

A high abundance of some species in the dataset used to calculate the DI-TP concentration caused some overestimations or underestimations compared with monitored TP (Wunsam and Schmidt 1995). The overestimated values corresponded to the time periods when the relative abundance of *Stephanodiscus* species was very high although the WA_{cla} model seemed to be less sensitive to this problem. DI-TP values were higher than expected between 1937 and 1945 in Lake Annecy, and in 1972 and between 1989 and 1996 in Lake Geneva. This over-estimation by the model was also observed by Marchetto et al. (2004) when *Stephanodiscus minutulus* was very abundant during the 1980s in Lake Maggiore. It should also be noted that between 1989 and 1996 in Lake Geneva, the abundance of *Stephanodiscus minutulus* might be overestimated by the fossil record (Fig. 2). *Cyclotella costei*, which was very abundant (in which cores?), may have the opposite effect as high abundances in the sediment appear to be associated with TP underestimations. Because of the weighs given to oligotrophic and eutrophic species, this reconstruction model does not account sufficiently for mesotrophic species (*Tabellaria floculosa* and *Diatoma tenuis*); their increasing abundances do not cause any progressive increase in DI-TP concentration in Lake Bourget and Geneva. The amplitude of the gradient and the uneven distribution of the lakes in the calibration dataset can explain this pattern, as shown in Fig. 4. As a result the optimum values of some species may be less precisely known in some parts of the gradient (Telford and Birks 2011). The lack of oligotrophic and ultra-oligotrophic lakes in the calibration dataset could also explain why reconstructions of low TP concentrations (<10 µg.l-1) in all three lakes before eutrophication are inaccurate.

Given the over-arching aim of the paper, you need to add a section to discuss your findings in relation to reference conditions. What are the baseline TP concentrations for your lakes and how do these compare with those set for this lake type in the WFD and with TP reference conditions for lakes elsewhere? See, for example, special issue on reference conditions in JOPL No 45 (2011).

5 CONCLUSIONS

By comparing limnological and paleolimnological datasets for Lake Geneva, it was shown that the preservation and representation? of diatom valves in the recent sediments of French pre-alpine lakes is good and fossil diatom records are, therefore, appropriate for assessing environmental change in these systems. In spite of some problems related to samples strongly dominated by certain species, the comparison between DI-TP and limnological monitoring data showed good agreement and allowed the trophic history of the three study lakes to be reconstructed and reference P concentrations to be identified.

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521 Tab.1 (Vincent Berthon, Aldo Marchetto, Frédéric Rimet, Emmanuelle Dormia, Jean-Philippe

522 Jenny, Cécile Pignol, Marie-Elodie Perga)

	species	p-value limnological dataset	p-value paleolimnological dataset	group
benthic species	*Achnanthidium minutissimum* (Kutzing) Czarnecki		0,0284	2
	Amphora pediculus (Kutzing) Grunow		0,0002	1
	Cocconeis placentula Ehrenberg var. *placentula*		0,0052	1
	Cymatopleura solea (Brebisson in Breb. & Godey) W.Smith var. *solea*		0,0018	1
	Diatoma moniliformis Kutzing		0,0016	1
	Encyonema minutum (Hilse in Rabh.) D.G. Mann		0,0002	1
	Fragilaria capucina Desmazieres var. *capucina*		0,0002	1
	Fragilaria capucina Desmazieres var. *radians* (Kutzing) Lange-Bertalot	0,0002		3
	Fragilaria capucina Desmazieres var. *vaucheriae* (Kutzing) Lange-Bertalot		0,0234	1
	Fragilaria mesolepta Rabenhorst		0,0022	1
	Gomphonema pumilum (Grunow) Reichardt & Lange-Bertalot		0,049	1
	Navicula cryptotenella Lange-Bertalot		0,011	1
	Navicula reichardtiana Lange-Bertalot var. *reichardtiana*		0,0258	1
	Navicula sp.	0,0002		3
	Navicula subrotundata Hustedt		0,004	1
	Nitzschia dissipata (Kutzing) Grunow var. *dissipata*		0,002	1
	Nitzschia fonticola Grunow in Cleve et Muller		0,0002	1
	Nitzschia sp.	0,0002		3
	Staurosira mutabilis (Wm Smith) Grunow		0,0006	1
planktonic species	*Asterionella formosa* Hassall	0,0002		4
	Aulacoseira ambigua (Grunow) Simonsen		0,0004	1
	Aulacoseira granulata (Ehr.) Simonsen var. *angustissima* (Muller) Simonsen	0,0002		3
	Aulacoseira islandica (Muller) Simonsen		0,0002	1
	Aulacoseira islandica (Muller) Simonsen subsp. *helvetica* (Muller) Simonsen	0,0002		3
	Cyclotella comensis Grunow in Van Heurck		0,0002	1
	Cyclotella delicatula Hustedt		0,0002	1
	Cyclotella sp.	0,0004		3
	Diatoma tenuis Agardh	0,0224		4
	Discostella pseudostelligera (Hustedt) Houk and Klee		0,0086	1
	Discostella stelligera (Cleve et Grun.) Houk and Klee		0,0002	1
	Fragilaria ulna (Nitzsch.) Lange-Bertalot var. *acus* (Kutz.) Lange-Bertalot	0,0002		3
	Fragilaria (Ulnaria) *ulna* Sippen *angustissima* (Grun.) Lange-Bertalot	0,0002		3
	Nitzschia acicularis (Kutzing) W.M.Smith	0,0002		3
	Stephanodiscus irregularis Druart. Reymond Pelletier and Gasse		0,0014	1
	Stephanodiscus parvus Stoermer and Hakansson		0,0002	1

Stephanodiscus species		0,0106	1

Tab. 2 (Vincent Berthon, Aldo Marchetto, Frédéric Rimet, Emmanuelle Dormia, Jean-Philippe Jenny, Cécile Pignol, Marie-Elodie Perga)

reconstruction method	r^2	RMSEP					
		Lake Annecy		Lake Bourget		Lake Geneva	
		calibration dataset	core basis	calibration dataset	core basis	calibration dataset	core basis
WA_{cla}	0.60	0.34	0.34	0.34	0.34	0.34	0.35
WA_{inv}	0.60	0.30	0.30	0.30	0.30	0.30	0.30
WAPLS	0.60	0.30	0.30	0.30	0.30	0.30	0.30
MAT	0.38	0.31	0.33	0.31	0.32	0.31	0.10

Fig.1 (Vincent Berthon, Aldo Marchetto, Frédéric Rimet, Emmanuelle Dormia, Jean-Philippe Jenny, Cécile Pignol, Marie-Elodie Perga)

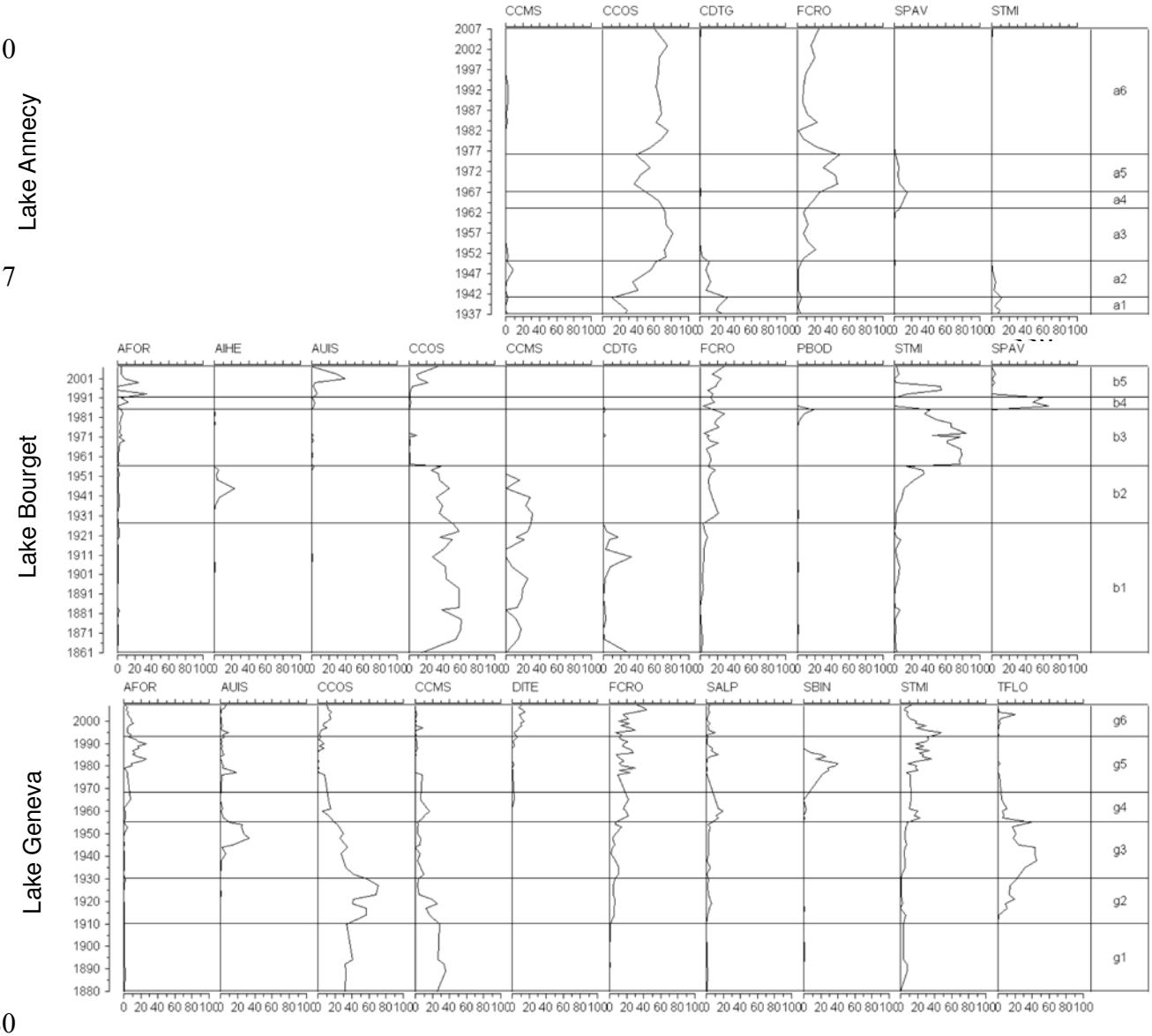


Fig. 2 (Vincent Berthon, Aldo Marchetto, Frédéric Rimet, Emmanuelle Dormia, Jean-Philippe Jenny, Cécile Pignol, Marie-Elodie Perga)

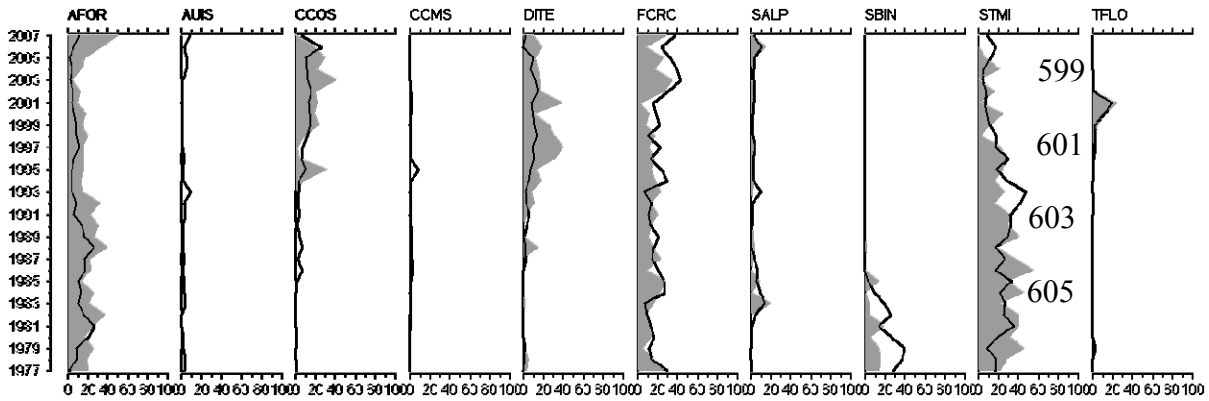


Fig. 3 (Vincent Berthon, Aldo Marchetto, Frédéric Rimet, Emmanuelle Dormia, Jean-Philippe Jenny, Cécile Pignol, Marie-Elodie Perga)

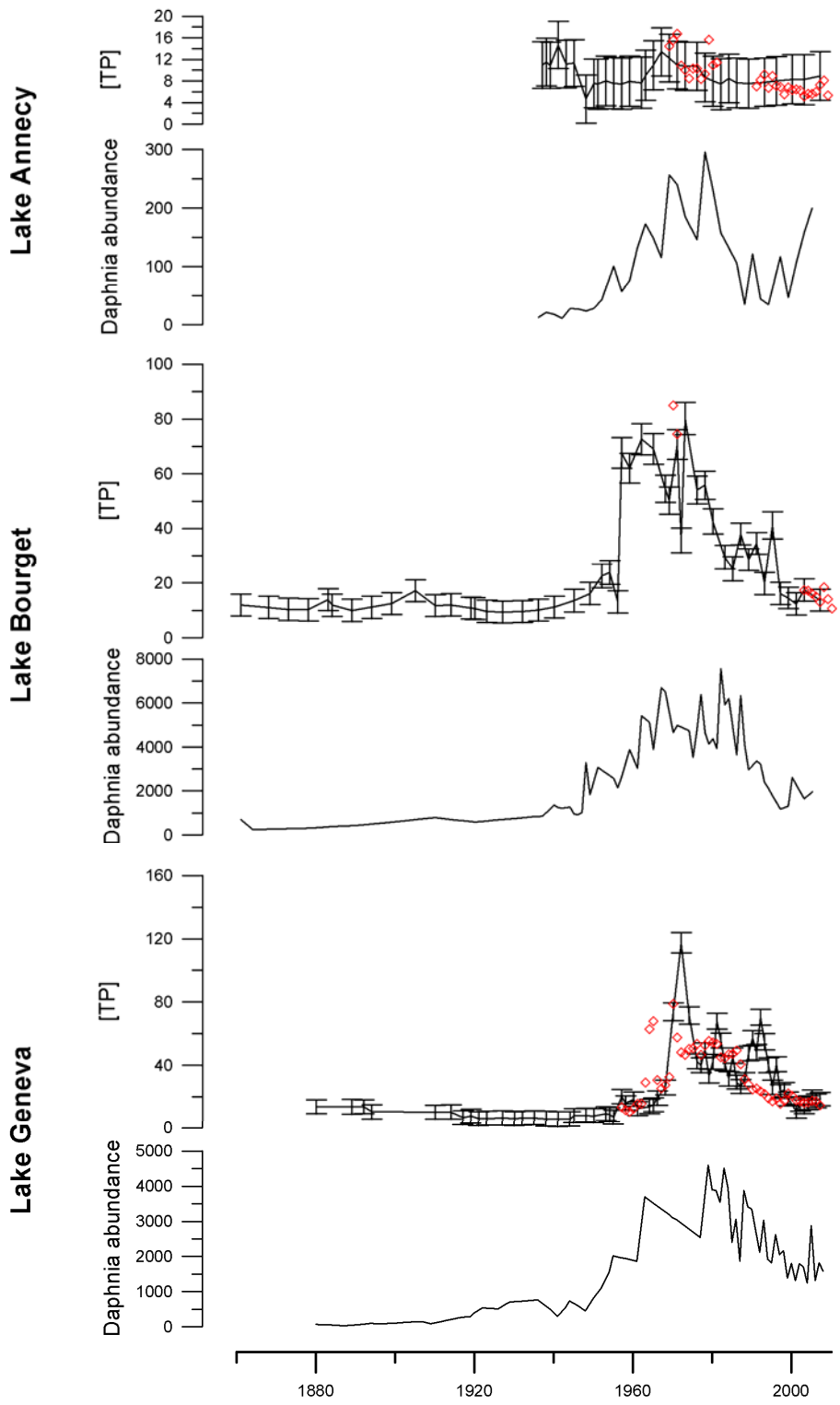
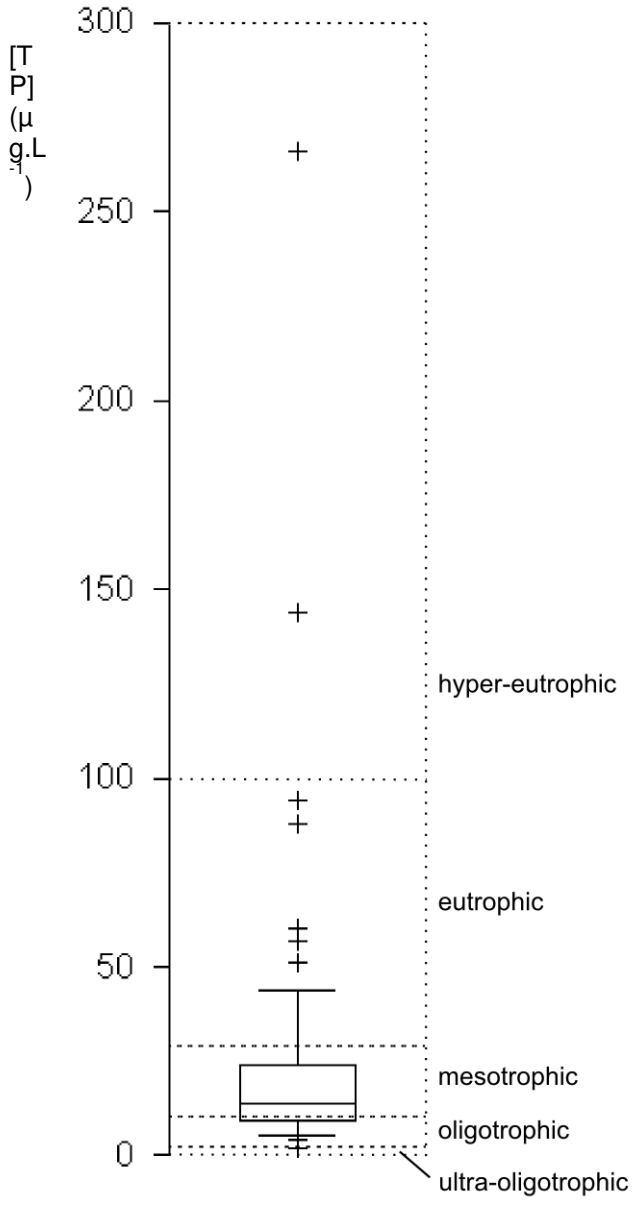


Fig. 4 (Vincent Berthon, Aldo Marchetto, Frédéric Rimet, Emmanuelle Dormia, Jean-Philippe Jenny, Cécile Pignol, Marie-Elodie Perga)



704 Table 1: Indval method results (needs a key to explain the groups and mention significance
705 (p) values
706
707 Table 2: Performance of the different tested transfer function models (r^2: Squared correlation
708 between inferred and observed values; RMSEP: Root mean squared error of prediction
709 (bootstrap RMSEP) – log units) (explain 'calibration set' and 'core basis')
710
711 Figure 1: Summary diatom stratigraphies of the most common taxa (relative abundances %)
712 in the three studied lakes (AFOR: *Asterionella formosa* Hassall ; AIHE: *Aulacoseira islandica*
713 (Muller) Simonsen subsp. *helvetica* (Muller) Simonsen ; AUIS: *Aulacoseira islandica*
714 (Muller) Simonsen ; CCOS: *Cyclotella costei* Druart & Straub; CCMS: *Cyclotella comensis*
715 Grunow in Van Heurck ; CDTG: *Cyclotella distinguenda* var. *distinguenda* Hustedt ; DITE:
716 *Diatoma tenuis* Agardh ; FCRO: *Fragilaria crotonensis* Kitton ; PBOD: *Puncticulata*
717 *bodanica* (Grunow in Schneider) Håkansson ; SALP: *Stephanodiscus alpinus* Hustedt in
718 Huber-Pestalozzi ; SBIN: *Stephanodiscus binderanus* (Kutzing) Krieger ; SPAV:
719 *Stephanodiscus parvus* Stoermer and Hakansson ; STMI: *Stephanodiscus minutulus* (Kutzing)
720 Cleve and Moller ; TFLO: *Tabellaria flocculosa* (Roth) Kutzing). The resolution of the fig is
721 very poor in my version so it is hard to comment on whether it needs improving. Need to say
722 what the y axis represents (Age AD ?)
723
724 Figure 2: Comparison of the major planktonic diatom species in the fossil diatom record and
725 the phytoplankton samples (relative abundances %) of Lake Geneva 1977-2007 (black line:
726 paleolimnological data ; grey silhouette phytoplankton data). See Fig 1. for taxon codes ?
727

728 Figure 3: Diatom-inferred total epilimnetic phosphorus (μg L^{-1}) (black line – and mention
729 error bars) compared with long-term measured epilimnetic P concentrations in the study lakes
730 (circle; annual values) and abundance of fossil *Daphnia* per gramme of dry sediment.
731
732 Figure 4: Distribution of the lakes of the calibration dataset in terms of their trophic state.