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Marked seasonality and high spatial variability of protist communities in shallow freshwater systems

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Small eukaryotes have key roles in aquatic ecosystems, influencing their local environment, global biogeochemical cycles and climate. Their impact depends on community structure, which varies along time. However, very few studies take into account temporal variation. This is especially true for small, shallow freshwater systems, which remain largely understudied despite their wide variety, global surface and intense microbial activity. We have monthly followed changes in the community structure of small microbial eukaryotes (0.2–5 µm cell diameter) for 2 years in four ponds and one brook located in North-Western France based on massive 18S rDNA amplicon 454 pyrosequencing. We detected a total of 3742 stringently defined operational taxonomic units (OTUs) encompassing all recognized eukaryotic supergroups and lineages of uncertain affiliation. Although geographically close, protist communities in the five ecosystems were contrasting, with very few shared OTUs, suggesting that environmental selection mainly drives community structure. The temporal dynamics of different high-rank taxa appeared complex and rapid at monthly scales. Despite this, a clear and reproducible seasonality was observed. As expected, low-abundance OTUs dominated the community. Although some of them appeared sporadically or remained at low frequencies during the survey, others occasionally reached relatively high abundances, sometimes recurrently. This shows that at least a fraction of low-abundance eukaryotes constitutes a seed bank. The annual proportion of primary producers, free-living heterotrophs and parasites appeared remarkably constant among the different ecosystems, suggesting underlying trends of ecosystem carrying capacity for these functional groups.
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Introduction

Microbial eukaryotes are versatile components of aquatic environments. They cover multiple roles in these ecosystems, from autotrophy (photosynthesizers) to heterotrophy (predators, decomposers, parasites), or mixotrophy (Zubkov and Tarran, 2008). Protists contribute to biogeochemical cycling, having special impact on global silica and carbon cycles (Li, 1994; Cloern, 1996; Jordan and Chamberlain, 1997; Jardillier *et al.*, 2010), and influence climate regulation (Simó, 2001). Despite the importance of microbial eukaryotes in the trophic web structure, protist community composition was only explored at very low resolution levels for a long time. Although large phytoplankton and zooplankton morphospecies were identified and counted under the microscope, most small, morphologically difficult-to-distinguish protists were consigned to the 'nanoflagellate' black box. This situation radically changed with the advent of molecular methods based on 18S rDNA

amplicon sequencing, first through cloning and Sanger sequencing (López-García *et al.*, 2001; Moon-van der Staay *et al.*, 2001), and then through direct high-throughput sequencing techniques (Sogin *et al.*, 2006). Such methods have expanded our capacity to describe community structure to an unprecedented resolution level, unveiling how widely diverse protists, specially the smallest ones, are, allowing the discovery of novel lineages (López-García *et al.*, 2001; Massana *et al.*, 2004; Lara *et al.*, 2010; Kim and Harrison, 2011) and setting the ground to test biogeography hypotheses (Martiny *et al.*, 2006).

Despite the progress achieved by molecular methods, comparative diversity and biogeography studies most often ignore time scales, leading to potentially biased conclusions (Nolte *et al.*, 2010). Yet, that microbial communities change through time has been known ever since Leeuwenhoek observed the succession and abundance shifts of 'animalcules' in a pepper infusion (Dobell, 1932) and phytoplankton blooms were regularly recorded (Giovannoni and Vergin, 2012). Changes in community composition have been observed at all time scales, within days (Bamforth, 1958; Mangot *et al.*, 2012), weeks (Vigil *et al.*, 2009; Nolte *et al.*, 2010) or months, that is, at annual frequency (Bamforth, 1958; Reid *et al.*, 1990;

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Hitchman and Jones, 2000; Lara *et al.*, 2011). Both abiotic (local rainfall, wind and solar irradiation, pH, temperature and nutrients) and biotic (predation, viral lysis, prey availability) parameters are thought to influence protist community dynamics (Kalff and Knoechel, 1978; Carrias *et al.*, 1998; Lepère *et al.*, 2006; Zhao *et al.*, 2011). However, few detailed, molecular surveys spanning long (>1 year) periods exist, and the parameters considered in these studies explain only very partially the community dynamics (Lepère *et al.*, 2006; Kim *et al.*, 2014). Understanding how environmental parameters influence seasonal variability and shape community structures at pluriannual scale is not only important for comprehensive diversity and biogeography studies but also is crucial to predict the evolution of community compositions on the long run, especially under climate-change scenarios.

Although much less studied than lakes (Richards *et al.*, 2005; Lefranc *et al.*, 2005; Zinger *et al.*, 2012), small, shallow, sometimes ephemeral, freshwater ecosystems (for example, ponds, brooks) appear excellent models to undertake this task for a variety of reasons. They are numerous, so that they cover at least the same surface as larger freshwater bodies (Downing *et al.*, 2006; Downing, 2010). They are diverse, largely because of the influence of benthic and littoral zones (Søndergaard *et al.*, 2005). Because of their limited water volume, they have little buffering capacity, being much more responsive to physicochemical changes (for example, meteorological fluctuations in temperature or solar radiation, nutrient, pesticide or fertilizer inputs, sediment resuspension by animals or storms). Ecologically, they seem very active and are thought to have a significant role in CO_2 fixation and, being generally less oxygenated, possibly also in CH_4 release (Downing, 2010). In addition, classical studies of the fauna, animal zooplankton and phytoplankton indicate that the number of species per surface unit is much higher in small water bodies (Downing, 2010). Indeed, available molecular studies suggest that this kind of systems constitute reservoirs of protist diversity (Šlapeta *et al.*, 2005; Lara *et al.*, 2011; Simon *et al.*, 2014). Finally, like lakes, because of their discreetness, they would behave as islands where allopatric speciation could in principle occur, being also suitable systems to test hypotheses on microbial biogeography.

In a recent study, we described highly diverse but very contrasting communities of small ($<5 \mu m$) protists in five shallow freshwater systems (4 ponds, 1 brook) located in a small geographic area (2–9.5 km distance) at the Natural Regional Park of the Chevreuse Valley, France (Simon *et al.*, 2014). Here, we analyze how protist community structure assessed by 454 pyrosequencing of 18S rDNA amplicons varied in these five systems over 2 years of monthly sampling, and apply multivariate statistics to see whether community dynamics correlate with a variety of regional and local environmental parameters measured in parallel. Despite these communities were spatially very different and underwent complex changes, they showed a clear seasonality.

Materials and methods

Sampling and measurement of physicochemical parameters

Samples were collected monthly from April 2011 to April 2013 in four geographically close (2–9.5 km) small shallow ponds and one brook, located at the Natural Regional Park of the Chevreuse Valley (South of Paris, France; http://www.parc-naturel-chevreuse. fr). These systems were characterized by different local environmental conditions (Supplementary Table S1; Simon *et al.*, 2014). Surface water was sampled using sterilized plastic carboys and processed immediately back in the laboratory. Samples were prefiltered through 5-μm pore-size Nucleopore membranes (Whatman, Amersham, UK) and small planktonic cells were collected onto 0.2-μm pore-size Nucleopore membranes. Membranes were immediately stored frozen at $-20\,°C$ until DNA extraction. Water temperature, conductivity, pH and dissolved oxygen concentrations were measured *in situ* using a multiparameter probe (Multi350i; WTW, Weilheim, Germany). The concentrations of dissolved orthophosphate (PO_4^{3-}), organic carbon (DOC), nitrate (NO_3^-), nitrite (NO_2^-) and ammonia (NH_3) in 0.2 μm filtered water were measured on the same day using colorimetric tests (Hach-Lange, Düsseldorf, Germany). Chlorophyll *a* concentration was determined by spectrometry after ethanol pigment extraction from plankton biomass harvested on GF/F filters (Whatman), as described in Simon *et al.* (2014). Rainfall, mean daily radiation, mean air temperature on the week before sampling and mean wind speed measured at 10 m above ground on the sampling day were obtained from MétéoFrance (https://donneespubli ques.meteofrance.fr/). Rainfallwas obtained by averaging the data recorded at the three closest meteorological stations: Choisel (48°41'00''N–2°00'00''E), le Perray (48°41'36''N–1°52'00''E) and Saint Arnoult (48° 34'18''N–1°55'54''E), whereas radiation, temperature and wind speed were measured at station Trappes (48°46'24''N–2°00'30''E).

DNA extraction, amplification and sequencing of 18S rDNA fragments

DNA was extracted using the PowerSoil DNA Extraction Kit (MoBio, Carlsbad, CA, USA) from biomass collected onto 0.2 μm pore-size filters. 18S rDNA fragments of ca. 550 bp, encompassing the V4 hypervariable region, were PCR amplified with primers EK-565F (5′-GCAGTTAAAAAGCTCGTAGT -3′; Simon *et al.*, 2014) and 18S-EUK-1134-R– UnonMet (5′-TTTAAGTTTCAGCCTTGCG-5′) biased against Metazoa (Bower *et al.*, 2004). These primers were tagged with twenty 10 bp MIDs (Molecular IDentifiers) to allow differentiation of amplicons in the pools of 20 samples multiplexed for sequencing.

PCRs were conducted in 25 µl reaction mix containing 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.3 µm primers, 0.3–3 µl eluted DNA and 0.5 U HotStart *Taq*-Platinum polymerase (Invitrogen, Carlsbad, CA, USA). Twenty-five amplification cycles (94 °C for 30 s, 58 °C for 45 s and 72 °C for 90 s) were preceded by a 3-min denaturation step (94 °C) and ended by 10 min at 72 °C for final extension. Five to eleven independent PCR products for each sample were pooled together. Amplicons were then purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). Equivalent amounts of purified amplicons (200–400 ng, depending on pools) for 20 different samples were pooled and pyrosequenced using the 454 GS-FLX Titanium technology from Roche (Beckman Coulter Genomics, Danvers, MA, USA).

454 Pyrosequence analysis

We obtained a total of 1 511 901 reads. To remove potential spurious sequences, several filters were applied using a modified local pipeline (Simon *et al.*, 2014). Short (< 350 bp) or too long reads (> 550 bp), reads containing errors in primers/MIDs or positions with undetermined bases and without the two flanking primer sequences were eliminated. At this stage, forward and reverse reads for each MID were separated and, for clustering purposes only, a single read from 100% identical sequences was retained. Pairwise flowgram alignments were clustered and treated with the PyroNoise program of AmpliconNoise (Quince *et al.*, 2011) to eliminate further PCR and pyrosequencing errors. A total of 1 272 748 reads from all samples were retained (Supplementary Table S2). Forward and reverse reads were assembled for each sample and primers eliminated, before their analysis by the SeqNoise program of AmpliconNoise. Sequences from all samples were then processed together and clustered into operational taxonomic units (OTUs; 98% similarity cutoff) using CD hit (Fu *et al.*, 2012), before the generation of OTU frequency tables for the different samples using previously retained read information. Singletons, that is, OTUs composed of only one read, were eliminated for precaution. The most abundant sequence in each OTU was used as reference and blasted against the PR2 database (Guillou *et al.*, 2013) to assign OTUs to taxonomic groups based on sequence similarity. OTUs affiliated to cryptophyte nucleomorphs were excluded from our analysis. Potential chimerical OTUs were eliminated using a stringent procedure with automated and manual steps as described in Simon *et al.* (2014). A total of 3742 OTUs were retained. Filtered sequences from these OTUs were then attributed to the different samples according to their MIDs.

Statistical analyses

All statistical analyses were conducted with the R software (http://cran.r-project.org) (R Development Core Team, 2013). Diversity and richness indices were determined using the 'Vegan' R-package (Oksanen *et al.*, 2013). Richness was evaluated by rarefaction analyses as the estimated number of OTUs in a random subsample of each sequence library, which is of the same size as the smallest one (Hurlbert, 1971). The Simpson index was calculated as $D' = 1 - \sum_{i=1}^{S} \left(f i^2 \right)$ (Simpson, 1949) and evenness as $e = \left(- \sum_{i=1}^{S} f i \ln(f i) \right) / \ln(S)$ (Pielou, 1966), with S being the observed number of OTUs and f_i the relative frequency of each OTU_i in the sample. Pairwise Bray–Curtis dissimilarities between samples were calculated on the basis of OTU relative frequencies, instead of raw sequence counts to remove bias due to variable sequence numbers per sample, or on OTU frequencies after Wisconsin standardization, to balance rare versus abundant OTU weight (Bray and Curtis, 1957). Boxplots were drawn with notches to compare the distribution of Bray–Curtis dissimilarities or diversity and richness estimators between different classes (Chambers *et al.*, 1983). Non-metric multidimensional scaling ordination analyses were conducted on Bray–Curtis dissimilarities based on square-root-transformed and Wisconsin-standardized OTU percentages, using the 'Vegan' R package. Ellipses grouping various classes were drawn using s.class from the 'Ade4' R package (Dray and Dufour, 2007). Permutational multivariate analyses of variance using distance matrices (Anderson, 2001) were conducted using Bray–Curtis dissimilarities calculated on OTU read percentages, with the Adonis function of the 'Vegan' package. Venn diagrams showing shared OTUs between ecosystems were drawn using the 'gplots' R-package (Bolker *et al.*, 2012) on pooled sequences from monthly samples. To study seasonality, pairwise Bray–Curtis dissimilarities between each sample from the first sampling year and the samples from the same system 1–12 months apart were calculated. Then, the mean and standard errors of Bray–Curtis dissimilarities between samples separated by the same number of months were computed. To compare sample's principal component analyses were computed on their centered and scaled environmental characteristics using the 'Ade4' R-package; missing values were replaced by the average value of the parameter. To distinguish 'not available' from 'very low' values for analysis, nutrient concentrations below kit detection limits were set as the limit value minus a small random number (normal distribution $\mu = 0$; $\sigma = 10^{-6}$). Canonical correspondence analyses (CCA) including the defined environmental parameters and the 37 most commonly abundant OTUs (those detected in at least 25% of samples, that is, 28 samples, and with $\geqslant 0.1\%$ mean relative abundance per sample; Supplementary Table S3) were calculated with 'Ade4' applying Wisconsin standardization. The proportion of community variance explained by environmental parameters was estimated as the ratio of the sum of eigenvalues from the CCA over the one of a CCA conducted on the same standardized OTU proportions (Borcard *et al.*, 1992).

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Results

Temporal variation of environmental parameters
The systems under study, four ponds and one brook, were initially chosen because of their contrasted physicochemical features (Simon *et al.*, 2014). During the sampling period (April 2011–April 2013), these ecosystems experienced large incoming radiation and water temperature variations, as expected in temperate areas (Supplementary Table S1 and Supplementary Figures S1 and S2). The Mare Gabard had the lowest pH (6.2 on average), high chlorophyll *a* and low nitrite concentrations. Saint Robert was characterized by episodic peaks of ammonia in July 2012 and January 2013 and very high chlorophyll *a* concentrations in the summer and autumn. The Etang des Vallées was usually the most oxygenated and had the lowest dissolved orthophosphate concentrations. Two systems underwent desiccation, the Ru Sainte Anne (brook), characterized by low chlorophyll *a* and high conductivity, in August and September 2012, and La Claye, characterized by low nitrite and ammonia, in July–December 2011 and September 2012 (Supplementary Table S1 and Supplementary Figures S1 and S2). Except for temperature and incoming radiation, the rest of the physicochemical parameters did not display apparent patterns of variation.

Overall composition of protist communities
We analyzed the diversity of small eukaryotes (0.2–5 μm cell-size fraction) in 112 water samples collected along 24 months in the five ecosystems based on 18S rDNA amplicon pyrosequencing. We obtained a total of 1 272 748 quality-filtered reads that grouped within 3742 OTUs (Supplementary Table S2), among which only two OTUs (represented by 164 and 145 quality-filtered reads) were affiliated to metazoans. Most OTUs (93.1%) occurred at low abundance (<0.05% reads per sample, on average). Only 26 OTUs (0.7% total) were relatively abundant (>0.5% reads per sample) making, on average, 51.4% reads per sample.

The phylogenetic diversity of OTUs covered all currently recognized supergroups (Adl *et al.*, 2012), Opisthokonta, Amoebozoa, Excavata, Archaeplastida, SAR (Stramenopiles–Alveolata–Rhizaria) and taxa of uncertain position in the eukaryotic tree (Cryptophyta, Centroheliozoa, Telonemida, Haptophyta, Katablepharida, Ancyromonadida and Apusozoa). In all, 10.6% of the OTUs could not be assigned to specific taxa based on sequence similarity (on average, 2.3% reads per sample). Stramenopiles, alveolates, cryptophytes, opisthokonts and archaeplastids were the most abundant groups (Figure 1). In addition to being abundant (up 35.1% reads per sample), stramenopiles were diverse, encompassing 956 OTUs (25.5% of all OTUs). Most of them affiliated to Chrysophyceae–Synurophyceae, the most abundant stramenopile group (~20.0% reads per sample). They were followed by Bacillariophyceae, Raphidophyceae, Dictyochophyceae,

Bicosoecida and Oomyceta (>1% reads per sample, on average) and other, less abundant groups, such as MAST lineages, Labyrinthulida or Xanthophyceae (Supplementary Figure S3C). Alveolates were the second most abundant supergroup, representing 20.9% reads per sample on average, and included members of the Perkinsea, Colpodellida, Dinophyta and Apicomplexa, although ciliates dominated both in terms of abundance (18.2% reads per sample, on average) and diversity (10.8% of all OTUs) (Figure 1 and Supplementary Figure S3C). The parasitic Perkinsea were occasionally highly abundant, reaching up to 32.4% of the reads in Etang des Vallées in June 2012 (Figure 1). Cryptophytes were as abundant as Alveolates (~20.8% reads per sample) but were little diverse (only 1.4% of OTUs).

Opisthokonts represented 9.9% of the reads per sample, on average, and were largely dominated by fungi (8.8% reads per sample, on average), especially chytridiomycetes, ascomycetes and basidiomycetes (Supplementary Figure S3C). Cryptomycota/Rozellids, choanoflagellates and ichthyosporeans were less abundant (<0.6 %), but occasionally reached higher proportions, for example, cryptomycota (5.7% reads in Mare Gabard, January 2013) or choanoflagellates (6.1% reads in Etang des Vallées, July 2012) (Figure 1). Archaeplastida, especially chlorophytes, were detected in relative high proportion (Supplementary Figure S3C). Several OTUs of streptophytes, rhodophytes and glaucophytes were occasionally recorded (Figure 1). Only OTU-2122 belonged to the rarely detected glaucophytes; it affiliated to *Cyanophora paradoxa* (99.6% sequence identity), and was detected twice at low relative proportion in Saint Robert. Rhizarians and katablepharids were detected in rather low proportion (ca. 2%), although the latter were always more relatively abundant in Etang des Vallées (4.8% reads on average) (Figure 1) and occasionally peaked at high proportions elsewhere (for example, 52.5% reads in Saint Robert, March 2013) (Figure 1). Rhizarians were only represented by the fairly diverse cercozoans (7.7% OTUs). Excavata were rather rare in general (Supplementary Figure S3C), with the exception of OTU-1147, affiliated to *Trimastix pyriformis* (98.6% sequence identity), which represented 6.5% of the reads in the Ru Sainte Anne in July 2011. Amoebozoan, haptophytes, centroheliozoans, telonemids, apusomonads and ancyromonads were also detected, although at lower diversity and abundances (Supplementary Figure S3C). Centroheliozoans could only be detected once in Saint Robert and La Claye and twice in the Ru Sainte Anne. Similarly, some OTUs affiliated to Conosa, Excavata, Rhodophyta and Apicomplexa were rarely detected (fewer than 10 samples).

Contrasted communities in the different ecosystems
Considered globally, protist communities strongly differed between the five shallow systems, confirming previous observations at a single time point
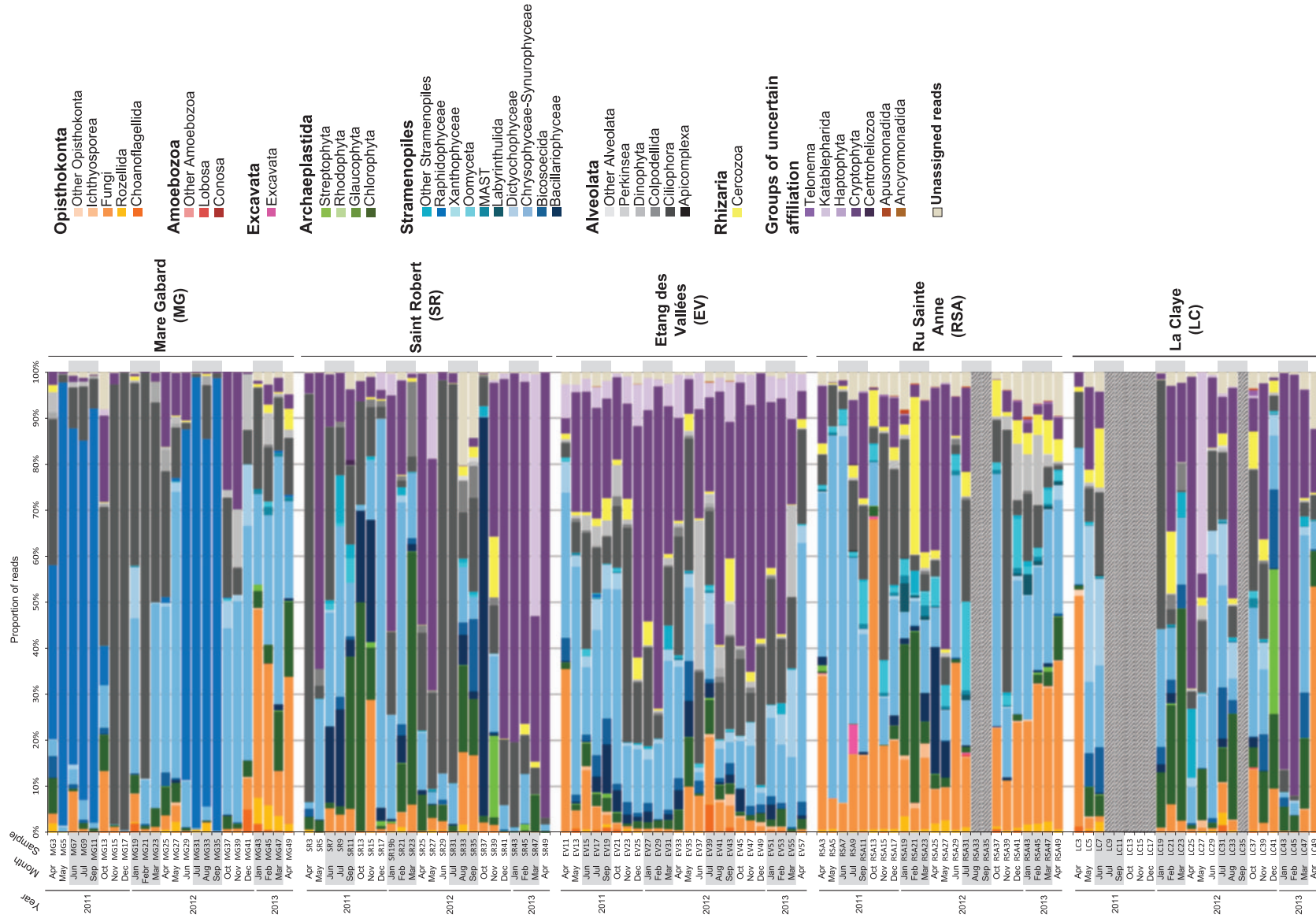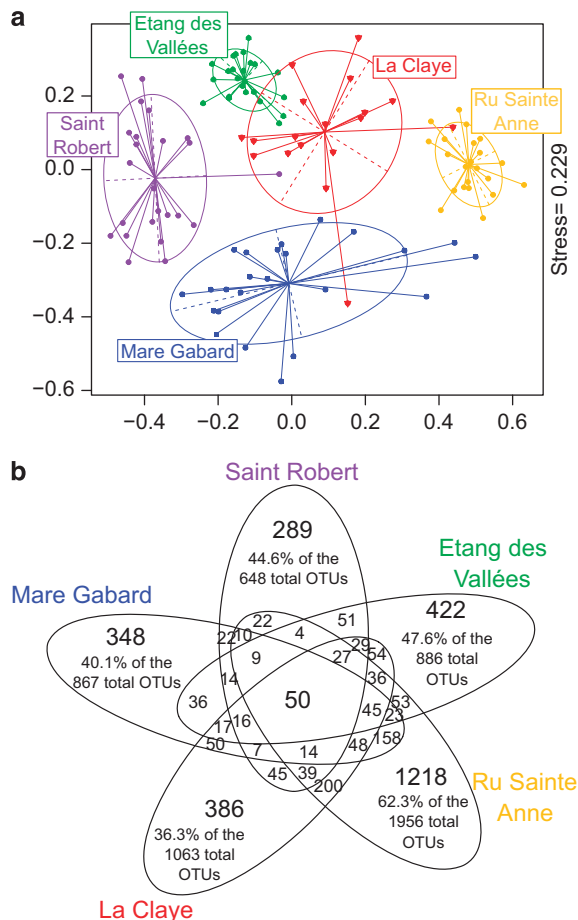
Figure 1 Histograms showing the relative abundance of 18S rDNA amplicon reads assigned to high-rank taxa, in all samples from the five ecosystems over the 2-years survey. Hatched bars correspond to missing data because of the drought periods: the Ru Sainte Anne was dried in August and September 2012, and La Claye from the end of July to December 2011 and in September 2012.

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(Simon *et al.*, 2014). Only 50 out of the 3742 OTUs recorded during the 24-month sampling were shared by the five ecosystems (Figure 2a). They were mostly fungi within opisthokonts, chlorophytes within archaeplastids, ciliates within alveolates, chryso-phytes within stramenopiles, cryptophytes, one cercozoan and one katablepharid. They were neither continuously abundant nor simultaneously present in all ecosystems (for example, ciliate OTU-356; Figure 3). From the 2663 OTUs (>71% of all OTUs) specific to one ecosystem, 1180 OTUs (31.5%) were detected at least twice. Remarkably, 62.3% of the OTUs retrieved in the Ru Sainte Anne were not detected elsewhere. Some OTUs reached high abundances in one system without being detected in their closest neighbor; for instance, the Raphido-phyceae OTU-15 in Mare Gabard (Figure 3).

The five ecosystems differed in richness, diversity and evenness. Richness was much higher in the brook than the ponds, while Mare Gabard and Saint Robert had the lowest richness (on average, 50.3 and

a

Stress= 0.229

b

Figure 2 Distinct communities in the five freshwater ecosystems. (**a**) Non-metric multidimensional scaling plot, built on square-root-transformed and Wisconsinstandardized Bray–Curtis dis-similarities between all samples. (**b**) Venn diagram showing the number of OTUs shared by several ecosystems or specific to an ecosystem. Proportions of OTUs specific to each ecosystem among all OTUs detected in this ecosystem are indicated. All samples from the 2-year survey are pooled for each ecosystem.

38.0 OTUs per sample, respectively) (Supplementary Figures S4 and S5). The same trend was observed for diversity and evenness estimates (Supplementary Figure S4).

Intersystem differences were clearly seen in non-metric multidimensional scaling analyses based on Wisconsin-standardized OTU frequencies (Figure 2b). More than 20% of the total variation between samples was owing to the variance between ecosystems, as estimated by permutational multivariate analyses of variance using a Bray–Curtis distance matrix (Table 1). Etang des Vallées and La Claye seemed to host the most similar communities. They harbored, along with Saint Robert, the densest cryptophyte communities. Mare Gabard was dominated by stra-menopiles (especially Raphidophyceae) and ciliates, and the Ru Sainte Anne by Chrysophyceae–Synur-ophyceae and fungi (Figure 1). Differences between ecosystems seemed to be due to the less frequent OTUs more than to the most abundant OTUs. Indeed, when frequencies for the different OTUs were not Wisconsin-standardized before non-metric multidi-mensional scaling (i.e. when the more an OTU is abundant in at least one sample, the more it influences sample cross-comparison), samples were no longer clustered per ecosystem (data not shown).

Overall temporal dynamics of protist communities
Within ecosystems, protist communities were highly dynamic, as shown by mean Bray–Curtis dissimila-rities between all pairs of samples per ecosystem, ranging from 0.71 (Etang des Vallées) to 0.89 (Saint Robert) (Supplementary Figure S6A). The dissimila-rities reached higher values after a Wisconsin standardization of OTU frequencies (0.88 for Etang des Vallées; 0.96 for Mare Gabard and Saint Robert; Supplementary Figure S6B), suggesting that infre-quent OTUs varied more in abundance than frequent OTUs. This observed change in community structure over the 2 years was already suggested by the strong variations in richness observed, for example, more than 10 times in Mare Gabard (Supplementary Figure S4). The most stable protist community over time seemed that of the Etang des Vallées, showing the smallest Bray–Curtis distances between all pairs of samples (Supplementary Figure S6).

Despite changes in community composition with time, eukaryotic communities in each system did not differ importantly between the 2 years of the survey. A maximum of 9.8% of the total variance was recorded between interannual samples (recorded in Mare Gabard; Table 1). A clear seasonal pattern was observed in each ecosystem (Figure 4). Pairwise Bray–Curtis dissimilarities were highest between samples separated by 5 to 6 months, that is, samples from opposite seasons, and lowest between samples separated by 12 months or contiguous samples, that is, samples from the same season (Figure 4). This pattern was less smooth for La Claye likely because of missing data because of the 6 months of drought.

Table 1 Variance partitioning between classes (Adonis)

Classes / Samples	Ecosystems		Seasons		Sampling years		Ecosystems ~ Seasons		Ecosystems ~ Sampling years		Sampling years ~ Seasons	
	$F.Model^a$	$R2_2^b$	$F.Model^a$	$R2_2^b$	$F.Model^a$	$R2_2^b$	$F.Model^a$	$R2_2^b$	$F.Model^a$	$R2_2^b$	$F.Model^a$	$R2_2^b$
All	8.3601	0.20631	2.5939	0.04738	2.0872	0.01271	2.1275	0.15545	1.9274	0.04694	1.2306	0.02246
Gabard pond			4.4853	0.35753	3.773	0.09807					1.6486	0.41586
Saint Robert pond			1.6863	0.19161	2.4289	0.092					0.9714	0.11038
Etang des Vallées			2.19928	0.24628	1.2112	0.04521					0.99371	0.11128
Sainte Anne brook			1.9582	0.23905	1.2828	0.0522					1.1392	0.13907
La Claye pond			1.5829	0.24404	1.1955	0.06658					1.2073	0.12408

Interactions between classes are indicated by 'class A~class B'.

$^aF.Model = \dfrac{Sum\ of\ squares\ of\ distances\ between\ classes\ /\ degrees\ of\ freedom}{Sum\ of\ squares\ of\ distances\ inside\ classes\ /\ degrees\ of\ freedom}$

$^bR^2 = 1 - \dfrac{Sum\ of\ squares\ of\ distances\ inside\ classes}{Sum\ of\ squares\ of\ distances\ between\ all\ sample\ pairs}$

Although seasonality was visible in all systems, its intensity varied for each ecosystem as attested by the proportion of community composition variance corresponding to variations between seasons ranging from 19.2% (Saint Robert) to 35.8% (Mare Gabard) (Table 1), and between seasons. Eukaryotic communities differed more between summer and winter than between autumn and spring, as revealed by non-metric multidimensional scaling analyses (Supplementary Figure S7).

Temporal variation of specific protist taxa
The relative proportions of diverse taxonomic groups fluctuated greatly and rapidly (as seen at a monthly scale) over the 2 years (Figure 1 and Supplementary Figure S8), most often because of changes in a few OTUs, which occasionally shifted proportions > 100 times in two consecutive months (for example, cryptophytes in La Claye in December 2012 and January 2013; Supplementary Figure S8). Globally, taxonomic groups were rarely detected at high relative proportions in more than two consecutive months. Successive peaks of relative abundances were either due to the rapid dynamics of the same OTUs (for example, katablepharids in Etang des Vallées) or to raises in proportions of distinct OTUs (for example, bicosoecids in Saint Robert). Low-frequency groups could occasionally peak at noticeable frequency. This was the case of excavates (OTU-1147) in Ru Sainte Anne, September 2011 (Figure 1 and Supplementary Figure S8).

To test whether environmental conditions affected the dynamics of the most abundant protists in our ecosystems, we carried out statistical analyses to see potential correlations between the 37 widespread OTUs representing at least 0.1% reads per sample, on average, and occurring in at least 25% samples (Supplementary Table S3) and various physicochemical parameters (our local measurements and meteorological data

recorded at the closest meteorological stations; see Materials and methods). Physicochemical data-only and both physicochemical plus climatologic parameters explained 18.7% and 21.9% of the OTU's variance, respectively. As shown by CCA, fungi positively correlated with conductivity, whereas ciliates showed a negative correlation (Figure 5). Chlorophyte frequency seemed to correlate with chlorophyll *a* (as expected) and phosphate. Cryptophytes seemed most abundant when dissolved oxygen and nitrite were higher, but their association with environmental variables was less clear.

At finer scales, individual OTUs showed variable patterns of temporal change. Very few OTUs were continuously recorded during the survey: 1, 0, 2, 1 and 7 OTUs in, respectively, Mare Gabard, Saint Robert, Ru Sainte Anne, La Claye and Etang des Vallées (for example, OTUs 55 and 356; Figure 3). Within low-frequency OTUs, two types of patterns were observed. In one pattern, displayed by 275 OTUs (7.3% total), OTUs always represented < 0.05% reads per sample, although they were detected in at least two samples of the whole survey, sometimes reproducibly in consecutive years (for example, OTU-2986, affiliated to stramenopiles; Figure 3). Approximately 23% of these OTUS were fungi. The second pattern corresponded to several OTUs at low abundance most of the time, but being able to occasionally reach high relative proportions (for example, OTU-369; Figure 3). Thus, 47 OTUs (fungi, stramenopiles, cercozoans, chlorophytes, alveolates, excavates and centroheliozoans) represented > 0.5% reads in one sample and were not detected elsewhere. Among the most abundant and frequent OTUs, some presented a marked seasonality. In particular, OTU 15, corresponding to the flagellate algae *Gonyostomum semen* (Raphidophyceae), bloomed repeatedly and persistently in summertime in the Mare Gabard, reaching up to 98% reads in September 2012 (Figure 3). Also, a few OTUs

remained abundant during the 2-year survey without apparent seasonal pattern: 3, 4, 6, 5 and 15 OTUs represented >0.5% reads in at least half of the samples in Mare Gabard, Saint Robert, Ru Sainte Anne, La Claye and Etang des Vallées (for example, OTU-55, Figure 3), respectively. These OTUs were affiliated to chrysophytes and fungi in Sainte Anne, and mainly to ciliates, cryptophytes and stramenopiles elsewhere. Finally, a small set of OTUs that were always abundantly detected in some systems were little abundant or episodic in other environments, for example, katablepharid OTU-55 (Figure 3).

Temporal dynamics of functional groups
To get an insight on temporal variation of major protist functions, we tentatively and roughly classified the detected OTUs in three major functional groups on the basis of their taxonomic affiliation: primary producers, free-living heterotrophs and parasites. Primary producers comprised obligate phototrophs (for example, chlorophytes) and mixotrophs (for example, cryptophytes), whereas free-living heterotrophs included phagotrophic predators (for example, ciliates, choanoflagellates) or osmotrophs (mostly fungi). Parasites included Perkinsea (Mangot *et al.*, 2010), LKM11-Rozellida-Cryptomycota, Ichthyosporea, Oomycota and Apicomplexa, often observed in freshwater systems (Richards *et al.*, 2005; Lepère *et al.*, 2008; Lara *et al.*, 2010; Jones *et al.*, 2011; Triadó-Margarit and Casamayor, 2012; Taib *et al.*, 2013) (see in Supplementary Figure S9 lineages included in each category). The relative proportions of the three groups varied differently among ecosystems. In Mare Gabard, primary producers were largely dominant in summer (mostly due to *Goniostomum semen* blooms) and heterotrophs in winter, whereas in other systems primary producers tended to dominate in spring (Supplementary Figure S9). Parasites had generally low relative proportions in Saint Robert, La Claye and Mare Gabard, with the exception of November 2012–February 2013 in Mare Gabard where Perkinsea and Cryptomycota-Rozellida were recorded at significant proportions. Perkinsids reached important proportions in the Etang des Vallées, whereas Oomycota, Perkinsea and Cryptomycota/Rozellids were non-negligible components of Ru Sainte Anne communities.

Interestingly, even if the relative proportions of the three categories varied differently, the integrated annual relative proportions of producers, free-living heterotrophs and parasites were remarkably similar between the five ecosystems, especially the ponds (Figure 6). Only the Ru Sainte Anne deviated somewhat from that proportionality, having a lower proportion of primary producers and a higher proportion of free-heterotrophs as compared with the other systems.
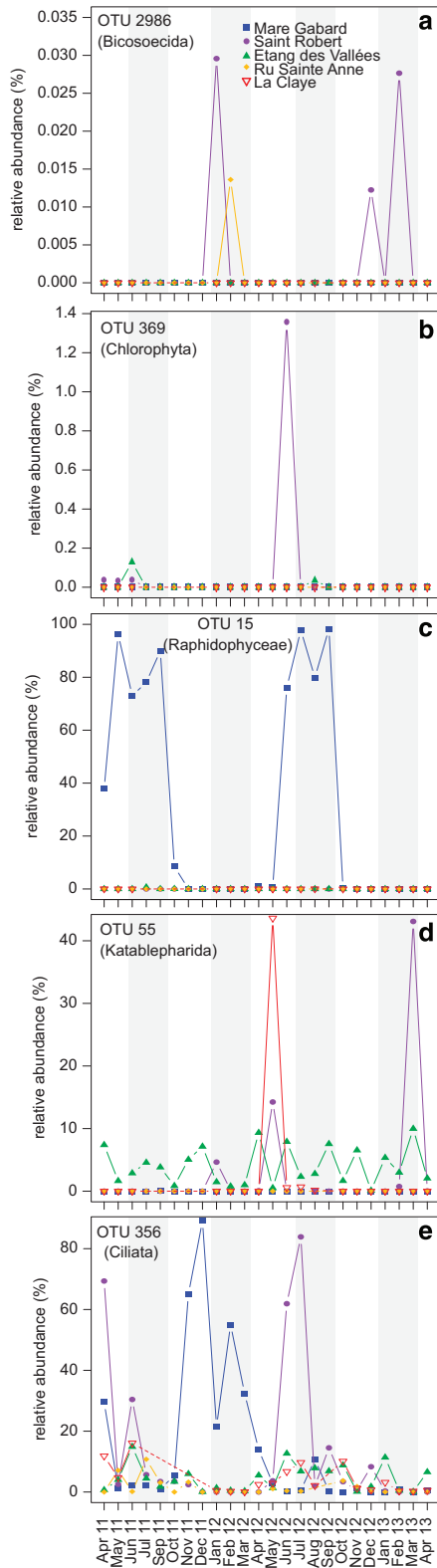
Discussion

Integrating time scales extends our perception of protist biodiversity
The diversity of small protists in the five shallow ecosystems studied over 24 months was large, including members of all eukaryotic supergroups and phyla of uncertain position (Figure 1). Stramenopiles, alveolates and cryptophytes generally dominated; green algae and fungi+cryptomycota were also common, as often observed in other freshwater systems (Lefranc *et al.*, 2005; Richards *et al.*, 2005; Šlapeta *et al.*, 2005; Lepère *et al.*, 2008; Mangot *et al.*, 2012; Triadó-Margarit and Casamayor, 2012; Taib *et al.*, 2013). We detected a total of 3742 stringently defined OTUs, which represent a number 6 to 11 times higher than that of OTUs observed at any discrete date (for example, 812 OTUs in April 2012; Simon *et al.*, 2014). Every month, previously unseen phylotypes were identified and saturation was not approached even after 24 months, especially for Ru Saint Anne, as the species accumulation curves appeared to be leveling off for the ponds but not the stream (Supplementary Figure S5).
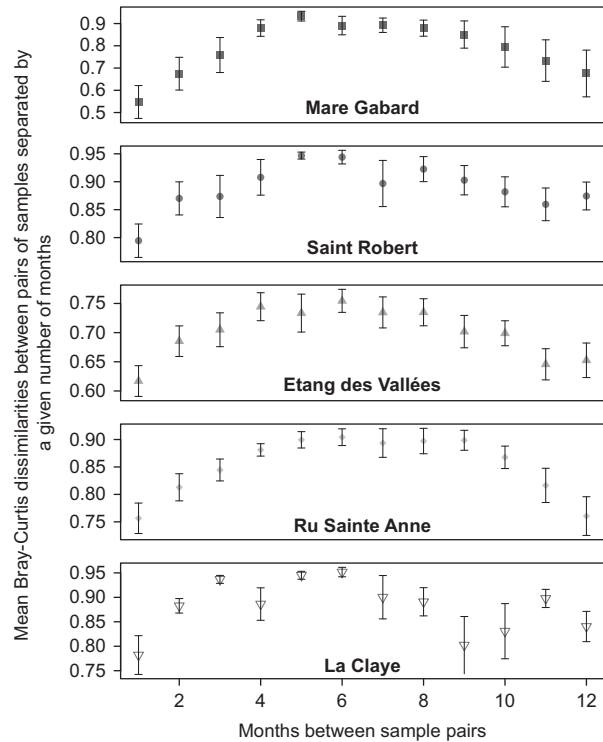
This not only supports previous observations suggesting that small freshwater systems, perhaps more than larger lakes, are reservoirs of protist diversity (Šlapeta *et al.*, 2005; Downing *et al.*, 2006; Downing, 2010; Simon *et al.*, 2014) but, critically, highlights the importance of including temporal scales in biodiversity and microbial ecology studies. The impact of doing so is manifold. Obviously, as temporal surveys allow detecting a wider diversity than time-point studies (Nolte *et al.*, 2010), this will influence comparative diversity analyses and conclusions about lineages specific to given habitats. For instance, in April 2012 we detected in these freshwater systems a few OTUs belonging to the 'typically marine' stramenopile lineages MAST-2, MAST-12, MAST-3 and MAST-6 (Massana *et al.*, 2014; Simon *et al.*, 2014). If we include data from the 2-year survey, a total of 52 OTUs branched within these lineages (or at the base of some of them), reinforcing the idea that they are not ocean-specific (Supplementary Figure S10).

As for comparative diversity studies, including time scales might significantly affect conclusions about biogeography. Unfortunately, most studies for marine and large freshwater systems still include punctual samples taken at disparate time points. In our case, the fact that our five physically close (2–9.5 km) freshwater ecosystems remained distinct over the two years (Figure 2) strongly argues in favor of environmental factors but not geographic distance as major community drivers, strengthening previous conclusions (Simon *et al*, 2014). Another hint pointing in that direction is that eukaryotic communities differed more between summer and winter than between autumn and spring (Supplementary Figure S7). This could be easily linked to seasonal variations of physico-chemical parameters, more

Figure 4 Pairwise Bray–Curtis dissimilarities between samples separated from 1 to 12 months. Each point represents the mean of pairwise Bray–Curtis dissimilarities between samples 1 to 12 months apart, plotted with standard errors. All means are based on 12 dissimilarities except for Ru Sainte Anne and La Claye pond because of missing data due to drought periods. The color reproduction of this figure is available on the *ISME Journal* journal online

Figure 3 Relative abundance of five OTUs showing different types of dynamics, in samples from the five ecosystems, over the 2-year survey. Each color corresponds to the relative abundance (proportion of reads) dynamics in one of the sampled systems (see legend in Figure 1). The name and taxonomic affiliations of OTUs are indicated at each panel. Ru Sainte Anne dried in August and September 2012, and La Claye pond from the end of July to December 2011 and in September 2012. Missing data are indicated by dashed lines.

similar in spring-autumn (Supplementary Figure S2). At a finer level, we could show some correlations between some taxonomic groups and measured environmental parameters (Figure 5). The clearest association was observed between fungi and high conductivity, which could result from their osmotrophic nutrition or from higher sediment input in waters. Cryptophytes correlated with oxygenated waters, as previously observed in ponds of different oxic levels (Šlapeta *et al.*, 2005). However, our set of environmental parameters explained only a fifth (22%) of the variation of the 37 most common OTUs tested. The detection of only few clear correlations may result from the lack of other influencing abiotic parameters but, most likely, biotic parameters, including predatory, mutualistic and/or parasitic interactions with prokaryotes, other eukaryotes (protists or metazoans) or viruses.

Finally, including temporal scales in diversity analyses also demonstrates that a perceptible component of the so-called 'rare biosphere' corresponds to organisms that experience temporal variation as a consequence of their lifestyle and/or that constitute a sort of 'seed bank' ready to respond to environmental cues (Pedrós-Alió, 2006; Caron and Countway, 2009; Lennon and Jones, 2011). Thus, even if in our systems most OTUs remained in low frequency

during the 2 years, over 300 low-frequency OTUs peaked to noticeable and even high abundances at least once or twice (for example, Figure 3). Longer surveys (and at varying temporal frequency) are expected to reveal even higher proportions of non-rare organisms whose abundance fluctuates over time.

### Marked seasonality despite complex patterns of temporal dynamics

Eukaryotic communities showed seasonal patterns, and were clearly differentiated in winter and summer (Table 1 and Supplementary Figure S7). However, the most striking observation of our study was that the seasonal pattern repeated during the second year in the five ecosystems (Figure 4) despite the fact that (i) the meteorology during the two was very different (2012 was hotter and drier), (ii) new OTUs were observed at each sampling point (Supplementary Figure S5) and (iii) that the patterns of variation of the different taxa, either at the level of phyla or OTUs, seemed generally complex and difficult to interpret (Figure 3 and Supplementary Figure S8). This implies that, despite observable differences in protist diversity, the overall community structure converges in similar periods in subsequent years, once again arguing in favor of an important role of environmental selection. Our results contrast with

those carried out in some lakes, where community patterns did not appear to repeat over consecutive years (Lepère et al., 2006). This is surprising given that small freshwater systems (Downing et al., 2006; Céréghino et al., 2008; Downing, 2010) are much more variable than larger water bodies.

Deciphering seasonal dynamics at finer levels seems difficult at this stage and will require additional sampling and analysis. Seasonality has been observed for various microbial eukaryotes (for example, Bamforth, 1958; Hitchman and Jones, 2000; Lara et al., 2011). In our case, despite the large data set, a clear seasonality for most of the high-rank taxonomic groups was not observed (Figure 1 and Supplementary Figure S8). Some groups (for example, choanoflagellates, labyrinthulids, dictyochophytes) exhibited seasonal-like dynamics, but not necessarily in several ecosystems, and the peaks were sustained by only one or a small set of OTUs. Indeed, as observed previously (Nolte et al., 2010), community temporal variability was not only due to relative abundance changes in frequently detected OTUs but also to the appearance and disappearance of many phylotypes (Figure 3). Whether the recurrent observation of new OTUs (Supplementary Figure S5) is due to immigration of foreign microbes or to perennial low-abundance
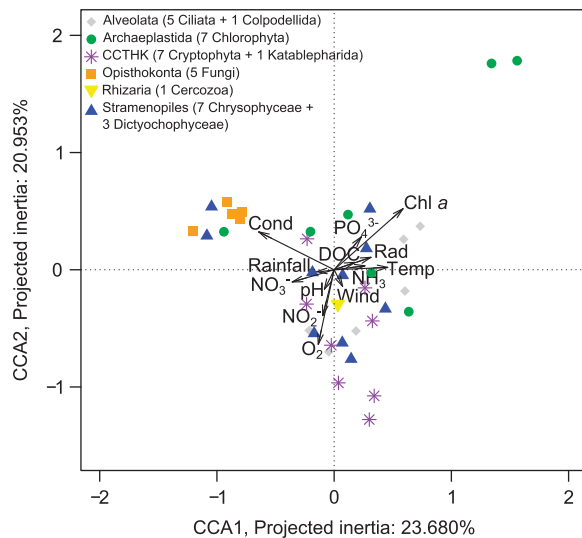
Figure 5 CCA plot. CCA was conducted on all samples and on the 37 OTUs detected in at least 25% of samples and having a mean relative abundance per sample of 0.1% or above, after a Wisconsin standardization. Both physicochemical parameters measured in the ecosystems' water and weather information were included in the analysis. Dots represent OTUs, with color and form indicating their taxonomic affiliation. CCTHK, Cryptophyta, Centroheliozoa, Telonema, Haptophyta, Katablepharida; Chl a, chlorophyll a; Cond, conductivity; DOC, dissolved organic carbon; Rad, mean daily solar radiation of the week before sampling; Rain, rainfall during the week before sampling; Temp, water temperature; Wind, mean wind speed at 10 m high during the sampling day.
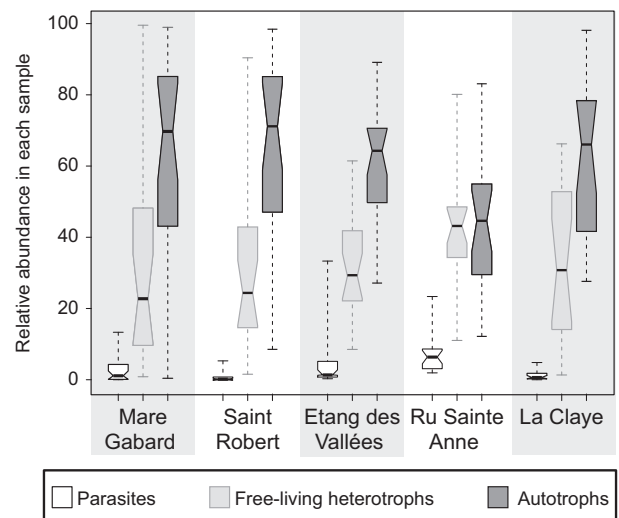
Figure 6 Distribution of relative abundances of reads affiliated to potential parasites, other heterotrophs and primary producers in all samples from the five ecosystems. The thickest line inside each box represents the median of the distribution, bottom and top borders of boxes correspond to the first and third quartiles and whiskers extend to minimal and maximal values. Notches are drawn to indicate whether medians of the three distributions can be considered as different for each ecosystem (Chambers et al., 1983, p. 62). Putative parasites: Apicomplexa, Rozellida, Oomyceta, Perkinsea, Ichthyosporea; potential other heterotrophs: Ancyromonadida, Apusomonadida, Bicosoecida, Centroheliozoa, Cercozoa, Choanoflagellida, Ciliophora, Colpodellida, Conosa, Discosea, Fungi, Katablepharida, Labyrinthulida, Lobosa, Malawimonadida, MAST, Metamonada, Telonema; putative primary producers: Bacillariophyceae, Chlorophyta, Chrysophyceae–Synurophyceae, Cryptophyta, Dictyochophyceae, Dinophyta, Glaucophyta, Haptophyta, Rhodophyta, Streptophyta, Xanthophyceae.

eukaryotes occasionally observed above detection limits remains unsettled. As mentioned above, several OTUs seem to correspond to this category, being detected more than once along the survey. However, immigration may be also an important component. Although the atmospheric transport of microbes in lakes may not be significant (Jones and McMahon, 2009), migration may be facilitated by animals (here essentially deer, wild boars and a variety of small mammals, birds and amphibians), especially at few kilometer scales. Animals also stir bottom sediment and surrounding soils, further contributing to the input of foreign microbes. Immigration may be particularly important in the Ru Sainte Anne, the brook, which harbors the highest diversity (Table 1 and Supplementary Figures S4 and S5), as local communities are connected to those in upstream locations via the continuous water flow.

Insight into the ecology of small freshwater bodies
In addition to repeated seasonal patterns of small eukaryotes observed in the ponds and brook, and despite the apparent complexity of individual taxon dynamics, other large-scale ecological indicators seem to emanate from our study. Analyzed collectively, some of them inform about, or correlate with, the trophic status of the different systems. For instance, the ponds Mare Gabard and Saint Robert have the lowest richness (and diversity estimates, although they are relatively high; Supplementary Table S1 and Supplementary Figure S4). However, protist diversity at Mare Gabard seems to be far from saturation, although that of Saint Robert seems to approach an asymptote (Supplementary Figure S5). This would be in agreement with Mare Gabard being a pristine forest pond, potentially more diverse, than Saint Robert, a highly eutrophic village pond highly impacted by agricultural and other associated human activities (Simon *et al*, 2014). On the opposite extreme, the Ru Sainte Anne and the Etang des Vallées have the largest richness and diversity indices, La Claye holding an intermediate position (Supplementary Table S1 and Supplementary Figure S4). However, while protist diversity at the Etang des Vallées seems to approach saturation, Ru Sainte Anne exhibits an exponential trend of OTU increase (Supplementary Figure S5). This may be partly explained by immigration being more abundant in the Ru Saint Anne. However, this seems also to imply that the Etang des Vallées, being the biggest, is the most stable of the five systems analyzed; its higher buffering capacity would explain a more longstanding diversity. Indeed, Bray–Curtis distances between sample pairs in the Etang des Vallées are significantly smaller than in the rest of ecosystems (Supplementary Figure S6).

The other interesting trend observed correspond to the repartition of functional categories (primary producers such as chlorophytes or cryptophytes,

free-living heterotrophs, parasites) through time. Interestingly, even if the relative proportions of the three categories varied differently along the survey (Supplementary Figure S9), annual relative proportions of producers, free-living heterotrophs and parasites were highly similar in the four ponds (Figure 6). The only exception was the Ru Sainte Anne, which, comparatively, had remarkable lower proportions of primary producers and higher proportions of free heterotrophs, suggesting that the food-web structure may be different in brooks. This might be explained both by a predominance of benthic primary production in a system characterized by running waters and by the presence of higher fungal abundances (osmotrophy) versus predators (phagotrophy), the latter dominating in the ponds. A higher osmotroph abundance might potentially be linked to incoming resources from runoff. Differences in the annual relative proportions of primary producers, free-living heterotrophs and parasites are thus suggestive of a fundamentally different food-web structure between lotic and lentic ecosystems, and would confirm differences previously observed at the bacterioplankton level (Portillo *et al.*, 2012). In all systems, parasites were at comparable relative low levels, between 2 and 7% on average, with very rare peaks over 10%, suggesting that this is the maximal parasite load that freshwater aquatic ecosystems can stably tolerate.

In summary, our study highlights the importance of analyzing temporal series for a comprehensive perception of microbial biodiversity, biogeography and ecology. Establishing microbial community pattern regularities and understanding their underlying causes, including functional constraints, as well as biotic and abiotic environmental determinants, will require considerable effort and constitutes the next important challenge in microbial ecology.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

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Table S1. Characteristics of freshwater systems studied and diversity and richness estimates for the different samples.

Sampled ecosystem	Mare Gabard (MG)	Mare Saint Robert (SR)	Etang des Vallées (EV)	Ru Sainte Anne (RSA)	Mare La Claye (LC)
Ecosystem type	Forest pond	Village pond	Large pond	Forest brook	Pond on peaty soil
GPS coordinates	48°39'15.83"N 1°55'20.26"E	48°39'54.82"N 1°56'45.28"E	48°41'23,0"N 001°54'59,2"E	48°36'45.91"N 1°58'16.61"E	48°36'31.72"N 1°56'17.33"E
Approximate surface (m²)	850 (210 x 75 m)	495 (20 x 28 m)	12 880 (210 x 75 m)	30-100 cm width at sampling point	265 (24 x 10 m)
Approximate depth (cm)	40-50	50-70	100-150	0-25	0-60
Water temperature [2] (°C)	10.8 (1.7-22.3)	11.8 (2.5-24.0)	12.6 (1.6-22.3)	9.3 (0.2-16.0)	10.0 (1.4-19.9)
pH [2]	6.2 (5.3-7.4)	7.2 (6.4-8.0)	7.2 (6.4-7.8)	7.0 (5.8-8.3)	7.1 (5.9-7.7)
Oxygen concentration [2] (%)	43.4 (8.1-78.9)	38.6 (0-102.3)	79.2 (30.4-118.8)	47.2 (0.0-84.1)	55.4 (11.5-91.2)
Conductivity [2] (μS/cm)	62.3 (45-91.4)	472.2 (409.0-577.0)	282.6 (234.0-312.0)	657.3 (0.6-1247.0)	389.3 (251.0-533.0)
Chlorophyll concentration [2] (mg/l)	132.4 (1.6-758.4)	207.8 (6.4-952.2)	21.7 (5.4-59.9)	1.3 (0.2-2.7)	10.3 (1.9-33.9)
Nitrate concentration [2] (mg/l NO_3^-)	1.9 (BL-3.2)	ND	4.2 (BL-7.7)	4.1 (BL-10.7)	2.9 (1.2-9.5)
Ammonia concentration [2] (mg/l NH_3)	0.09 (BL-0.57)	0.63 (BL-2.65)	0.14 (BL-0.34)	0.10 (BL-0.38)	0.07 (BL-0.27)
Nitrite concentration [2,2] (mg/l NO_2^-)	ND	0.043 (BL-0.152)	0.051 (0.011-0.11)	0.030 (BL-0.088)	ND
DOC concentration [2] (mg/l)	47.2 (14.7-183.5)	37.2 (14.6-126.0)	52.3 (9.8-370.0)	40.1 (17.4-143.0)	44.9 (14.9-113.5)
Orthophosphate concentration [2] (mg/l PO_4^{3-})	0.11 (BL-0.27)	0.11 (BL-0.37)	0.05 (BL-0.17)	0.07 (BL-0.28)	0.08 (BL-0.49)
Observed number of OTUs all dates taken together	867	648	886	1956	1063
Observed number of OTUs per sample [2]	81.2 (21-242)	70.5 (26-144)	152.1 (67-240)	257.7 (134-487)	130.0 (42-288)
Richness per sample [1,2]	50.3 (7.3-186.8)	38.0 (10.4-91.2)	90.2 (47.5-142.2)	138.0 (74.8-227.1)	70.6 (24.8-184.8)
Diversity: Simpson Index per sample [2]	0.61 (0.03-0.98)	0.67 (0.27-0.95)	0.90 (0.54-0.98)	0.90 (0.65-0.98)	0.78 (0.18-0.97)
Evenness per sample [2]	0.44 (0.04-0.87)	0.46 (0.17-0.76)	0.67 (0.38-0.81)	0.68 (0.45-0.86)	0.54 (0.14-0.80)

[1] Expected number of OTUs in random subsamples of the size of the smallest sequence library (793 reads in Gabard pond in January 2013).

[2] Mean of values measured over the 2-years survey. Minimal and maximal values are given in brackets.

Mean nutrient concentrations are calculated on values above the method detection limit. Minimum values below limit are indicated as 'BL'. (Detection limit for nitrite: 0.002 mg/l NO_2^--N or 0.006 mg/l NO_2^-, nitrate: 0.23 mg/l NO_3^--N or 1.0 mg/l $NO_3^{-'}$, ammonia: 0.01 mg/l NH_3-N or 0.01 mg/l NH_3, orthophosphate: 0.02 mg/l PO_4^{3-}.)

No mean is calculated when concentrations were below the detection limit in half the water samples or more. Those cases are indicated as 'ND': Not Determined.

No Dissolved Organic Carbon (DOC) concentrations are available for the 5 first months.

Table S2. Names, numbers of reads, OTUs and diversity estimates obtained for the different samples.

Ecosystem	Sample name	Sampling date	Number of reads before filtering	Number of quality-filtered reads	Observed number of OTUs	Richness (Standard error)	Diversity (Simpson index)	Evenness
Mare Gabard	MG3	April11	2936	2028	48	41.56 (2.10)	0.76	0.54
Mare Gabard	MG5	May11	31486	30291	35	10.68 (1.92)	0.07	0.07
Mare Gabard	MG7	June11	12060	11272	50	27.45 (2.45)	0.46	0.33
Mare Gabard	MG9	July11	7870	6923	44	28.652 (2.47)	0.38	0.30
Mare Gabard	MG11	September11	36269	34294	61	19.66 (2.41)	0.19	0.14
Mare Gabard	MG13	October11	14500	12086	93	55.46 (3.40)	0.92	0.68
Mare Gabard	MG15	November11	7087	6697	38	17.91 (2.38)	0.54	0.34
Mare Gabard	MG17	December11	15251	13771	24	9.20 (1.77)	0.20	0.15
Mare Gabard	MG19	January12	11502	9739	183	83.09 (5.14)	0.88	0.56
Mare Gabard	MG21	February12	5093	4781	46	32.85 (2.48)	0.60	0.38
Mare Gabard	MG23	March12	22666	21074	68	27.52 (2.68)	0.75	0.44
Mare Gabard	MG25	April12	15020	13882	92	48.03 (3.31)	0.87	0.58
Mare Gabard	MG27	May12	6923	6222	122	76.60 (4.171)	0.87	0.62
Mare Gabard	MG29	June12	23807	22650	61	25.29 (2.90)	0.40	0.24
Mare Gabard	MG31	July12	13157	12547	21	8.80 (1.82)	0.04	0.05
Mare Gabard	MG33	August12	10165	9587	45	22.29 (2.42)	0.35	0.24
Mare Gabard	MG35	September12	41945	40567	26	7.32 (1.45)	0.03	0.04
Mare Gabard	MG37	October12	6827	6424	58	41.30 (2.65)	0.86	0.62
Mare Gabard	MG39	November12	11747	8343	37	26.23 (2.11)	0.82	0.57
Mare Gabard	MG41	December12	10213	8849	66	41.15 (2.92)	0.82	0.56
Mare Gabard	MG43	January13	1391	793	87	87 (0)	0.96	0.87
Mare Gabard	MG45	February13	4335	2528	237	186.79 (5.26)	0.98	0.86
Mare Gabard	MG47	March13	7360	5683	165	101.62 (4.93)	0.93	0.67
Mare Gabard	MG49	April13	4633	2823	242	179.824 (5.65)	0.96	0.79
Mare Saint Robert	SR3	April11	6386	5278	47	31.16 (2.55)	0.50	0.34
Mare Saint Robert	SR5	May11	16158	14839	48	17.77 (2.43)	0.53	0.30
Mare Saint Robert	SR7	June11	21510	18212	71	34.20 (2.81)	0.83	0.53
Mare Saint Robert	SR9	July11	6382	5739	49	38.76 (2.27)	0.81	0.59
Mare Saint Robert	SR11	September11	20697	18111	91	54.75 (3.01)	0.94	0.71
Mare Saint Robert	SR13	October11	25461	12378	44	26.71 (2.32)	0.73	0.53
Mare Saint Robert	SR15	November11	8781	4075	79	58.24 (3.17)	0.89	0.67
Mare Saint Robert	SR17	December11	15664	14331	59	28.48 (2.81)	0.32	0.24
Mare Saint Robert	SR19b	January12	33246	30434	70	24.34 (2.86)	0.66	0.35
Mare Saint Robert	SR21	February12	7816	6318	128	78.68 (4.27)	0.92	0.66
Mare Saint Robert	SR23	March12	2406	2035	48	43.09 (1.85)	0.90	0.74
Mare Saint Robert	SR25	April12	85803	79069	144	35.02 (3.42)	0.83	0.44
Mare Saint Robert	SR27	May12	6260	4472	71	49.99 (3.13)	0.88	0.62
Mare Saint Robert	SR29	June12	10976	10461	55	34.44 (2.66)	0.59	0.40
Mare Saint Robert	SR31	July12	17576	16724	79	26.93 (3.39)	0.29	0.19
Mare Saint Robert	SR33	August12	7075	5847	115	80.93 (3.69)	0.95	0.76
Mare Saint Robert	SR35	September12	11437	9173	138	91.23 (4.11)	0.94	0.72
Mare Saint Robert	SR37	October12	26754	23288	89	32.58 (3.46)	0.27	0.19
Mare Saint Robert	SR39	November12	10594	8624	64	38.87 (2.89)	0.82	0.54
Mare Saint Robert	SR41	December12	19009	16332	51	20.17 (2.62)	0.38	0.25
Mare Saint Robert	SR43	January13	26375	23625	41	12.77 (2.22)	0.33	0.17
Mare Saint Robert	SR45	February13	16785	14467	50	22.55 (2.50)	0.67	0.42
Mare Saint Robert	SR47	March13	17079	14322	36	19.39 (1.98)	0.75	0.51
Mare Saint Robert	SR49	April13	29652	26902	26	10.38 (1.59)	0.39	0.24
Etang des Vallees	EV11	April11	4353	3369	144	114.49 (3.99)	0.97	0.81
Etang des Vallees	EV13	May11	7855	6561	145	92.69 (4.49)	0.92	0.68
Etang des Vallees	EV15	June11	6602	5418	158	109.24 (4.45)	0.95	0.76
Etang des Vallees	EV17	July11	14164	12358	240	142.20 (5.51)	0.98	0.79
Etang des Vallees	EV19	September11	7407	6040	194	128.57 (5.22)	0.95	0.75

Etang des Vallees	EV21	October11	8972	7982	157	94.35 (4.65)	0.93	0.68
Etang des Vallees	EV23	November11	16241	13631	178	90.77 (4.89)	0.86	0.60
Etang des Vallees	EV25	December11	9474	8231	143	80.83 (4.55)	0.90	0.64
Etang des Vallees	EV27	January12	8344	7248	137	84.67 (4.36)	0.89	0.65
Etang des Vallees	EV29	February12	13625	11890	135	66.38 (4.28)	0.54	0.38
Etang des Vallees	EV31	March12	11678	10275	142	79.65 (4.36)	0.90	0.64
Etang des Vallees	EV33	April12	22314	19612	206	107.18 (5.07)	0.96	0.72
Etang des Vallees	EV35	May12	7474	6462	161	107.78 (4.44)	0.97	0.79
Etang des Vallees	EV37	June12	7557	6900	88	58.37 (3.50)	0.89	0.63
Etang des Vallees	EV39	July12	7230	6337	186	123.60 (4.92)	0.97	0.80
Etang des Vallees	EV41	August12	33322	22650	199	87.08 (5.09)	0.88	0.59
Etang des Vallees	EV43	September12	8670	7560	157	104.298 (4.45)	0.97	0.78
Etang des Vallees	EV45	October12	8229	7527	108	68.86 (3.88)	0.90	0.64
Etang des Vallees	EV47	November12	13608	11075	149	77.42 (4.52)	0.91	0.63
Etang des Vallees	EV49	December12	10909	9612	118	62.85 (4.13)	0.86	0.57
Etang des Vallees	EV51	January13	10613	8807	126	72.57 (4.18)	0.90	0.63
Etang des Vallees	EV53	February13	16872	13918	140	73.86 (4.09)	0.86	0.59
Etang des Vallees	EV55	March13	7820	6752	67	47.52 (2.78)	0.91	0.68
Etang des Vallees	EV57	April13	14115	11663	172	90.58 (4.77)	0.88	0.62
Ru Sainte Anne	RSA3	April11	3917	2186	162	130.83 (4.40)	0.96	0.77
Ru Sainte Anne	RSA5	May11	10615	9128	162	80.98 (4.81)	0.86	0.57
Ru Sainte Anne	RSA7	June11	7148	5735	134	74.78 (4.65)	0.65	0.45
Ru Sainte Anne	RSA9	July11	5399	4014	143	97.82 (4.50)	0.95	0.72
Ru Sainte Anne	RSA11	September11	12232	9111	301	149.19 (6.53)	0.95	0.71
Ru Sainte Anne	RSA13	October11	5761	4158	188	122.81 (5.24)	0.94	0.73
Ru Sainte Anne	RSA15	November11	9207	6560	183	109.25 (5.18)	0.92	0.69
Ru Sainte Anne	RSA17	December11	10297	7838	349	176.18 (7.15)	0.95	0.73
Ru Sainte Anne	RSA19	January12	11424	8945	340	168.31 (6.88)	0.93	0.71
Ru Sainte Anne	RSA21	February12	18584	14698	264	93.14 (6.04)	0.77	0.45
Ru Sainte Anne	RSA23	March12	23308	17498	421	175.98 (7.15)	0.94	0.71
Ru Sainte Anne	RSA25	April12	37766	30979	487	149.02 (7.30)	0.91	0.60
Ru Sainte Anne	RSA27	May12	6404	4883	196	113.88 (5.67)	0.74	0.54
Ru Sainte Anne	RSA29	June12	6258	3811	158	108.77 (4.81)	0.90	0.66
Ru Sainte Anne	RSA31	July12	3914	2895	184	136.59 (4.97)	0.94	0.74
Ru Sainte Anne	RSA37	October12	11610	9472	296	128.71 (6.69)	0.82	0.56
Ru Sainte Anne	RSA39	November12	9869	6713	198	113.33 (5.47)	0.79	0.58
Ru Sainte Anne	RSA41	December12	7503	4523	224	151.16 (5.57)	0.98	0.82
Ru Sainte Anne	RSA43	January13	2241	1238	151	144.19 (2.39)	0.97	0.86
Ru Sainte Anne	RSA45	February13	10134	6443	392	217.44 (7.38)	0.98	0.82
Ru Sainte Anne	RSA47	March13	10136	6598	422	227.10 (7.73)	0.98	0.82
Ru Sainte Anne	RSA49	April13	9585	6871	314	166.94 (6.86)	0.97	0.77
Mare La Claye	LC3	April11	9435	7448	42	29.85 (1.92)	0.81	0.58
Mare La Claye	LC5	May11	8979	7546	161	91.96 (4.88)	0.85	0.59
Mare La Claye	LC7	June11	7202	6375	133	79.35 (4.56)	0.90	0.62
Mare La Claye	LC19	January12	9582	8713	73	38.89 (3.30)	0.68	0.42
Mare La Claye	LC21	February12	15405	13672	146	66.01 (4.18)	0.90	0.61
Mare La Claye	LC23	March12	33414	24865	158	64.04 (4.27)	0.92	0.61
Mare La Claye	LC25	April12	21283	19912	101	41.34 (3.40)	0.67	0.39
Mare La Claye	LC27	May12	6794	5691	81	48.03 (3.50)	0.78	0.53
Mare La Claye	LC29	June12	10085	9127	84	45.90 (3.36)	0.79	0.52
Mare La Claye	LC31	July12	5883	4066	173	121.67 (4.82)	0.97	0.80
Mare La Claye	LC33	August12	9217	8052	135	72.51 (4.48)	0.84	0.55
Mare La Claye	LC37	October12	6516	4215	288	184.81 (6.46)	0.97	0.79
Mare La Claye	LC39	November12	10715	7495	126	77.31 (4.04)	0.92	0.66
Mare La Claye	LC41	December12	10669	8741	191	97.31 (5.26)	0.87	0.60
Mare La Claye	LC43	January13	14175	12762	57	27.72 (2.94)	0.28	0.20
Mare La Claye	LC45	February13	16673	14455	60	24.76 (2.85)	0.18	0.14
Mare La Claye	LC47	March13	17759	13350	162	84.06 (4.53)	0.91	0.64
Mare La Claye	LC49	April13	13230	11178	169	75.10 (5.19)	0.74	0.45

Table S3. OTUs detected in at least 25% of samples (i.e. 28 samples) and representing at least 0.1% of reads by sample, in mean

| OTU identification number	Taxonomic affilliation	First blast hit (% identity)	Ecosystems	Mean relative abundance by sample (min-max)
25	Ascomycota	Dothideomycetes_X sp. (100%)	MG, SR, EV, RSA, LC	0.3% (0 - 5.84)
36	Chytridiomycota	Cladochytriales_X sp. (99.4%)	MG, SR, EV, RSA, LC	0.2% (0 - 3.49)
182	Ascomycota	Leotiomycetes_X sp. (100%)	MG, SR, EV, RSA, LC	0.72% (0 - 9.33)
195	Ascomycota	Dothideomycetes_X sp. (100%)	MG, EV, RSA, LC	0.14% (0 - 5.22)
208	Basidiomycota	Agaricomycetes_X sp. (100%)	MG, SR, EV, RSA, LC	0.13% (0 - 2.55)
1	Chlorophyta	Chlorellales_X sp. (99.2%)	MG, SR, EV, RSA, LC	0.78% (0 - 48.69)
14	Chlorophyta	Choricystis sp. (100%)	MG, SR, EV, RSA, LC	0.13% (0 - 5.65)
52	Chlorophyta	*Monoraphidium convolutum* (100%)	MG, SR, EV	0.24% (0 - 7.03)
100	Chlorophyta	CW-Chlamydomonadales_X sp. (99.0%)	MG, EV, RSA, LC	1.34% (0 - 36.25)
353	Chlorophyta	*Crustomastix* sp. (98.1%)	MG, SR, EV, RSA, LC	0.18% (0 - 3.76)
368	Chlorophyta	Sphaeropleales_X sp. (100%)	MG, SR, EV, LC	0.12% (0 - 1.77)
2036	Chlorophyta	*Chloromonas subdivisa* (99.6%)	MG, SR, RSA, LC	0.26% (0 - 15.92)
158	Chrysophyceae-Synurophyceae	Chrysophyceae-Synurophyceae_XXX sp. (96.1%)	MG, SR, EV, RSA, LC	0.18% (0 - 3.81)
185	Chrysophyceae-Synurophyceae	Clade-C_X sp. (97.5%)	MG, SR, EV, RSA, LC	0.49% (0 - 23.34)
427	Chrysophyceae-Synurophyceae	*Chrysosaccus* sp. (98.6%)	MG, SR, EV, LC	0.23% (0 - 3.93)
669	Chrysophyceae-Synurophyceae	*Spumella*_X sp. (100%)	MG, SR, EV, RSA, LC	1.59% (0 - 40.36)
710	Chrysophyceae-Synurophyceae	*Synura petersenii* (99.6%)	MG, SR, EV, RSA, LC	1.95% (0 - 36.83)
761	Chrysophyceae-Synurophyceae	Clade-C_X sp. (99.8%)	MG, EV, RSA, LC	0.2% (0 - 4.13)
2	Chrysophyceae-Synurophyceae	Clade-D_X sp. (99.2%)	MG, SR, EV, LC	0.43% (0 - 23)
24	Dictyochophyceae	Pedinellales_X sp. (99.2%)	MG, SR, EV, RSA, LC	0.48% (0 - 13.36)
91	Dictyochophyceae	Pedinellales_X sp. (99.4%)	MG, SR, EV, RSA, LC	0.15% (0 - 2.63)
178	Dictyochophyceae	*Pseudopedinella* sp. (99.6%)	MG, EV, RSA, LC	0.49% (0 - 11.76)
46	Ciliophora	Choreotrichia_X sp. (100%)	MG, SR, EV, RSA	1.37% (0 - 30.31)
107	Ciliophora	Oligotrichia_X sp. (99.8%)	MG, SR, EV, RSA, LC	1.09% (0 - 33.99)
356	Ciliophora	Stichotrichia_X sp. (99.6%)	MG, SR, EV, RSA, LC	7.55% (0 - 89.15)
462	Ciliophora	Peritrichia_X sp. (100%)	MG, SR, EV, RSA, LC	0.13% (0 - 9.3)
481	Ciliophora	Prostomatea_XXX sp. (99.6%)	MG, SR, EV	0.11% (0 - 4.96)
7	Colpodellidae	Colpodellidae_XX sp. (99.2%)	MG, SR, EV, RSA, LC	0.18% (0 - 5.88)
20	Cercozoa	Novel-clade-10_XXX sp. (99.8%)	MG, SR, EV, RSA, LC	0.31% (0 - 13.37)
18	Cryptophyta	Cryptomonadales_X sp. (99.4%)	MG, EV, RSA, LC	6.34% (0 - 79.98)
50	Cryptophyta	Cryptomonadales_X sp. (100%)	MG, SR, EV, RSA, LC	7.91% (0 - 90.52)
65	Cryptophyta	Basal environmental Cryptophyceae_X sp. (99.8%)	SR, EV, RSA, LC	0.5% (0 - 11.76)
142	Cryptophyta	Cryptomonadales_X sp. (99.8%)	SR, EV, LC	0.39% (0 - 4.03)
326	Cryptophyta	*Komma caudata* (99.4%)	SR, RSA, LC	1.11% (0 - 25.72)
347	Cryptophyta	*Cryptomonas obovoidea* (100%)	MG, SR, EV, RSA, LC	1.02% (0 - 24.1)
400	Cryptophyta	Cryptomonadales_X sp. (100%)	SR, EV, LC	1.43% (0 - 22.55)
55	Katablepharidophyta	Katablepharidales_XX sp. (99.8%)	MG, SR, EV, RSA, LC	1.82% (0 - 43.61)

MG: mare Gabard, SR: Saint Robert, EV: Etang des Vallées, RSA: Ru Sainte Anne, LC: La Claye
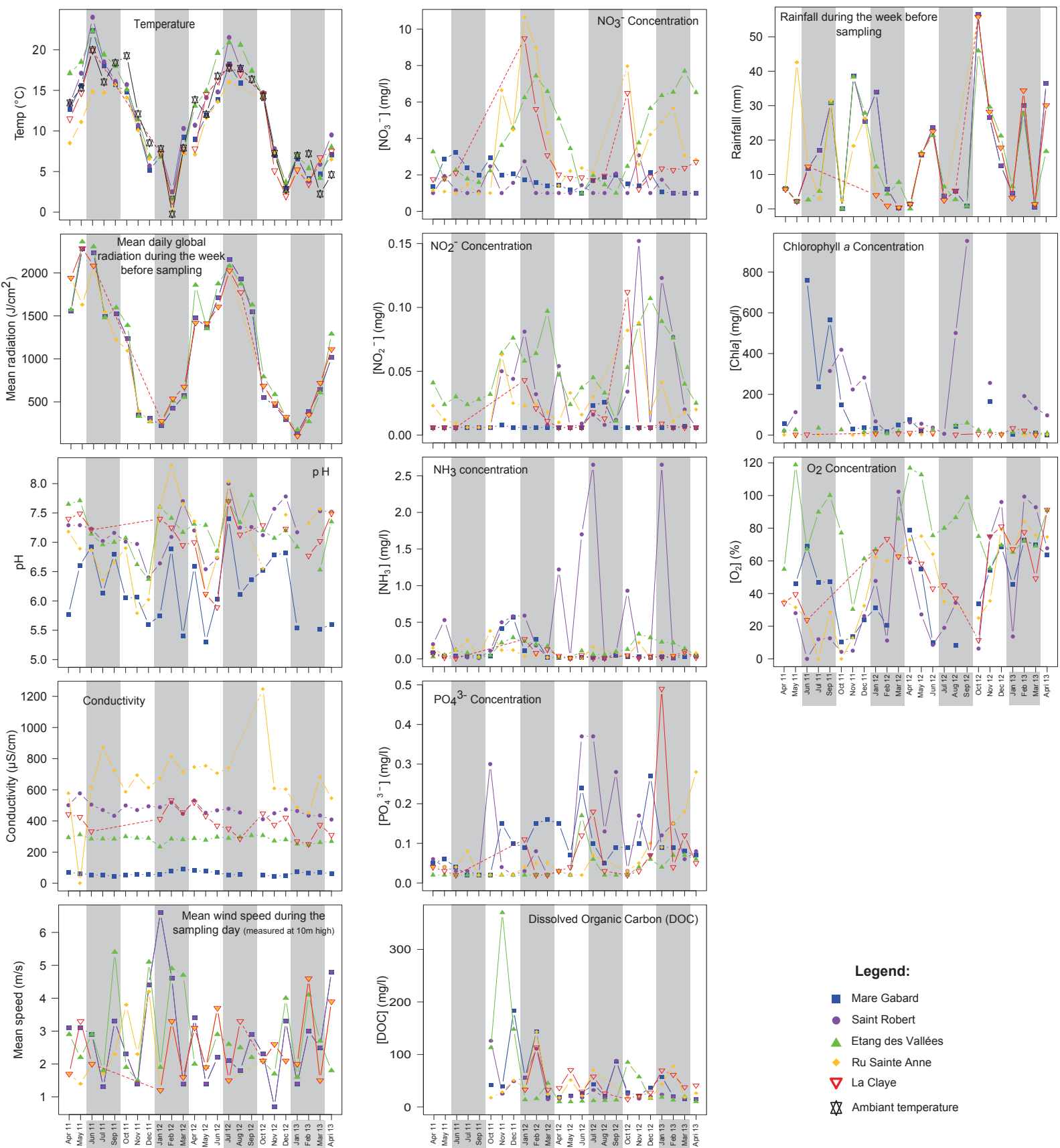
Figure S1 Dynamics of the environmental parameters included in our analysis, over the 2-years survey. Temperature, conductivity, pH and O_2 concentration were measured *in situ*. Nutrient concentrations (NO_3^-, NO_2^-, NH_3, PO_4^{3-}, organic carbon) were estimated in water prefiltered through 0.2μm pore-sized filters. Chlorophyll *a* concentration was measured by spectroscopy on pigments extracted from cells harvested on filters. Rainfall, radiation, wind speed and ambient temperature (mean temperature of the week before sampling) were obtained from the closest weather stations (Météo France). Dashed lines correspond to missing data because of the drought periods: Ru Sainte Anne was dried in August and September 2012, and La Claye from the end of July to the beginning of December 2011, and in September 2012.
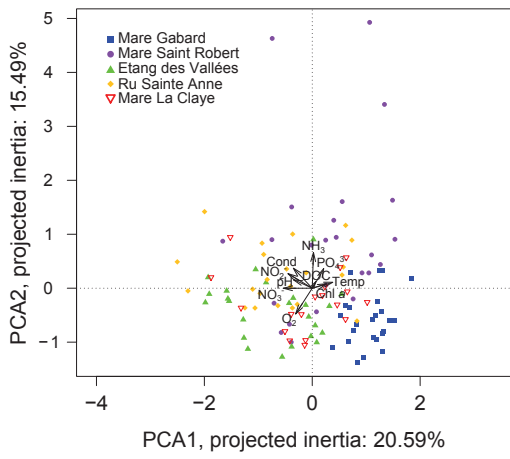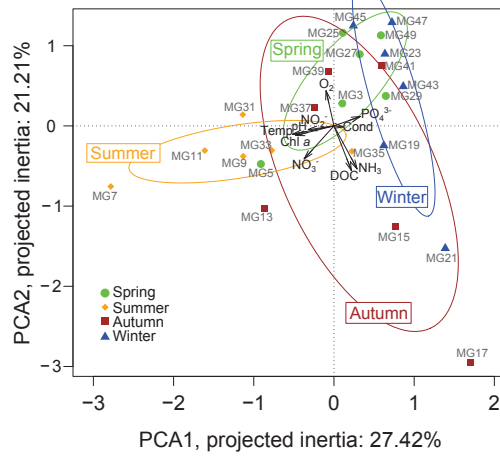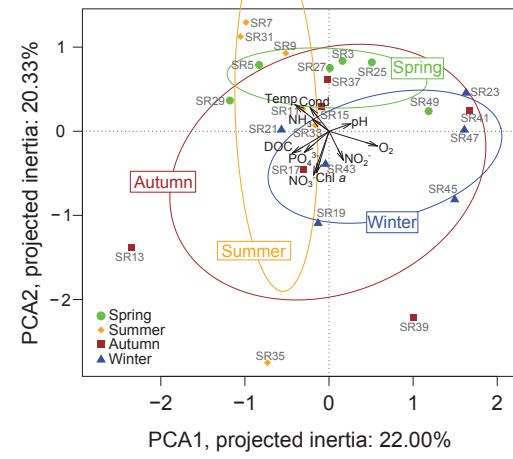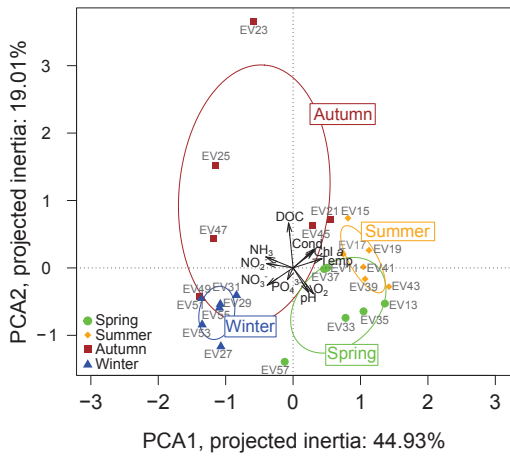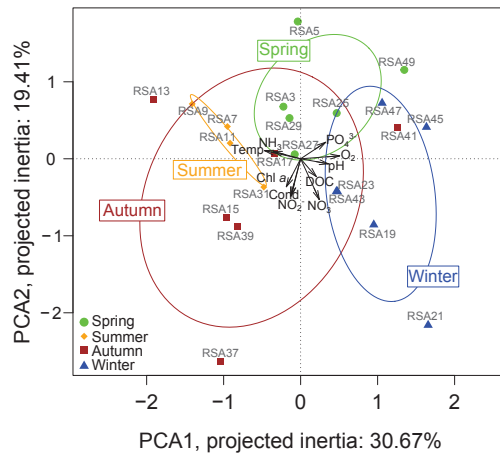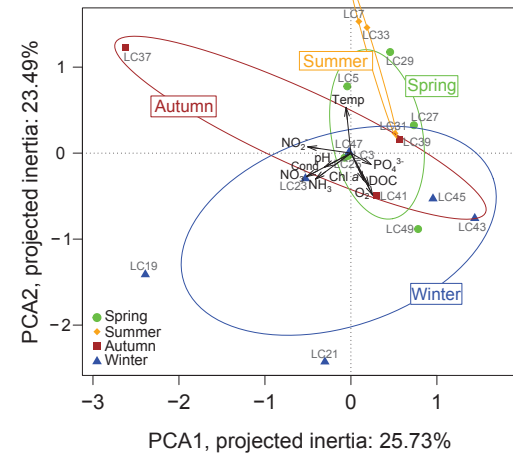
Figure S2 Principal component analysis (PCA) plot of the physico-chemical parameters measured in the ecosystems. PCAs were conducted for the 5 systems and 24 dates (A), or for each ecosystem separately (B to F). Samples are represented by points coloured according to the ecosystems they came from (A) or to the season when they were sampled (B to F), and their names are indicated in grey (B to F). Ellipses were drawn to show the region of the PCA where samples from the same season cluster (B to F). Cond: Conductivity, Temp: Temperature, Chl *a*: Chlorophyll *a*, DOC: Dissolved Organic Carbon.
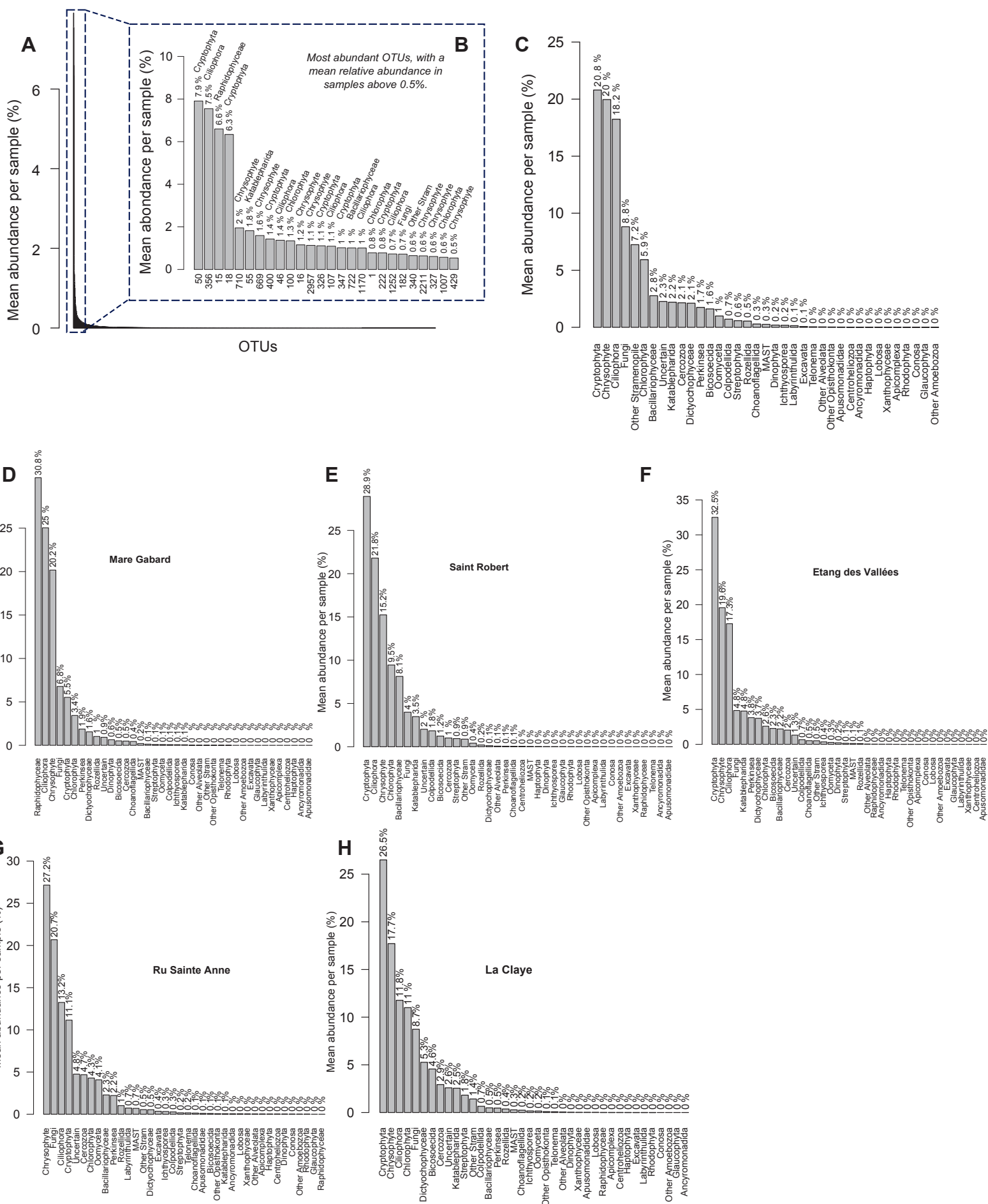
Figure S3. General molecular diversity of small eukaryotes in the five ecosystems over the two-year survey. Rank-abundance curves of all OTUs (A), OTUs with a mean relative abundance in samples of at least 0.5% (B) or taxonomic groups (C), are shown. D to F: rank-abundance curves of taxonomic groups among all samples from each ecosystem. Taxonomic groups and OTUs are sorted based on their mean percentage of reads ; those proportions are indicated on the top of the bars. In B, the identification number of the OTUs and their taxonomic affiliation are shown below and on the top of the bar, respectively.
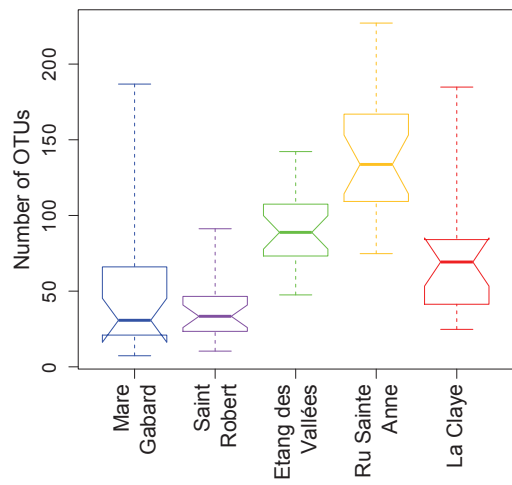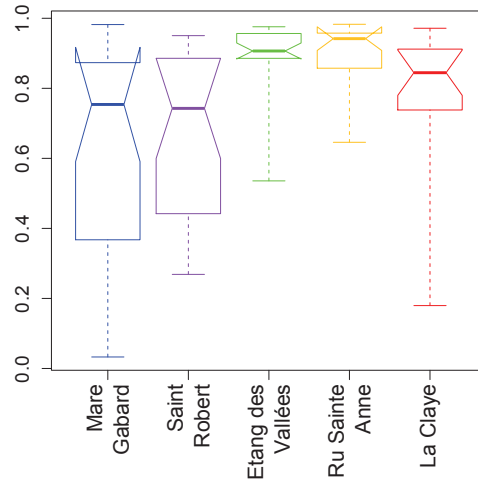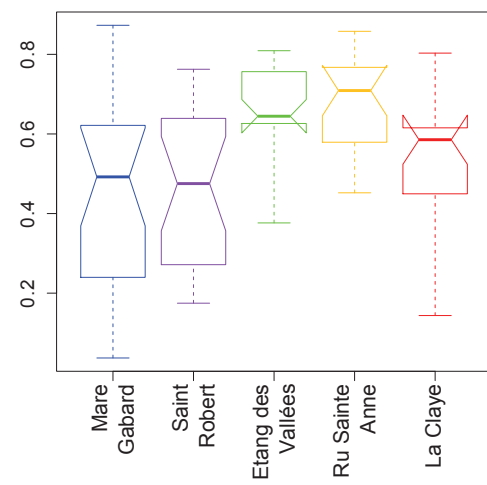
Figure S4. Richness and diversity estimators computed for the 24 samples from Mare Gabard, Saint Robert and Etang des Vallées, the 22 samples from Ru Sainte Anne and for the 18 samples from La Claye. Richness is expressed as the expected number of OTUs in random subsamples of the size of the smallest sequence library (793 reads in Mare Gabard in January 2013). The thickest line inside each box represents the median on the distribution, bottom and top borders of boxes correspond to the first and third quartiles and whiskers extend to minimal and maximal distances. Notches are drawn to indicate whether medians from the five ecosystems can be considered as different (Chambers *et al.*, 1983, p.62).
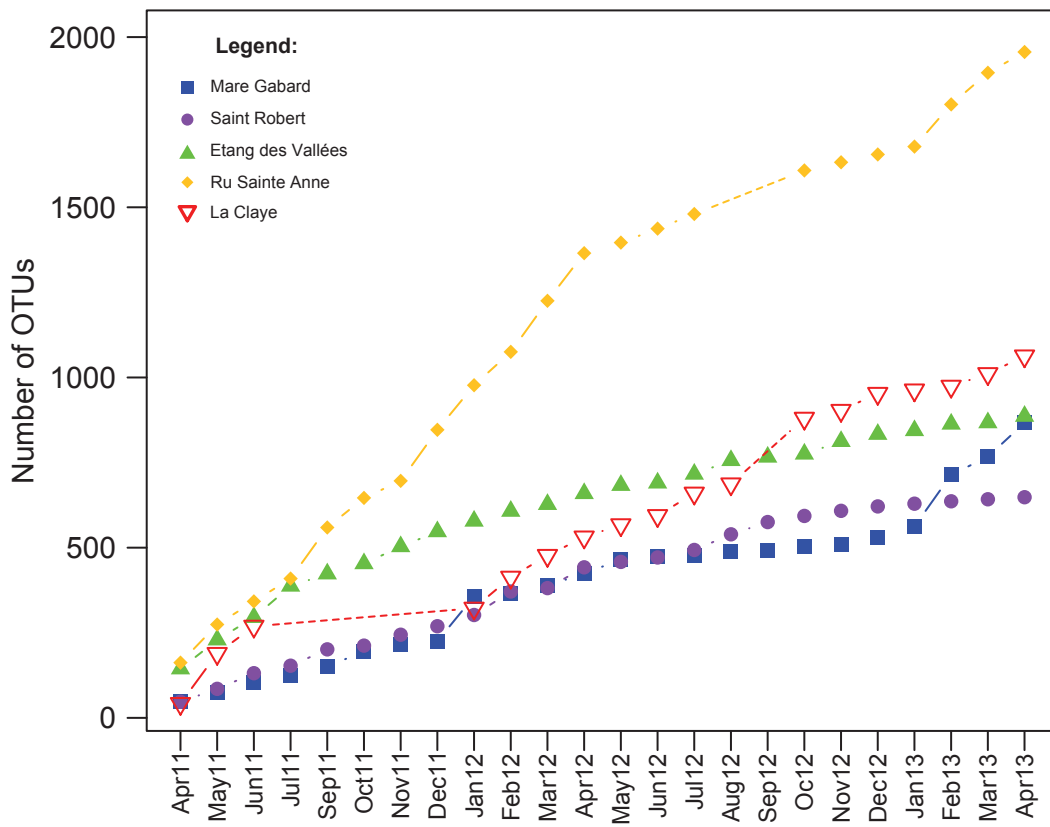
Figure S5. Accumulation curves showing the total number of OTUs detected in each ecosystem until the beginning of the survey. Dashed lines indicate missing data due to the drought: Ru Sainte Anne was dried in August and September 2012, and La Claye from the end of July to December 2011 and in September 2012.
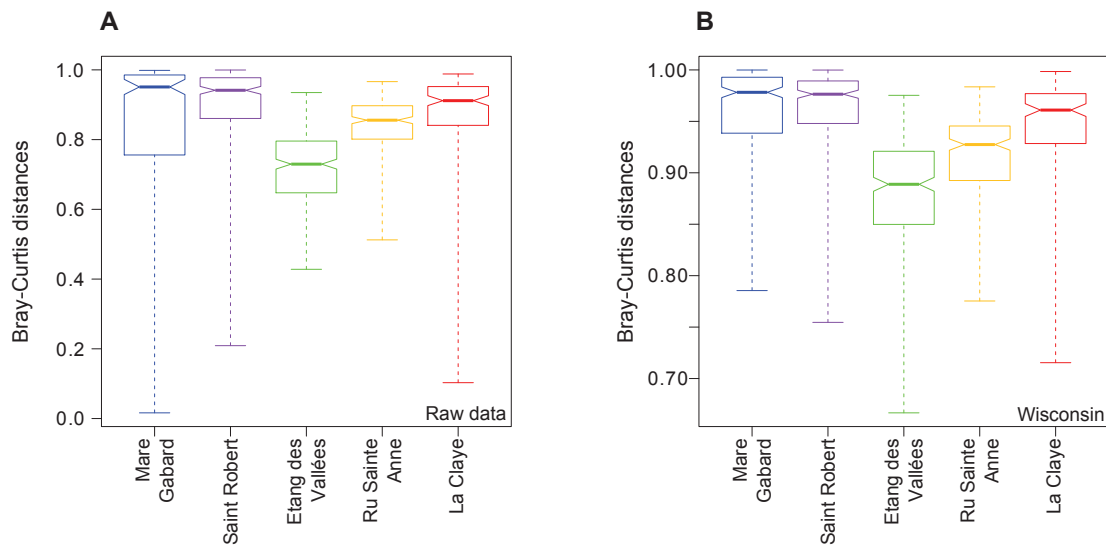
Figure S6. Box plot of Bray-Curtis distances between all pairs of samples from each ecosystem, calculated on raw (A) or Wisconsin standardized (B) percentage of OTUs in samples. The thickest line inside each box represents the median on the distribution, bottom and top borders of boxes correspond to the first and third quartiles and whiskers extend to minimal and maximal distances. Non overlapping notches are a strong evidence that median of Bray-Curtis distance distributions in ecosystems differ (Chambers *et al.,* 1983, p.62).
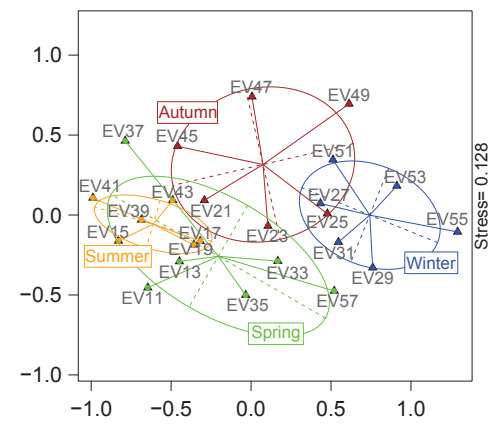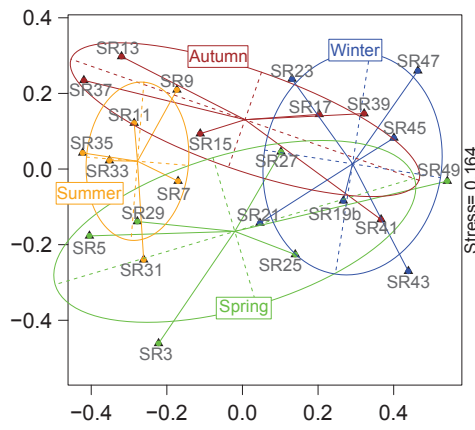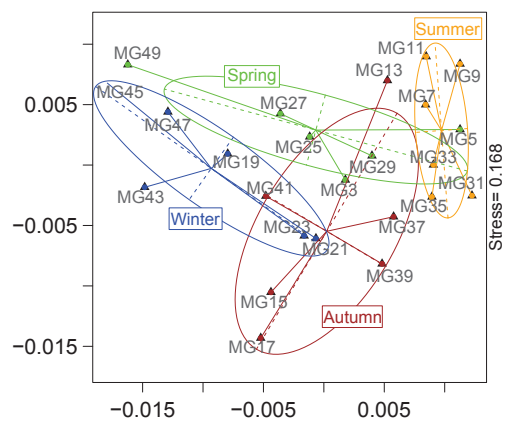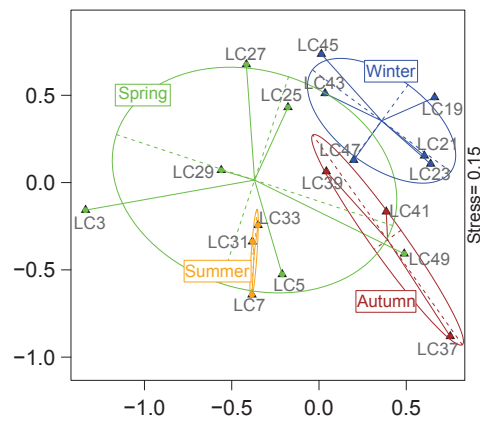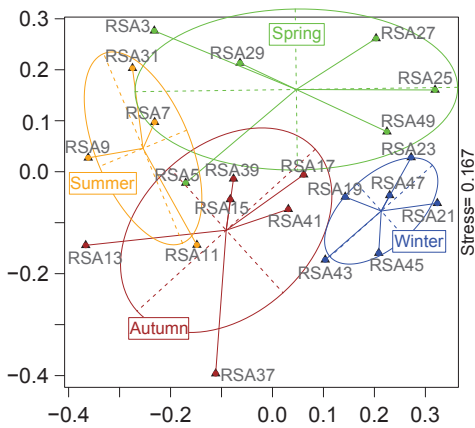
Figure S7. Non-metric MultiDimensional Scaling (NMDS) plots, based on Bray-Curtis distances computed after Wisconsin standardization and square-root transformation of all OTU frequences in samples from Mare Gabard (A), Saint Robert (B), Etang des Vallées (C), Ru Sainte Anne (D) and La Claye (E). Samples are indicated in grey, and grouped by season. Stress value of each NMDS is displayed on the right border of the corresponding plot.
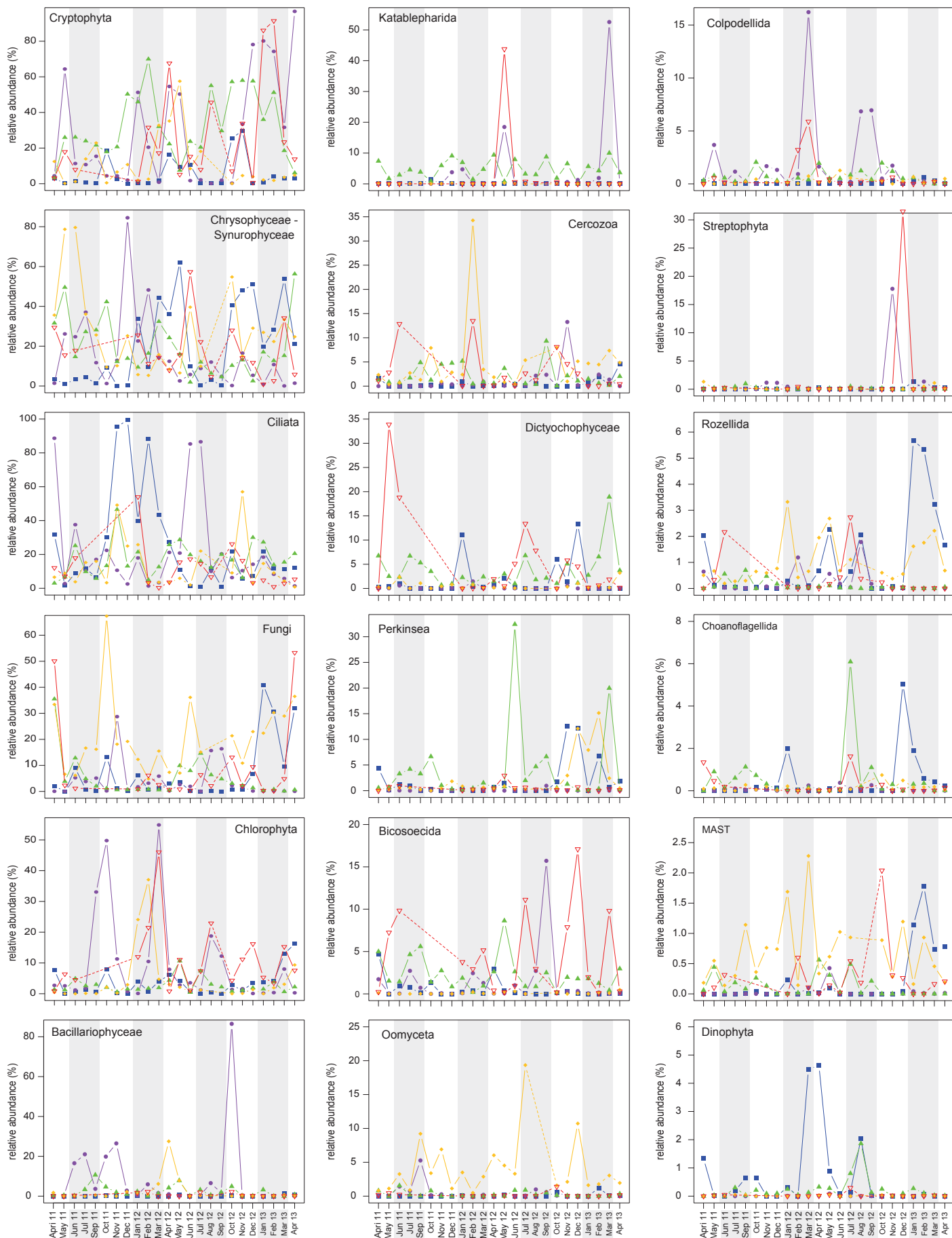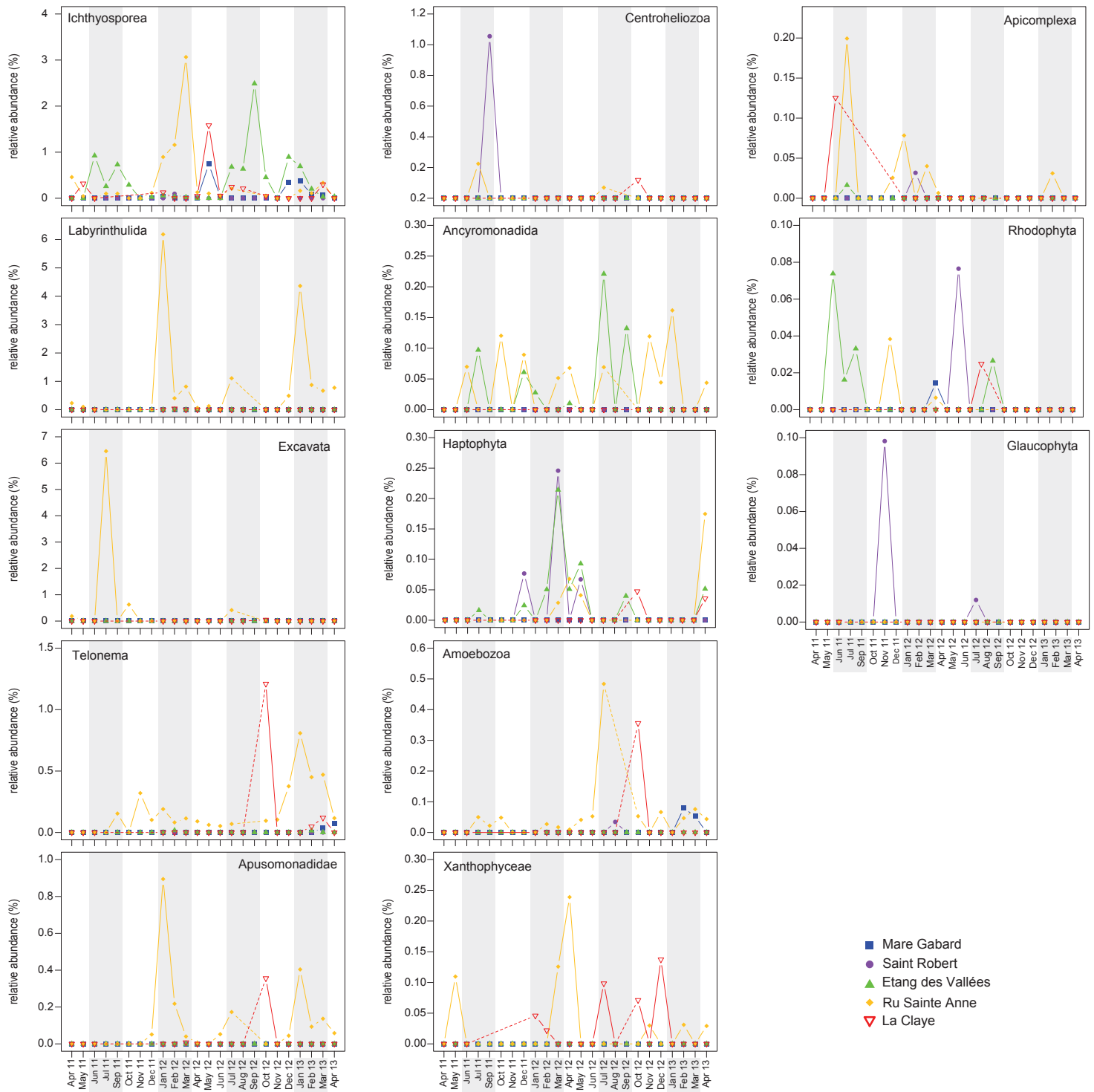
Figure S8. Part 1

Figure S8. Part 2. Temporal dynamics of detected taxonomic groups in the five sampled ecosystems. Each color corresponds to a distinct system. Abundances are estimated as the proportion of reads affiliated to each taxa in the total number of reads in the corresponding sample. Ru Sainte Anne was dried in August and September 2012, and La Claye from the end of July to December 2011 and then in September 2012. Corresponding missing data are indicated by dashed lines.
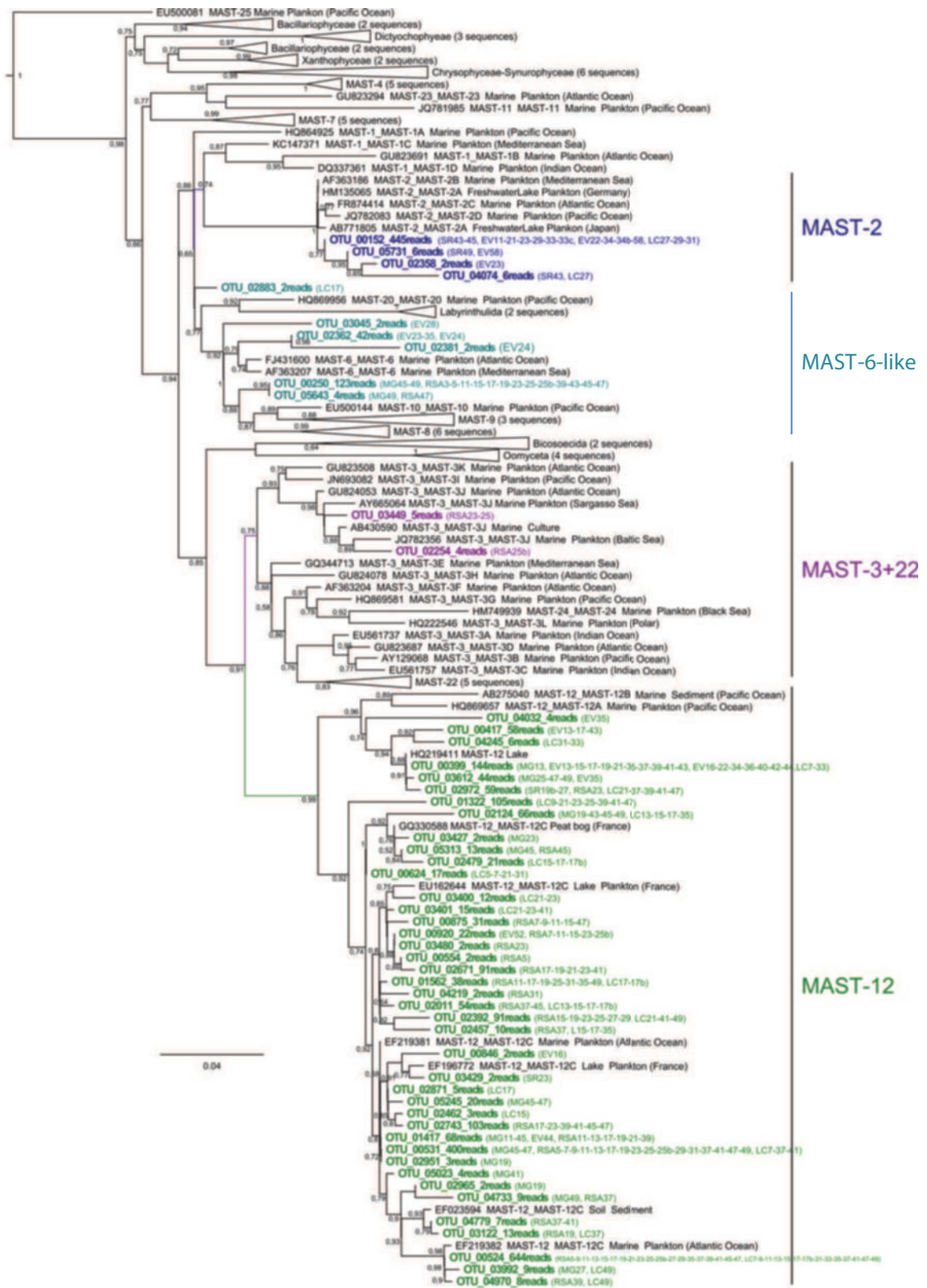
Figure S9. Approximate maximum likelihood phylogenetic tree of 18S rDNA amplicons cumulated over 24 months affiliating to MAST clades within the Stramenopiles. 367 informative positions were used to reconstruct the phylogenetic tree. 2 ciliate sequences were used as outgroup (not shown). Sequences from this work are shown in color. Only bootstrap values higher than 50% are shwon at nodes. The names of samples where they were found and the number of reads are indicated for each OTU.