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High mycobacterial diversity in recreational lakes

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Abstract Although nontuberculous mycobacteria (NTM) are natural inhabitants of freshwater ecosystems, few studies have focused on their distribution in these habitats. Thus, the knowledge about the abundance as well as the composition of NTM remains limited and patchy in these environments. In this context, a prospective study was performed to identify favourable habitats for mycobacteria in two recreational lakes. Mycobacterial density and diversity were measured using quantitative real-time PCR and the MiSeq Illumina platform. For both lakes, five compartments were investigated, i.e. water column, air–water interface, sediment, epilithon and epiphyton biofilms. Nontuberculous mycobacteria were detected in all compartments in large densities and displayed a remarkable diversity. NTM were dominated by fast-growing species. Lakes and compartments appeared to shape mycobacteria assemblage composition as well as their densities. In both lakes, some OTUs assigned to the species level were identified as related to known opportunistic pathogens.

Keywords Nontuberculous mycobacteria · Lake · Quantitative real-time PCR · High-throughput sequencing

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Introduction

The genus *Mycobacterium* contains more than 170 species, mostly described as nontuberculous mycobacteria (NTM) (Euzéby 1997). Although some species are recognized as opportunistic pathogens, many of these bacteria are saprophytic and therefore are natural inhabitants of terrestrial and aquatic environments (Collins et al. 1984; Falkinham 2002; Hruska and Kaevska 2012). However, studies focusing on NTM in natural ecosystems remain rare in comparison with drinking water distribution systems (e.g., Covert et al. 1999; Falkinham et al. 2001; Vaerewijck et al. 2005) or hospital water supply networks (e.g., du Moulin et al. 1988; Fox et al. 1992; Fujita et al. 2002). These two types of habitats have been particularly well studied (Kazda et al. 1999), owing to the increased susceptibility of immunodeficient persons to mycobacteriosis following contact with waters harbouring mycobacteria.

The lack of studies exploring the environmental habitats of mycobacteria could partly be explained by the difficulties in isolating slow-growing bacteria from natural environments using classical culture methods (Falkinham 2002). Cultivation approaches also provide a limited view of NTM density and diversity. However, improvements in extracting DNA from natural samples (Guo and Zhang 2013) as well as PCR-based methods, which have been developed over the last decades, should solve some of these biases. Nevertheless, studies describing the entire mycobacterial assemblage in ecosystems using molecular tools are still scarce. These few published papers mainly concentrated on soil habitats (e.g., Niva et al. 2006; Pontiroli et al. 2013). Moreover, the majority of studies investigating freshwater habitats either focused on particular species (e.g., Stinear et al. 2000; Pickup et al. 2005, 2006; Gauthier et al. 2010) or on cultivable species (e.g., Viallier and Viallier 1973; Kirschner et al. 1992; Iivanainen et al. 1993; Bland et al. 2005). Only few studies considering the whole NTM assemblage have been performed in aquatic ecosystems (Niva et al. 2006; Parashar et al. 2009; Debruyn et al. 2009; Khera 2012). Consequently, it appears necessary to improve our knowledge in terms of abundance, diversity and potential niches of mycobacterial species in surface freshwater ecosystems, especially since NTM probably acquired their virulence traits under selective pressures in their natural habitat, as have other environmental pathogens (Dyble et al. 2008; Adiba et al. 2010).

Among freshwater ecosystems, lakes appear to be interesting models to investigate the distribution of NTM. Indeed lakes are common ecosystems in urban areas, where they attract a high number of visitors due to the cultural and recreational services they provide. Moreover, according to the literature, lakes could provide relevant ecological niches for mycobacteria. In boreal lakes, Niva et al. (2006) observed that the mycobacterial assemblages constituted a large part of the *Actinobacteria* phylum, one of the dominant phyla in these ecosystems (Newton et al. 2011). Moreover, numerous NTM species (e.g., *M. avium*, *M. chelonae*, *M. xenopi*) are able to persist or develop in aquatic environments within biofilms (Hall-Stoodley and Lappin-Scott 1998; Dailloux et al. 2003; Williams et al. 2009) or at water interfaces (Alavi et al. 2006) due to their hydrophobic cell walls. Sediment seems also to constitute a suitable habitat for NTM (Pickup et al. 2005; Debruyn et al. 2009; Gauthier et al. 2010).

This prospective study investigated the spatial distribution of the mycobacterial assemblages in two shallow lakes with different trophic status (eutrophic and mesotrophic) in order to evaluate the density and diversity of NTM in different compartments. First, we sought to determine the main reservoirs of NTM among a set of different compartments (water column, air–water interface, sediment and biofilms). Second, we evaluated to what extent mycobacterial density could be impacted by spatial parameters. We hypothesized that NTM would be preferentially found at the air–water interface and in biofilms, and that the species composition would differ among the different compartments in both lakes. For that purpose, an approach combining a quantitative and a compositional analysis of NTM based on recent molecular tools (Pontiroli et al. 2013; Radomski et al. 2013) was used. Mycobacterial densities were quantified using quantitative real-time PCR and NTM diversity was assessed using MiSeq Illumina high-throughput sequencing.

Materials and methods

Sampling sites

Créteil Lake and Daumesnil Lake are two shallow lakes separated by less than 10 km. They are located in the Paris area (France), near the confluence of the Seine and Marne Rivers. Créteil Lake (Suppl. Material 1) is a 0.40 km^2 mesotrophic lake that was originally a sandpit. This lake is mainly supplied by an alluvial groundwater, however the water quality of Créteil Lake can be affected by the presence of a storm sewer outlet, which drains 1 km^2 of a residential area. Daumesnil Lake (Suppl. Material 1) is a 0.12 km^2 eutrophic lake that was dug for recreational purposes in the 1860s in the Bois de Vincennes (Paris). Daumesnil Lake is supplied by water pumped in the Ourcq Channel.

Sample collection

Lakes were sampled in August and October 2012. For both lakes, five stations (C1–C5 and D1–D5 for Créteil and Daumesnil Lake respectively) were surveyed

(Fig. 1). For each station, five compartments were collected: water column, surface microlayer (air–water interface), sediment, epilithic and epiphytic biofilms. For each station, water column samples were collected at three depths (depending on the depth of the water column) using a Niskin bottle (General Oceanics Inc., Miami, USA). The surface microlayer was collected using a metal screen as described in detail by Agogué et al. (2004). Pooled sediment samples were constituted with the aerobic top sediment layer (~1 cm) from three cores. Epilithic biofilms (epilithon) were removed from rock surface by scraping 20 cm^2 biofilm area with a sterile syringe-toothbrush sampler inspired from Steinman et al. (2006). Epiphytic biofilms (epiphyton) were collected by harvesting submerged leaves sheath of phragmites (*Phragmites australis*) and milfoil (*Myriophyllum spicatum)* within Créteil and Daumesnil lakes respectively. All compartments were stored in sterile containers and placed at 4 °C until return to the laboratory in less than 10 h.

Sample processing

One liter of water sample (water column and surface microlayer) was centrifuged (7500×*g*, 4 °C) and the resulting cell pellet was collected into a 2 mL sterile tube. Before centrifugations, 1 mL of Tween 80 (final concentration, 0.01 % vol/vol) was added to each sample in order to facilitate pellet resuspension. Sediments and biofilms were immediately frozen, then lyophilized. All samples were stored at −20 °C until DNA extraction.

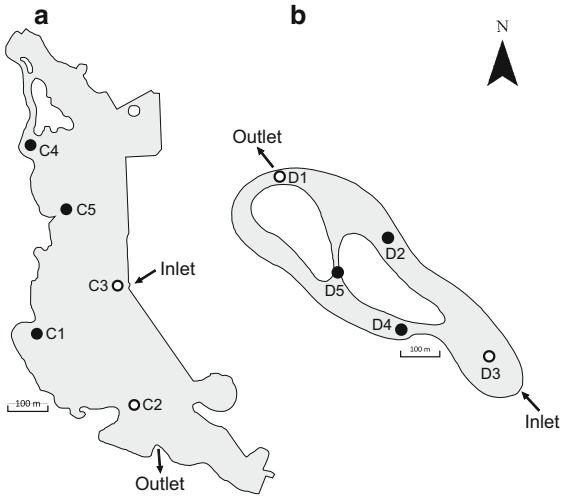

Fig. 1 Location of the five stations in Créteil Lake (**a**) and Daumesnil Lake (**b**). Nontuberculous mycobacteria composition was analyzed in the stations symbolized by *open circles*

DNA extraction

For water samples, pellets were resuspended in 400 µL of sterile water. Extractions were performed using a MagNA Pure Compact system (Roche Applied Science, Bâle, Switzerland) and the MagNA Pure Compact Nucleic Acid Isolation Kit I using the Bacteria V3 protocol, according to the manufacturer's instructions. Prior to extraction, samples underwent freeze–thaw cycles that consisted in three cycles of 1 min in liquid nitrogen and 5 min at 90 °C. Approximately 250 mg of sediment, 50 mg of dry epilithic biofilms and 40 mg of dry leaves were extracted using the FastDNA® SPIN Kit (Qbiogene, Carlbad, CA, USA) according to the manufacturer's instructions. Two modifications to this protocol were applied: cells were lysed in a FastPrep bead beater three times for 30 s at 4.0 ms^{-1} and the SPIN filters were washed twice. DNA was recovered in 50 µL eluent buffer and DNA quality and quantity were analyzed at 230, 260, and 280 nm by spectrophotometry before storage at −20 °C.

Quantitative real-time PCR

To quantify the abundance of *Mycobacterium* in the five compartments, TaqMan® real-time PCR assays targeting the *atpE* gene were carried out as previously described by Radomski et al. (2013). The assay was performed using the forward primer F*atpE* 5′-CGGYGCCGGTATCGGYGA-3′, the reverse primer R*atpE* 5′-CGAAGACGAACARSGCCAT-3′ and the probe P*atpE* 5′-ACSGTGATGAAGAACGGBG-TRAA-3′ labeled with the fluorescent dyes 6-carboxyfluorescein (5′ end) and Black Hole Quencher (3′ end). Contaminations in PCR mix were checked using negative controls. Moreover, the presence of PCR inhibitors in DNA templates was verified using a non-competitive exogenous internal control that was included in the PCR buffer. This internal control was made of a partial sequence of the *human β-actin* gene cloned in pGEM-T-easy vector (Promega, Madison, WI, USA) (Wurtzer et al. 2014). Absence of

significant PCR inhibition of the *atpE* assay was confirmed based on the average C_q values (±SD) obtained from 100 repeated PCR reactions containing 1000 copies of *human β-actin*.

The *atpE* copy numbers concentration was estimated from crude extracts using a *Mycobacterium chelonae* standard curve from 1.0×10^1 to 1.0×10^6 copies/µL. The PCR method presented a high amplification efficiency (82 %) and the standard curve had a good linearity ($R^2 = 0.998$). Results were expressed as *atpE* gene copies per liter for water samples, per gram (dry weight) for sediments and per square centimeter for biofilms. Relationships between biofilm area and mass are presented in the Supplementary Material 2.

Illumina sequencing of the V2–V3 region of the 16S rRNA gene

For the study of mycobacterial diversity in each compartment, twenty samples from the summer campaign were analyzed. The samples were selected from two stations corresponding to the inlet and the outlet of both lakes: C2 and C3 for Créteil Lake; D1 and D3 for Daumesnil Lake (Fig. 1).

PCR libraries were created by amplifying a fragment of the 16S rRNA gene (*rrs*) including the V2–V3 hypervariable regions using mycobacterial primers JSY16SF 5′-TGGGAAACTGGGAAACTGGGTCTAATA-3′ and JSY16SR 5′-CCCGCACGCCCAAGTTAAGCTGTGAG-3′ (Pontiroli et al. 2013). PCR and sequencing were performed by Research and Testing Laboratory (Lubbock, TEXAS) using the Illumina MiSeq platform (Illumina, Inc.). All DNA extracts were adjusted to 120 ng/µL prior to PCR reactions.

Quality control and sample processing were performed using QIIME v. 1.8.0-20140103 (Caporaso et al. 2010). Sequences of poor quality were removed using the default parameters (quality score < 25, length < 200 nt and the presence of at least six ambiguous bases or six successive homopolymers). Chimeric sequences were then identified with USEARCH 6.0 using the UCHIME algorithm in *de novo* mode (Edgar et al. 2011) implemented in the online service FunGene pipeline and ignored for further analysis. Filtered sequences were clustered into classical 97 % similarity-based operational taxonomic units (OTU). A clustering cutoff at 99 % rather than 97 % would lead to a six-fold increase of the OTU number (data not shown). Each OTU represented by a single sequence was removed from the analysis. Rarefaction curves are presented in the Supplementary Material 3. OTUs were assigned to the genus level with the SILVA 111 database (Quast et al. 2013) using UCLUST (Edgar 2010). Assignment to the species level was performed using blastn 2.2.31 search (Morgulis et al. 2008) provided by GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Query coverage was set to at least 97 %. A 97 % threshold was chosen based on Pontiroli et al. (2013) who did not notice a change of the most preponderant species identities or the overall numbers of sequences affiliated to these species using the more stringent cutoff of 99 %. Sequence data have been deposited in the NCBI Sequence Read Archive (SRA) and can be accessed through accession number SRP061716.

Data analysis

All statistical analyses and indices computing were conducted using the statistical environment R version 3.1.1 (R Development Core Team 2014) and the 'vegan' package (Oksanen et al. 2013). The default statistical significance was based on a $P \leq 0.05$ level.

Quantitative data analysis

Paired Wilcoxon or *t* tests were used to compare the data between August and October for each compartment and each lake. An intra-lake analysis was conducted to identify significant differences between the water compartments and between the biofilms. For that purpose, linear models were performed with stations as covariables. An inter-lake analysis was also conducted to characterize differences between the two lakes for all of the five compartments using linear models. All statistical analyses were performed with log-transformed data.

Mycobacterial composition analysis

First, OTU richness, diversity and evenness indices were calculated. Theoretical richness was estimated according to the non-parametric model of Chao-1 (Chao 1987). Alpha diversity was estimated using the exponential form of Shannon entropy. Evenness was calculated using the Simpson's evenness index (Smith

and Wilson 1996). Results of evenness ranged from 0 (when one OTU is predominant) to 1 (when all OTUs are equally abundant). Second, variations in the NTM community assembly were explored. For each lake, a Mann–Whitney test was conducted with Bray-Curtis dissimilarities to determine if the variability in the community structure within each compartment was significantly different from the variability between compartments. The dissimilarity of NTM assemblages among compartments (beta diversity) was evaluated using the Sørenson dissimilarity index. The beta diversity was then partitioned following the framework proposed by Baselga using the 'betapart' package (Baselga 2010; Baselga et al. 2013) to quantify the fraction of dissimilarity explained by OTUs replacement (based on the Simpson's dissimilarity index) and from pure random richness variation of the OTUs (Azeria et al. 2011). To evaluate the influence of lakes and compartments in the composition of the NTM communities, a redundancy analysis was performed on Hellinger-transformed data (Legendre and Gallagher 2001). Finally, samples were associated depending of their NTM community composition similarity using the unweighted-pair group method with UPGMA clustering (bootstrap = 999).

Results

Quantification of nontuberculous mycobacteria

The *atpE* gene was successfully amplified from all the samples with copy numbers of *atpE* gene ranging from 5.0×10^2 to 1.1×10^5 per liter of water samples, from 2.2×10^6 to 5.3×10^8 per gram of dry sediment and from 1.0×10^3 to 6.9×10^5 per square centimeter of biofilms (Fig. 2). No significant difference in the *atpE* gene copy number was found between August and October for each compartment and each lake, except for the epiphytic biofilm from Créteil. For this compartment, the *atpE* gene copy number was significantly higher in October than in August (paired *t* test, $P = 0.006$), with values of $4.4 \pm 2.4 \times 10^4$ copies/cm^2 in summer against $1.3 \pm 0.9 \times 10^5$ copies/cm^2 in autumn. Despite this unique difference, the remaining analysis was performed by combining data from the two sampling dates for all the compartments.

Intra-lake analysis indicated that for both lakes, the *atpE* gene copy numbers were significantly higher in the surface microlayer compared to the water column (linear models, $P = 0.014$ for Créteil Lake and $P = 0.007$ for Daumesnil Lake) (Fig. 2a). The copy numbers of *atpE* gene in epilithic biofilms were significantly higher than the copy numbers in the epiphytic biofilms (linear model, $P < 0.001$ within Créteil and Daumesnil Lake) (Fig. 2c). For each compartment, an inter-lake comparison revealed that the *atpE* gene copy numbers in the water column was significantly lower in Créteil compared to Daumesnil Lake (linear model, $P = 0.010$), whereas for the epiphytic biofilm the copy number was significantly higher in Créteil Lake (linear model, $P < 0.001$). No significant difference was observed between the two lakes for the NTM densities in the surface microlayer, the epilithic biofilm and the sediment (linear models, $P = 0.555$, $P = 0.191$ and $P = 0.748$ respectively). Interestingly, Fig. 2b showed high NTM densities for the two sediment samples collected in front of the storm sewer outlet of the Créteil Lake (Fig. 1). After removal of these two outliers from the analysis, the *atpE* gene copy numbers were significantly higher in Daumesnil than in Créteil Lake (linear model, $P < 0.001$).

Richness of nontuberculous mycobacteria

For each lake, ten samples collected in summer at two stations were sequenced, which produced a total of 503,164 sequences with an average length of 418 bp. Trimming and chimera check removed 39,939 sequences (Table 1). For Créteil Lake, two samples from different compartments were removed from the analysis due to a problem in amplification and sequencing (Table 1), thus the rest of the analysis was performed on 18 samples. After removal of the singleton sequences, 317,923 sequences were identified as belonging to the *Mycobacterium* genus (Table 1). These sequences were clustered into 658 OTUs. The observed richness covered on average 73 % of the estimated richness (Chao-1 index). A median of 125 OTUs was encountered in the different samples. Only six percent of these OTUs (37/658), presented in Fig. 3, had a relative abundance higher than 1 % in at least one of the 18 samples. This result suggests that the mycobacterial assemblages were dominated by few OTUs and mainly composed of rare OTUs, as underscored by the low values of the evenness index (Table 1).

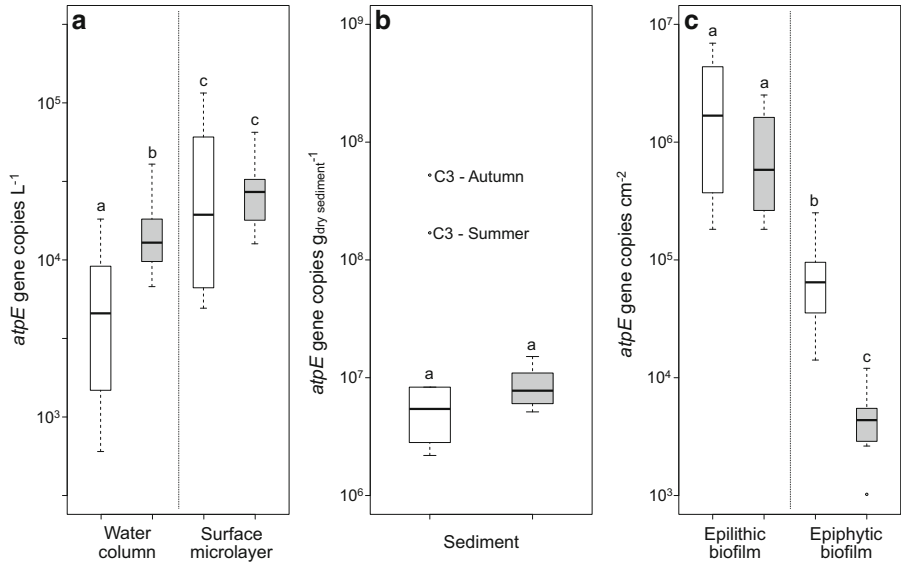

Fig. 2 Comparison of the *atpE* gene copy numbers between Créteil Lake (*white boxplot*) and Daumesnil Lake (*grey boxplot*) for water (**a**), sediment (**b**) and biofilms compartments (**c**) (n = 10). *Different letters* indicate a significant difference between lakes or compartments (linear models)

Although Créteil and Daumesnil Lakes have different trophic status, no significant difference was encountered between these lakes regarding the observed and the estimated richness (*t* tests, $P = 0.966$ and $P = 0.435$ respectively). However, the exponential form of Shannon entropy was significantly higher (*t* test, $P = 0.002$) within Créteil Lake (11.6 ± 9.3 OTUs) compared to Daumesnil samples (3.3 ± 2.3 OTUs). Epilithic biofilms from Créteil possessed the highest diversity (Table 1).

Composition of the mycobacterial assemblages

The blastn algorithm allowed a putative assignment of 364 out of 658 total OTUs to *Mycobacterium* species, each belonging to the NTM group (see Suppl. Material 4). The remaining OTUs were not assigned or were assigned to unclassified mycobacteria. Among the 364 identified OTUs, 94 were assigned to several mycobacterial species without any possible discrimination. Among the 270 remaining OTUs, 141 were assigned to a single species, 71 to two species and 58 OTUs to three species. When only considering these 270 OTUs, a large part (68 OTUs) was affiliated to *M. moriokaense*, 35 to the undifferentiated species *M. neglectum/tusciae*, 34 to the undifferentiated species *M. bacteremicum/frederiksbergense/sacrum*, 12 to *M. rhodesiae* and 9 to *M. asiaticum*.

The majority of all the sequences (87 %) were assigned to fast-growing mycobacteria. These fast-growing species were mostly identified as *M. moriokaense/barrassiae* (41 %), *M. frederiksbergense* (11 %) or *M. austraoafricanum* (5 %). Some OTUs related to fast-growing mycobacterial species dominated some of the compartments. Such was the case for the otu1026, which represented up to 99 % of all the sequences retrieved from the Daumesnil sediments (Fig. 3).

Potential opportunistic pathogens

When considering the 270 OTUs (Suppl. Material 4), 93 were affiliated with at least one potential pathogen species listed by Katoch (2004) and Tortoli (2009). However, few of these 93 OTUs (16 OTUs) were exclusively assigned to opportunistic pathogen species. Among these 16 OTUs a large fraction (9 OTUs) were identified as *M. asiaticum*. The remaining OTUs were affiliated to *M. fortuitum*, *M. immunogenum*, *M. kansasii, M. lentiflavum, M. smegmatis, M. thermoresistibile* and *M. vaccae*. All the OTUs were assigned with an identity close to 97.0 % except for *M. vaccae*

Table 1 Comparison of sequenced libraries, including the number of reads pre- and post-trimming, the observed richness of OTUs, the estimated richness, the diversity index and the evenness index for each sample

Sample name[a]	No. of raw sequences	No. of filtered sequences	Belonging to the *Mycobacterium* genus		Richness estimator (Chao-1)	Diversity index (Exp. form of Shannon)	Evenness index (Simpson evenness)
			No. of sequences[b]	No. of OTUs[b]			
C2W	3548	3128	2708	87	108.7	10.69	0.07
C3W	7961	6862	6616	105	181.6	9.11	0.06
C2 SML[c]	135	99	19	3	3.0	2.76	0.85
C3 SML	6963	5504	5466	64	77.6	7.21	0.07
C2 S	14,052	13,182	13,092	85	114.3	3.84	0.02
C3 S[c]	159,736	136,815	116,827	402	435.5	19.47	0.02
C2 El	6992	5681	5630	167	226.6	26.92	0.09
C3 El	20,789	17,377	16,699	268	332.5	26.60	0.05
C2 Ep	14,817	14,097	13,997	139	160.1	4.20	0.01
C3 Ep	10,589	10,010	9816	101	117.0	5.17	0.02
D1 W	3079	2969	2556	51	61.9	4.23	0.05
D3 W	16,007	15,354	12,842	110	129.4	5.15	0.03
D1 SML	2564	2490	2056	59	92.0	7.57	0.07
D3 SML	3764	3684	2744	78	109.2	5.67	0.03
D1 S	126,217	123,967	123,587	147	184.1	1.10	0.01
D3 S	16,974	16,682	16,578	61	204.5	1.09	0.02
D1 El	22,332	20,907	20,422	222	315.8	3.04	0.01
D3 El	40,176	38,979	38,359	158	246.4	1.89	0.01
D1 Ep	14,808	13,917	13,257	79	102.4	1.92	0.02
D3 Ep	11,661	11,521	11,498	14	23.3	1.23	0.08

OTUs operational taxonomic units; *C* Créteil Lake; *D* Daumesnil Lake; *SML* surface microlayer; *W* water column; *S* sediment; *Ep* epiphytic biofilm; *El* epilithic biofilm

[a] Numbers correspond to the stations numbers

[b] Without singleton sequences

[c] Samples removed from the analysis due to a problem of amplification (C2 SML) and sequencing (C3 S)

and *M. thermoresistibile* with identities of 98.1 and 99.4 % respectively. *M. asiaticum* was mainly encountered in the epilithic biofilm for both lakes and also in the sediment of Créteil Lake. These 16 OTUs were detected in both lakes, in almost all compartments except in waters. However, for all these OTUs characterized as potential opportunistic pathogens, the prevalence in each sample never exceeded 0.34 %.

Comparison of the mycobacterial assemblages

For both lakes, the intra-compartment variations of the NTM assemblages, evaluated using Bray-Curtis distances, were significantly lower than the inter-compartment variations (Mann–Whitney, $P = 0.006$). Overall, mycobacterial community structure variations between compartments, i.e. beta diversity, was about 72 % of dissimilarity. The beta diversity partitioning showed that the variation in the mycobacterial assembly was mainly due to lake or compartment particularities (83 %) and only 17 % was explained by pure random fluctuation of the OTUs richness. Besides, a redundancy analysis performed on the 18 samples with a goodness-of-fit of 0.73 (adjusted R-squared) revealed that the NTM assemblages were significantly shaped by the lake ($P < 0.001$) and the type of compartment (Fig. 3).

Finally, we observed that the majority of the 37 dominant OTUs were present in both lakes and in almost all the compartments (Fig. 3). Inversely, two OTUs were only detected in a single sample: otu547 in

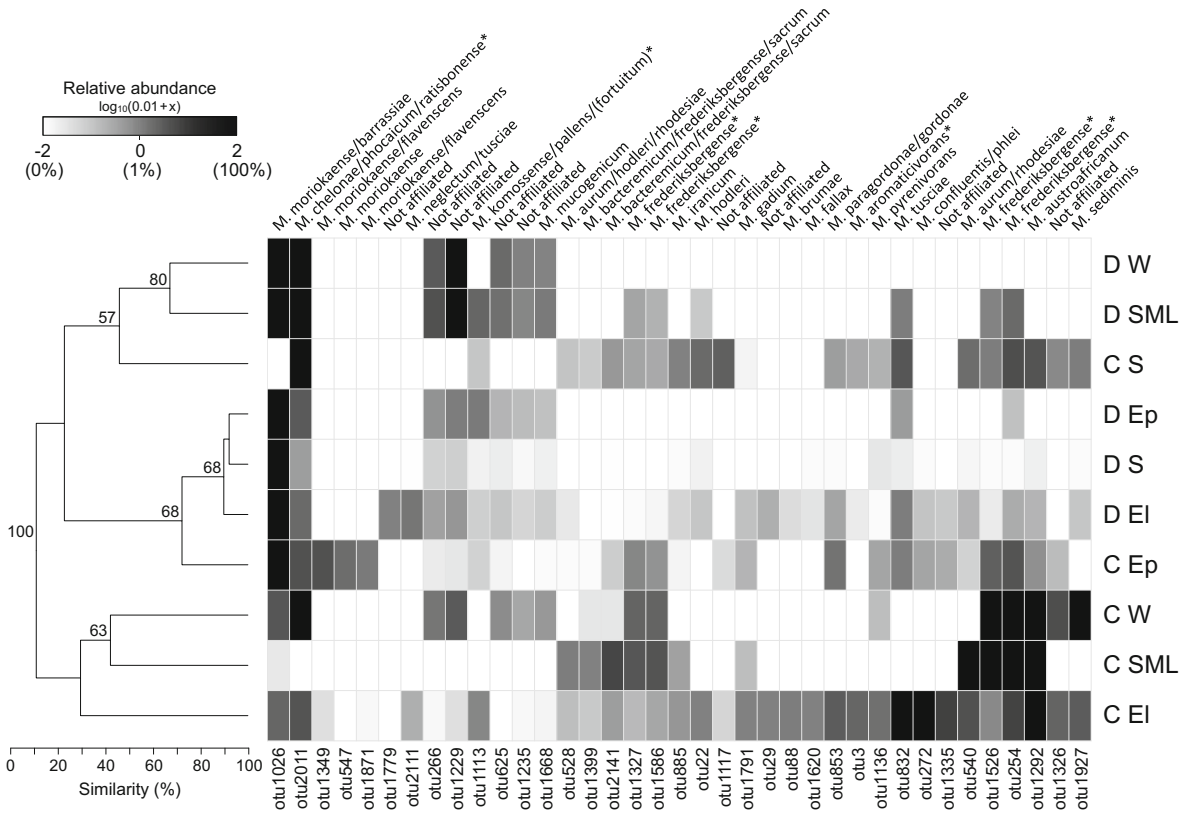

Fig. 3 Heatmap dendrogram illustrating similarities of nontuberculous mycobacteria assemblage composition for the five compartments (*W* water; *SML* surface microlayer; *S* sediment; *El* epilithic and *Ep* epiphytic biofilms) in Créteil (C) and Daumesnil (D) Lakes. The OTU ID as well as species to which they were assigned are presented in abscissa (only OTUs with relative abundances >1 % in at least one compartment were represented). *Asterisk* indicates an assignation for a single OTU to more than three species

epilithon from Créteil Lake and otu1779 in epiphyton from Daumesnil Lake. Otu29 and otu88 were identified in both lakes but exclusively in epilithic biofilms. Only one OTU (otu1399) was present in all the compartments but only in Créteil Lake.

Discussion

This study aimed to prospect the distribution of nontuberculous mycobacteria and identify their natural reservoirs in two recreational lakes. For this purpose, NTM assemblages were conjointly quantified and characterized within five compartments (water column, surface microlayer, sediment, epilithic and epiphytic biofilms) using quantitative real-time PCR and high-throughput sequencing. To date, this sequencing approach has already been applied in soils (Pontiroli et al. 2013) or in drinking water systems (van der Wielen et al. 2013), but as far as we are aware it is the first time that these analyzes have been performed in recreational lakes and in different compartments within the same lake. The results reveal that NTM are ubiquitous and diverse.

Ubiquity of nontuberculous mycobacteria

In natural habitats, NTM have been more frequently identified in extreme environmental conditions, such as in alkaline or acid brown-water and in highly polluted soils and sediments or in environments with low oxygen concentration (Brooks et al. 1984; Kirschner et al. 1992; Iivanainen et al. 1993; Bland et al. 2005; Leys et al. 2005). However, in this study, NTM were detected at high levels in both lakes, in all the collected samples. This result suggests that even in

non-extreme environments, mycobacteria are able to persist and/or grow in the five investigated compartments at relatively high diversity and abundance. However, it is difficult to compare the densities measured in this study with the literature, since no other study has used the same extraction and amplification methodologies. Nevertheless, some studies using quantitative real-time PCR targeting the entire NTM assemblages reported similar values in a coastal lagoon (Jacobs et al. 2009), in freshwater samples in Ethiopia (Khera 2012) or in sediments from Lake Erie (Debruyn et al. 2009).

Nontuberculous mycobacteria dominated by fast-growing species

High-throughput sequencing of the hypervariable regions V2–V3 from the 16S rRNA gene allowed an in depth-analysis of the NTM composition. With 41 % of the OTUs assigned to one, two or three *Mycobacterium* species, the percent of unclassified mycobacteria was comparable to that reported by Pontiroli et al. (2013) who used the same primers to characterize soil samples. The large proportion of unassigned sequences suggests, as already known, that the *rrs* gene does not have enough information to properly identify NTM to the species level (Kim et al. 2005) and/or that many NTM species are still unknown or poorly described. Indeed, partly due to the improvement of the isolation and identification techniques, the number of described species belonging to the *Mycobacterium* genus is steadily growing, with 50 identified species in 1997 and up to 170 presently (Euzéby 1997, Accessed on August 2015).

For all the analyzed samples, NTM assemblages were mainly composed of rare OTUs and were dominated by fast-growing species. Similar results were encountered in studies focusing on water and soil samples (Khera 2012; Pontiroli et al. 2013), which also identified NTM by targeting the *rrs* gene with the same primer set. This prevalence could suggest that fast-growing species possess more plasticity to environmental changes and/or are more competitive compared to slow-growing species. It could also be a PCR artifact due to a difference in *rrs* gene copy numbers between fast and slow-growers (Bercovier et al. 1986) and/or it may also be due to the preferential binding of the primer set to the *rrs* gene in fast-growing mycobacteria. Besides, two previous culture-based studies that investigated freshwater ecosystems (Viallier and Viallier 1973; Bland et al. 2005) did not find any noticeable dominance of the fast-growing species.

Among the fast-growing OTUs identified in the present study, numerous species have already been identified for their potential role in polycyclic aromatic hydrocarbon degradation. Indeed, *M. austroafricanum* or *M. frederiksbergense* have been characterized for their abilities to degrade anthracene or pyrene (Willumsen et al. 2001; Wick et al. 2003; Leys et al. 2005; Uyttebroek et al. 2006). The dominant OTU, identified as *M. moriokaense* and *M. barrassiae* (up to 99 % of the identified OTUs for the sediment in Daumesnil Lake), have been previously observed in Japanese and Ethiopian soils (Tsukamura et al. 1986; Pontiroli et al. 2013), in German hospital tap water (Hussein et al. 2009) or in a French water distribution system (Dubrou et al. 2013), but this is the first time it was reported in lakes.

Mycobacterial densities and diversity differed among compartments

The abundance and diversity of mycobacterial assemblage was significantly shaped by the compartments, potentially due to differences in physicochemical properties and type of substrates.

In water compartments, NTM densities were significantly higher in the surface microlayer compared to the water column. This result is consistent with Parker et al. (1983) who experimentally found a concentration of *M. intracellulare* up to 15,000 higher in aerosols (formed from the surface microlayer) compared with the bulk water. Even if the surface microlayer is exposed to high levels of UV, the enrichment of mycobacteria in the lipid microlayer could be due to the hydrophobicity of the mycobacteria, which can be concentrated at the surface by preferential binding to air bubbles rising in the water column (Blanchard 1964). Moreover the high NTM concentration in this compartment could be due to high concentrations of hydrophobic compounds such as hydrocarbons (Cincinelli et al. 2001; Wurl and Obbard 2004; Manodori et al. 2006) that could constitute a substrate for mycobacteria.

In the sediment, large differences among assemblages were observed between the different samples. This result could be due to differences in the quantity and/or the quality of the nutrients available for the

bacteria. The high densities of NTM in the sediment near the storm sewer outlet in the Créteil Lake were in agreement with Pickup et al. (2006), who found that densities of *M. avium* subsp. *paratuberculosis* in river were well predicted by rainfall events. This result suggests that significant densities of NTM are transported by runoff effluents and settle in the lake sediment close to the storm sewer outlet.

Mycobacteria are well known to colonize biofilms, as they tend to easily stick to surfaces and to clump together. Indeed, high mycobacterial densities and diversity were found in the epilithic and epiphytic biofilms. Moreover, the density and composition of NTM differed between the epilithic and epiphytic biofilms, which may be related to the difference of biofilm ages. Epilithic biofilms were established since several decades, while phragmites and milfoils regrew every year. It may also be due to the colonization of specific algal species in the epilithon or epiphyton (Danilov and Ekelund 2000) that could interact differently with the bacteria. The mycobacterial densities were significantly higher in the phragmite biofilms compared to the milfoil biofilms, although the mycobacterial assemblages showed similar OTU compositions. This result indicates that the plant species did not exhibit a strong influence on the NTM assemblage composition, contrary to a previous result obtained for the total bacterial community (Hempel et al. 2008). This discrepancy could potentially be due to the microaerophile nature of NTM. However, plant biofilms had lower *Mycobacterium* density compared with epilithic biofilms. One potential explanation could rely in the capacity of *Myriophyllum spicatum* to produce polyphenols that can affect the bacterial growth (Walenciak et al. 2002; Hempel et al. 2009).

Potential opportunistic pathogens

Several OTUs were identified as potential opportunistic pathogens (*M. asiaticum, M. fortuitum, M. immunogenum, M. kansasii, M. lentiflavum, M. segmatis, M. thermoresistibile and M. vaccae*), known to be responsible for a large range of diseases including pulmonary, cutaneous and soft tissue infections (Katoch 2004; Griffith et al. 2007; Tortoli 2009). All these species have previously been isolated in terrestrial or aquatic habitats (Viallier and Viallier 1973; Engel and Berwald 1980; Covert et al. 1999; Narang et al. 2009; Pontiroli et al. 2013; Klanicova et al. 2013). Although most of these pathogenic species were detected in both lakes in almost all compartments except in waters, some species were preferentially present in particular compartments. For instance, *M. asiaticum* was only identified in epilithon and sediment.

Due to methodological aspects, it is possible that some species could be either underestimated or overestimated. On one hand, it is likely that the number of potential pathogens was underestimated owing to the highly conservative sequence of the *rrs* gene. On the other hand, it is possible that this number was overestimated due to the poor discrimination of the 16S rDNA sequences at the species levels using the 97 % similarity threshold. Drancourt et al. (2000) recommended using 99 % as identity cutoff to assign OTU sequences to bacterial species. When we applied this recommendation, only one OTU could be identified as an opportunistic pathogen species: *M. thermoresistibile*.

Sequencing genes encoding for *hsp65* and *rpoB* are necessary for a proper identification of mycobacterial species (Adékambi and Drancourt 2004; Kim et al. 2005). Although *rrs* sequencing may not be a suitable tool to reliably identify mycobacterial species in environmental samples, it could be an initial screen for dominant NTM groups that could later be studied in detail using quantitative real-time PCR or DNA microarrays.

To conclude, our results showed that a molecular approach combining quantification and characterization of the bacterial composition is suitable to screen for NTM in complex aquatic ecosystems. High-throughput sequencing of the *rrs* gene offered sufficient depth to investigate variations in the mycobacterial assemblages between lakes and compartments. Although the 16S rRNA gene fragment had a too low resolution to assign all the OTUs at the species level, this approach represents a useful tool to prospect for potential pathogenic species that should be studied in detail with appropriate methods.

This study emphases the ubiquity of NTM in natural aquatic environments and the high diversity of mycobacterial assemblages. However, although they were detected in all five compartments, large variations shaped by compartments and lakes were observed in the mycobacterial densities and diversity. Moreover, the density of NTM in the water column and sediment was significantly greater in the eutrophic Daumesnil Lake. These results could be due to the

difference of quantity and/or quality of nutrients available for bacteria (Suppl. Material 1). The difference in epilithic mycobacteria between the two lakes could be also due to differences in the substratum nature (rocks in Créteil and concrete in Daumesnil Lake). However, it is difficult to conclude about the environmental parameters that structure the NTM community since the two lakes were different in terms of trophic status. To establish a clear relationship between limnological properties and mycobacteria density and diversity, it would be necessary to perform a study on a larger number of lakes in order to perform a robust statistical analysis. Despite all these discrepancies, NTM assemblages were mainly composed of fast-growing species, regardless of compartment. Finally, it would be interesting to take into account biotic reservoirs, such as amoebae (Delafont et al. 2014), fishes (Mrlik et al. 2012) or insects (Marsollier et al. 2002), which could represent relevant vectors of mycobacteria and favour the survival of these bacteria in environment.

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